

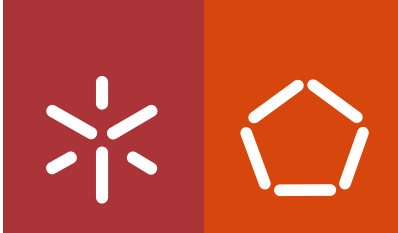


Universidade do Minho
Escola de Engenharia

Leping Yan

Silk Fibroin-Based Scaffolds, Hydrogels and Calcium-Phosphate Filled Materials Aimed for Regenerative Medicine Applications

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Tese de Doutoramento em Engenharia de Tecidos,
Medicina Regenerativa e Células Estaminais

Trabalho realizado sob a orientação do

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É AUTORIZADA A REPRODUÇÃO PARCIAL DESTA TESE APENAS PARA EFEITOS DE INVESTIGAÇÃO, MEDIANTE DECLARAÇÃO ESCRITA DO INTERESSADO, QUE A TAL SE COMPROMETE

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To my wife Shaohong Lin

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Silk Fibroin-Based Scaffolds, Hydrogels and Calcium-Phosphate Filled Materials Aimed for Regenerative Medicine Applications

Abstract

Bone and cartilage defects derived from trauma or disease are major problems in orthopedics. Tissue engineering and regenerative medicine provides promising strategies for the regeneration of damaged tissues. Biomaterials, processed into porous scaffolds and hydrogels, have been playing a crucial role in the tissue regeneration. Controlling the physicochemical properties of biomaterials is important for inducing proper cellular response towards tissue formation, thus facilitating the regeneration procedure. While the ideal tissue regeneration outcome has not yet been achieved, great progress had been made in the last decades, in terms of the application of biomaterials for tissue regeneration.

The aim of this thesis is to develop novel silk fibroin (SF) based porous scaffolds and hydrogels with adequate properties and controlled conformations for different tissues regeneration. Several strategies were used in this thesis, including the improvement of scaffolds' strength, biomimetic of the tissue composition and stratified structure, and development of stimuli-responsive hydrogels with injectable or spatial tunable properties. SF derived from *Bombyx mori* cocoons was chosen as the matrix material because it has many advantages. It is a biodegradable protein based biomaterial with superior *in vitro* and *in vivo* biocompatibility. Moreover, its mechanical properties and degradation profile can be tuned by the processing approach. SF can be processed into different shapes and architectures, and it is a readily available supply.

Salt-leached SF scaffolds with superior mechanical properties were produced by using highly concentrated aqueous SF solutions. The compressive and storage moduli of the scaffolds were significantly enhanced with increasing the concentration of SF solution. The developed scaffolds were of macro/microporous structure, high porosity and interconnectivity, and presented a homogeneous porosity distribution. The obtained scaffolds present adequate properties for cartilage and meniscus regeneration.

Mimicking the composition of natural bone, composite scaffolds composed of SF and calcium phosphate were developed for bone regeneration. Nano calcium phosphate particles were incorporated in the concentrated SF solution using an *in-situ* synthesis method following salt-leaching to develop the silk-nano calcium phosphate (Silk-NanoCaP). These scaffolds maintained the superior mechanical properties of SF scaffolds but demonstrated *in vitro* bioactivity. The NanoCaP particles were homogeneously distributed in the silk matrix, at both macroscopic and microscopic levels. The leachables of the scaffolds were non-cytotoxic as determined by *in vitro* cytotoxicity assays.

The *in vitro* and *in vivo* biological performance of both SF and Silk-NanoCaP scaffolds was further evaluated. These scaffolds supported the viability and proliferation of human adipose tissue derived stromal cells. The formed extracellular matrix improved the mechanical properties of the cell-laden scaffolds or constructs. *In vivo*, both scaffolds have supported *de novo* bone formation and ingrowth's and induced no acute inflammatory response. The Silk-

NanoCaP scaffold was osteoconductive as new bone grew directly on its surface. This group induced higher amount of new bone formation than the SF group. The Silk-NanoCaP scaffolds can be used in bone regeneration.

Considering the stratified/composition characteristics of osteochondral tissue, bilayered scaffolds composed of a SF layer and a Silk-NanoCaP layer were produced for osteochondral defects (OCD) regeneration. The *in vitro* bioactivity was only observed in the Silk-NanoCaP layer *i.e.*, the bone-like layer. When seeded with marrow mesenchymal stromal cells, the bilayered scaffolds promoted cell viability and proliferation, and the Silk-NanoCaP layer induced a higher alkaline phosphatase level as compared to the SF layer (cartilage-like layer). *In vivo* subcutaneous implantation showed that the scaffolds supported tissue infiltration and no granulation tissue or acute inflammation were observed. When implanted in the rabbit OCD, the bilayered scaffolds supported cartilage regeneration in the SF layer and promoted bone ingrowths in the Silk-NanoCaP layer. Therefore, they demonstrated to be promising candidates for OCD regeneration.

Besides the development of SF based scaffolds, another approach explored in this thesis was to develop injectable and enzymatically cross-linked SF hydrogels that could be suitable for cartilage regeneration. The SF hydrogels were prepared by peroxidase mediated cross-linking of the tyrosine groups in the backbone of SF. These hydrogels could be formed in a few minutes under physiological conditions. Dominant amorphous conformation was presented in these hydrogels. These hydrogels were ionic strength and pH stimuli responsive. Cells were successfully encapsulated into these hydrogels. Subcutaneous implantation showed that these hydrogels did not induce any acute inflammatory reaction. After *in vitro* cell culture or *in vivo* implantation, β -sheet conformation was observed in these hydrogels. The developed SF hydrogels can be used as an injectable material for filling tissue defects (such as bone or cartilage) or as a drug delivery system.

Finally, SF hydrogels with spatially controllable properties were generated. Core-shell SF hydrogels consisted in a β -sheet conformation in the shell layer and mainly an amorphous conformation in the core layer. These were prepared by the controlled immersion of the peroxidase mediated SF hydrogels in methanol. The thickness of the shell layer and the mechanical properties of the core-shell SF hydrogels increased with increasing the immersion time. When incorporating albumin as a model drug, the core-shell SF hydrogels presented slower and more controllable release profile as compared to the SF hydrogel. The core-shell SF hydrogels can be used as a controlled release system, tissue substitute or both.

In this thesis, different strategies for developing novel SF based scaffolds and enzymatically cross-linked hydrogels were explored. In both cases remarkable properties and functions for tissue engineering and regenerative medicine applications were achieved, as well as a high reproducibility of the systems. The SF based scaffolds and enzymatically cross-linked SF hydrogels provided herein can be promising candidates for cartilage, meniscus, bone, and osteochondral regeneration, as well as drug delivery systems or tissue substitutes.

Matrizes Tridimensionais Porosas, Hidrogéis e Materiais Reforçados com Fosfatos de Cálcio à Base de Fibroína de Seda para Aplicação em Medicina Regenerativa

Resumo

Os defeitos ósseos e de cartilagem resultantes de trauma ou de um processo degenerativo são os principais problemas em ortopedia. As áreas da engenharia de tecidos e a medicina regenerativa têm permitido propor um conjunto de estratégias promissoras para a regeneração dos tecidos danificados. Neste âmbito, os biomateriais quando processados sob a forma de matrizes tridimensionais porosas e hidrogéis têm desempenhado um papel crucial na regeneração dos tecidos. Mas o controlo das propriedades dos biomateriais é também importante, uma vez que influenciam a resposta celular e a formação do novo tecido. Assim sendo, a melhoria das propriedades físico-químicas dos biomateriais como estratégia para a optimização do processo de regeneração de tecidos, científicas de maior relevo por forma a facilitar o processo de regeneração constitui uma questão científica relevante.

Esta tese tem como objetivo o desenvolvimento de novas matrizes tridimensionais porosas e hidrogéis à base de fibroína de seda (FS) com propriedades adequadas e conformações controladas para regeneração de diferentes tecidos. Neste contexto, foram utilizadas várias estratégias, incluindo a melhoria das propriedades mecânicas, utilização de composições biomiméticas e abordagens de estratificação no processo de produção das matrizes tridimensionais porosas, e desenvolvidos hidrogéis injetáveis e sensíveis a estímulos com propriedades ajustáveis. A FS derivada de casulos da espécie de *Bombyx mori* foi escolhida como o material base, dado que possui inúmeras vantagens. É um biomaterial de natureza protéica e biodegradável, e cuja biocompatibilidade tem sido demonstrada *in-vitro* e *in-vivo*. Além disso, as propriedades mecânicas e o perfil de degradação podem ser optimizados durante as várias etapas de processamento. A FS pode ser ainda processada em diferentes formas e arquiteturas, sendo uma fonte prontamente disponível.

Inicialmente, foram produzidas matrizes tridimensionais porosas através do processo de “salt-leaching”. Estas matrizes apresentam propriedades mecânicas superiores, uma vez que foram usadas soluções aquosas de FS altamente concentradas. Os módulos de elasticidade e de compressão das estruturas obtidas foram significativamente melhorados com o aumento da concentração da solução de FS. As matrizes desenvolvidas apresentam uma estrutura com macro- e micro-porosidade, interconectividade, e distribuição homogênea dos poros. As matrizes obtidas apresentam propriedades adequadas para a regeneração de cartilagem e menisco.

Por forma a mimetizar a composição natural do osso e promover a regeneração óssea, foram desenvolvidas estruturas compósitas de FS e fosfato de cálcio. Assim, foi desenvolvido uma matriz tridimensional porosa e compósita de seda-nanofosfato de cálcio (Seda-NanoCaP), através da síntese *in-situ* de nanopartículas de fosfato de cálcio na solução de FS concentrada, seguido do método de “salt-leaching”. Estas matrizes possuem propriedades mecânicas superiores, e apresentam bioatividade *in-vitro*. As partículas de NanoCaP foram homogeneamente distribuídas na matriz de seda, ao nível macroscópico e microscópico. Verificou-se que os materiais lixiviados destas estruturas não são citotóxicos, tal como demonstrado em ensaios de avaliação da citotoxicidade *in-vitro*.

O desempenho biológico de ambas as matrizes de FS e seda- NanoCaP foi ainda avaliado *in-vitro* e *in-vivo*. Estas matrizes tridimensionais porosas suportaram a viabilidade e proliferação de células obtidas do estroma de tecido adiposo humano. A matriz extracelular produzida, permitiu melhorar as propriedades mecânicas dos dois tipos de matrizes tridimensionais porosas de SF. *In-vivo*, ambas as

matrizes permitiram a formação de novo osso sem indução de resposta inflamatória aguda. No entanto, a matriz de seda-NanoCaP tem capacidade osteocondutora superior, uma vez que suportou o crescimento de novo osso directamente na sua superfície. Estas estruturas tridimensionais porosas e compósitas induziram uma maior quantidade de formação de novo osso em comparação com a matriz de FS. Desta forma as matrizes de seda-NanoCaP são as mais adequadas para serem utilizadas na regeneração óssea.

Considerando as características do tecido osteocondral e a sua composição estratificada foram desenvolvidas matrizes tridimensionais porosas em bicamada, constituídas por uma camada de SF e uma camada compósita de Seda-NanoCaP para a regeneração de defeitos osteocondrais (DOC). A bioatividade *in-vitro* foi observada apenas na camada Seda-NanoCaP, a camada semelhante ao osso. Quando cultivada com células mesenquimais isoladas da medula óssea, as matrizes de bicamada promoveram a viabilidade e proliferação celular. A camada de seda-NanoCaP induziu uma expressão mais elevada da fosfatase alcalina em comparação com a camada de FS. *In-vivo*, as matrizes tridimensionais porosas de bicamada permitiram a infiltração de tecido e não foi observado tecido de granulação ou inflamação aguda, após a implantação subcutânea. Quando implantado num DOC crítico em modelo de coelho, as matrizes de bicamada permitiram a regeneração da cartilagem na camada de FS e o crescimento ósseo na camada de Seda-NanoCaP. Desta forma, estas matrizes mostraram ser muito promissoras na regeneração osteocondral.

Além do desenvolvimento de matrizes tridimensionais porosas à base de FS para a regeneração da cartilagem, foram também produzidos com sucesso hidrogéis injectáveis, reticulados por via enzimática. Os hidrogéis de FS foram preparados a partir da reticulação dos grupos de tirosina na cadeia principal da FS, recorrendo à actividade da enzima peroxidase. Estes hidrogéis formam-se em poucos minutos e em condições semelhantes às condições fisiológicas. Apresentam uma conformação amorfa dominante, e são sensíveis a estímulos de força iónica e pH. Permitem também o encapsulamento de células. Através de um implante subcutâneo foi possível demonstrar que os hidrogéis não induzem reacção inflamatória aguda, em modelo de ratinho. Após cultura de células *in-vitro* ou implantação *in-vivo*, foi observada a alteração de conformação para folha β nestes hidrogéis. Os hidrogéis desenvolvidos podem ser utilizados como um material injectável para o preenchimento de defeitos em tecidos, tais como osso e cartilagem ou como um sistema de libertação controlada de fármacos.

Finalmente, foram desenvolvidos hidrogéis de FS com propriedades controláveis ao nível espacial. Estes hidrogéis de FS apresentam uma camada externa em conformação de folha β e um núcleo amorfo, e foram preparados por imersão controlada do gel em metanol, após reticulação enzimática. A espessura da camada de invólucro e as propriedades mecânicas dos hidrogéis de FS aumentam ao longo do tempo de imersão em metanol. Em estudos de prova de conceito usando a albumina como um fármaco-modelo, os hidrogéis revestidos apresentam um perfil de libertação mais lento e controlável, em comparação com o hidrogel de SF não tratado com metanol. Os hidrogéis com diferentes conformações podem ser usados como sistemas de libertação controlada, substitutos de tecidos ou ambos.

Nesta tese, foram exploradas diferentes estratégias para o desenvolvimento de matrizes tridimensionais porosas e hidrogéis reticulados por via enzimática. Em ambos os casos foram obtidas propriedades e funções notáveis para aplicações em diferentes abordagens da engenharia de tecidos e medicina regenerativa, bem como uma elevada reprodutibilidade dos sistemas. As matrizes tridimensionais porosas de FS e os hidrogéis reticulados enzimaticamente aqui propostos constituem candidatos promissores para a regeneração de cartilagem, menisco, osso e tecido osteocondral, podendo actuar simultaneamente como um sistema para a libertação controlada de fármacos.

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List of Abbreviations

(B)	layered scaffolds/hydrogels;	C	
(G)	growth factors or bioactive reagents incorporated scaffolds/hydrogels;	CAD/CAM	computer-aided design and computer-aided manufacturing;
(I)	studies on regeneration of osteochondral interface;	CaP	calcium phosphate;
3D	three dimensional;	cm	centimeter;
A		CMCh	carboxymethyl chitin;
AAOS	the American Academy of Orthopaedic Surgeons;	CO ₂	carbon dioxide;
ACI	autologous chondrocyte implantation;	Col	collagen;
ACP	amorphous calcium phosphate;	CPM	continued passive motion;
ADSCs	adipose tissue derived stem cells;	CPP	calcium polyphosphate;
AFSCs	amniotic fluid-derived stem cells;	CS-MA	chondroitin sulfate-methacrylate;
Albumin-FITC	the albumin-fluorescein isothiocyanate conjugate;	D	
ALP	alkaline phosphatase;	DCBM	decellularized cancellous bone matrix;
α -MEM	alpha-minimum essential medium;	DCM	decellularized cartilage matrix;
ANOVA	one-way analysis of variance;	DDM	demineralized dentin matrix;
AOFAS	the American Orthopaedic Foot and Ankle Society;	DDS	drug delivery systems;
APPACDM	the Portuguese Association of Parents and Friends of Mentally Disabled Citizens;	Dex	dexamethasone;
ASCs	adipose tissue derived stromal cells;	DMA	dynamic mechanical analysis;
ATDC-5	a chondrogenic cell line derived from mouse teratocarcinoma cells;	DMEM	Dulbecco's modified Eagle's medium;
ATR	attenuated total reflectance;	DNA	deoxyribonucleic acid;
B		dsDNA	double-stranded DNA;
BCP	biphasic calcium phosphate;	E	
β -GP	beta-glycerol phosphate;	ECM	extracellular matrix;
β -sheet	beta-pleated sheet;	EDTA	ethylenediaminetetraacetic acid;
bFGF	basic fibroblast growth factor;	EDX	energy dispersive X-Ray detector;
BMP	bone morphogenetic protein;	EPC	endothelial progenitor cells;
BMSCs	bone marrow mesenchymal stromal cells;	ESCs	embryonic stem cells;
		F	
		FBS	fetal bovine serum;
		FDM	fused deposition modeling;
		FFI	the Foot Function Index;
		FTIR	Fourier transform infra-red spectroscopy;

G		L-pore	the macro pores in the prepared salt leached silk fibroin scaffolds;
G''	the loss moduli;		
G'	the storage moduli;		
GAG	glycosaminoglycan;		
GAGAGS	repetitive amino acid sequence glycine-alanine-glycine-alanine-glycine-serine;	M	
		M	molar;
		MACI	matrix-induced autologous chondrocyte implantation;
GF	growth factor(s);	MASI	matrix-induced autologous stem cells implantation;
GFP	green fluorescent protein;		
GP	glycerol phosphate;	μ-CT	micro computed tomography;
		μg	microgram;
H		μg/mL	microgram per milliliter;
H&E	haemotoxylin and eosin;	mg	milligram;
HA	hydroxyapatite;	MGCs	multinucleated giant cells;
hASCs	human adipose tissue derived stromal cells;	Micro-CT	micro-computed tomography;
		mL	milliliter;
hBMSCs	human BMSCs;	mM	millimolar;
HCl	hydrochloric acid;	mm	millimeter;
HFIP	hexafluoroisopropanol;	MOCART	Magnetic Resonance Observation of Cartilage Repair Tissue;
H ₂ O	water;		
H ₂ O ₂	hydrogen peroxide;		
HRP	horseradish peroxidase	MPa	megapascal;
Hz	hertz;	MRI	magnetic resonance imaging;
		uS	microsiemens;
I		MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophynyl)-2H-tetrazolium);
IAM	intermittent active motion;		
ICRS	International Cartilage Repair Society;		
IGF	insulin-like growth factor;	MWCO	molecular weight cut off;
IKDC	International Knee Documentation Committee;	N	
Imm	immobilization;	Na ₂ CO ₃	sodium carbonate;
iPS	induced pluripotent stem cells;	NaCl	sodium chloride;
		nano-CaP	nano-sized calcium phosphate particles;
ISS	isotonic saline solution;	NaOH	sodium hydroxide;
K		n-HA	nano-Hydroxyapatite;
kDa	kilo daltons;	NMR	nuclear magnetic resonance;
KOOS	Knee injury and Osteoarthritis Outcome Score;	O	
		OATS	OC autograft transplantation;
kPa	kilopascal;	°C	degree celsius;
L		OC	osteocondral;
L	liter;	OCD	osteocondral defect;
LiBr	lithium bromide;	OD	optical density;
		OLTs	OC lesion of the talus;
		OPF	poly(ethylene glycol) fumarate hydrogel;

P		rhIGF	recombinant human insulin-like growth factor;
P0	passage 0;		
P1	passage 1;	S	
P2	passage 2;	S16	salt-leached silk fibroin scaffolds derived from 16 wt.% aqueous silk fibroin solution;
P3	passage 3;		
P4	passage 4;		
PA6	polyamide 6;		
PBS	phosphate buffered saline;	SBF	simulated body fluid;
PBT	poly(butylenes terephthalate);	SC16	salt-leached silk fibroin/nano-CaP scaffolds, derived from 16 wt.% aqueous silk fibroin solution and with 16 wt.% theoretically incorporated calcium phosphate (CaP:Silk, by wt.);
PCL	poly(ϵ -caprolactone);		
PDLA	poly(D-lactic acid);		
PDLLA	poly(DL-lactide);		
PEC	polyelectrolyte composite;		
PEGDA	poly(ethylene glycol) diacrylate;		
PEGDAM	poly(ethylene glycol) dimethacrylate;	SCID	severe combined immunodeficiency;
PEOT/PBT	poly(ethylene oxide terephthalate) and poly(butylenes terephthalate) copolymer;	SD	standard deviation;
		SDSCs	synovium-derived stem cells;
		SEM	scanning electron microscopy;
PEOT	poly(ethylene oxide terephthalate);	SF	silk fibroin;
PGA	poly(glycolic acid);	SF-36	patient outcome scores;
PHEMA	poly(hydroxyethyl methacrylate) hydrogel;	Shh	sonic hedgehog;
		Silk/CaP-4	silk/nano-CaP scaffold with 4 wt.% initially theoretically incorporated CaP (divided by the mass of silk fibroin);
PLA	poly(lactic aci);		
PLGA	poly(glycolic-co-lactic acid);		
PLLA	poly(L-lactic acid);	Silk/CaP-8	silk/nano-CaP scaffold with 8 wt.% initially theoretically incorporated CaP (divided by the mass of silk fibroin);
PMMA	poly(methyl methacrylate);		
pNP	<i>p</i> -nitrophenol;		
pNPP	<i>p</i> -nitrophenyl phosphate disodium;	Silk/CaP-16	silk/nano-CaP scaffold with 16 wt.% initially theoretically incorporated CaP (divided by the mass of silk fibroin);
POC	poly(1,8-octanediol-co-citrate);		
PRP	platelet-rich plasma;		
PU	polyurethane;	Silk/CaP-25	silk/nano-CaP scaffold with 25 wt.% initially theoretically incorporated CaP (divided by the mass of silk fibroin);
PVA	poly(vinyl alcohol);		
PVA-MA	poly(vinyl alcohol)-methacrylate;		
PVP-I	polyvinylpyrrolidone-iodine;	Silk/nano-CaP	scaffolds composed of silk fibroin and nano-sized calcium phosphate;
R			
RBMSCs	rabbit BMSCs;	Silk/Silk-NanoCaP	bilayered scaffolds composed of a silk and a Silk-NanoCaP layer;
Ref	reference(s);		
rhBMP	recombinant human bone morphogenetic protein;		

Silk-8	silk fibroin scaffolds prepared with 8% (by wt.) aqueous silk fibroin solutions;	X XRD	X-ray diffraction;
Silk-10	silk fibroin scaffolds prepared with 10% (by wt.) aqueous silk fibroin solutions;		
Silk-12	silk fibroin scaffolds prepared with 12% (by wt.) aqueous silk fibroin solutions;		
Silk-16	silk fibroin scaffolds prepared with 16% (by wt.) aqueous silk fibroin solutions;		
Silk-II	antiparallel β -pleated sheet conformation in silk fibroin;		
Silk-NanoCaP	scaffolds composed of silk fibroin and nano-sized calcium phosphate;		
SPCL	starch-PCL composite;		
S-pore	the micro pores in the prepared salt leached silk fibroin scaffolds;		
SVF	stromal vascular fraction;		
T			
Tan δ	loss factor;		
TCP	tricalcium phosphate;		
TCPS	tissue culture polystyrenes plate;		
TGA	thermal gravimetric analysis;		
TGF- β	transforming growth factor β ;		
TRE	tetracycline-responsible element;		
Tris-EDTA	tris(hydroxymethyl)aminomet hane-ethylenediaminetetraacetic acid buffer;		
U			
U	unit;		
UCMSCs	umbilical cord mesenchymal stromal cells;		
UV	ultra-violet;		
V			
vol	volume;		
W			
Wt	weight;		

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List of Publications

The research work performed during the PhD period resulted in the following publications.

International Journals with Referee

1. **Yan LP**, Silva-Correia J, Correia C, da Silva Morais A, Sousa RA, Oliveira AL, Oliveira JM, Reis RL. A Novel Silk Fibroin Hydrogel for Tissue Engineering and Regenerative Medicine Applications. 2014, Submitted.
2. **Yan LP**, Oliveira JM, Oliveira AL, Reis RL. Core-shell Silk Fibroin Hydrogels: Modulating the Release of Bioactive Molecules through Controlled Spatial Conformation. 2014, Submitted.
3. **Yan LP**, Oliveira JM, Oliveira AL, Reis RL. Tissue Engineering Strategies for the Treatment of Osteochondral Lesions: From Clinical Studies to Preclinical Challenges. 2014, Review, Submitted.
4. **Yan LP**, Oliveira MB, Vilela C, Pereira H, Sousa RA, Mano JF, Oliveira AL, Oliveira JM, Reis RL. Bilayered Silk/Silk-NanoCaP Scaffolds for Osteochondral Tissue Engineering: *In Vitro* and *In Vivo* Assessment of Biological Performance. 2014, Submitted.
5. **Yan LP**, Oliveira JM, Oliveira AL, Reis RL. *In Vitro* Evaluation of the Biological Performance of Macro/Microporous Silk Fibroin and Silk-Nano Calcium Phosphate Scaffolds. 2014, Submitted.
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2. **Yan LP**, Oliveira JM, Oliveira AL, Reis RL. Development of A Bilayered Scaffold Based on Silk Fibroin and Silk Fibroin/Nano-Calcium Phosphate for Osteochondral Regeneration. TERM STEM 2012, Guimaraes, Portugal, October 09-13th, 2012.

3. **Yan LP**, Oliveira JM, Oliveira AL, Caridade SG, Mano JF, Reis RL. Macro/Micro Porous Silk Fibroin Scaffolds Obtained via Combined Methodologies for Articular Cartilage and Meniscus Tissue Engineering. The 9th International Symposium on Frontiers in Biomedical Polymers (FBPS 2011), Funchal, Portugal, May 09-12th, 2011.

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Poster Communications

1. **Yan LP**, Correia C, Pereira DR, Sousa RA, Oliveira JM, Oliveira AL, Reis RL. Injectable Silk Fibroin Hydrogels with Ionic Strength and pH Response for Tissue Engineering and Regenerative Medicine Applications. TERM STEM 2013, Porto, Portugal, October 07-12th, 2013.

2. **Yan LP**, Silva-Correia J, Caridade SG, Fernandes EM, Mano JF, Sousa RA, Oliveira JM, Oliveira AL, Reis RL. Preparation and Characterization Of Macro/Micro Porous Silk Fibroin /Nano-Sized Calcium Phosphate Scaffolds for Bone Tissue Engineering. The 3rd TERMIS World Congress 2012, Vienna, Austria, September 5-8th, 2012.

3. **Yan LP** , Silva-Correia J, Correia C, Caridade SG, Oliveira JM, Oliveira AL , Mano JF, Reis RL. Characterization and Cytotoxicity of Macro/Micro Porous Silk and Silk/Nano-CaP Scaffolds for Bone Tissue Engineering Applications. The 9th World Biomaterials Congress, Chengdu, China, June 1st-5th, 2012.

4. **Yan LP**, Oliveira JM, Oliveira AL , Caridade SG, Mano JF, Reis RL. Preparation And Characterization of Water-Soluble C60/Silk Fibroin Nanocomposite for Cartilage Regeneration Application. The XXXVIII Congress of the European Society for Artificial Organs (ESAO 2011) and IV Biennial Congress of the International Federation on Artificial Organs (IFAO 2011), Porto, Portugal, October 19-21th, 2011.

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5. Pereira H, Silva-Correia J, **Yan LP**, Frias AM, Oliveira AL, Oliveira JM, Reis RL, Espregueira-Mendes JD. Silk-Fibroin Scaffolds for Meniscus Cell-Based Tissue Engineering Therapy. The 20th Anniversary Annual Meeting of European Orthopaedic Research Society (EORS 2012), 2012.

6. Silva-Correia J, Pereira H, **Yan LP**, Miranda-Gonçalves V, Oliveira AL, Oliveira JM, Reis RM, Espregueira-Mendes JD, Reis RL. Advanced Mimetic Materials for Meniscus Tissue Engineering: Targeting Segmental Vascularization. TERM STEM 2012, Guimaraes, Portugal, October 09-13th, 2012.

Introduction to the Thesis Format

This thesis contains mainly two parts, namely the preliminary part concerning the authorship and general information of the thesis, and the second part constituting the body of the thesis. The second part is divided into five sections which comprise ten chapters. The first section is composed of a review paper presenting the current state-of-the-art on osteochondral tissue engineering. The second section describes the materials and methods used for the experimental works mentioned in section three and four. Section three (five chapters) and four (two chapters), together with section one are based on a series of papers published or submitted for publication. These are presented in the format of a manuscript, *i.e.* abstract, introduction, materials and methods, results, discussion, conclusions, and references. The major conclusions and future remarks are addressed in section five. The following is a brief description on the content of each section.

Section 1

Chapter I presents a comprehensive literature overview on osteochondral tissue engineering. In this review, clinical trials and pre-clinical studies employing tissue engineering strategies for osteochondral regeneration from the last decade are summarized. The important advances in osteochondral tissue engineering are highlighted, including novel scaffolds development, stem cells differentiation, application of bioactive compounds, and new techniques in clinical studies. Besides, the promising new trends and new directions for osteochondral tissue engineering are proposed, covering nanotechnologies and re-programmed cells, also expanding to customized-design of the scaffolds and post-operation stimulus.

Section 2

Chapter II describes the materials, the experimental work, and the protocols used in the research works of this thesis. Additionally, it provides the rationale for the selection of the materials, the processing approaches for scaffolds and hydrogels, and the physicochemical and biological evaluation methodologies.

Section 3

This section includes five chapters related with silk-based porous scaffolds and based on research work already published or submitted for publication.

Chapter III describes the feasibility of production of salt-leached silk fibroin (SF) scaffolds with robust mechanical properties using highly concentrated aqueous SF solution. This work overthrows the previous viewpoint that it was impossible to prepare salt-leached SF scaffold with more than 10 wt.% aqueous SF solution. The developed scaffolds are aimed for articular cartilage and meniscus tissue engineering. The physicochemical properties of these scaffolds were fully characterized by different techniques.

Chapter IV reports the development of silk/nano-sized calcium phosphate scaffolds (Silk-NanoCaP) aimed for bone tissue engineering application. The novelty of this work is that the calcium phosphate nanoparticles were introduced into the concentrated aqueous SF solution via an *in-situ* synthesis approach. Thus, the synthesized calcium phosphate particles presented nano size and homogeneous dispersion, in the SF solution and the final salt-leached Silk-NanoCaP scaffolds. The physicochemical properties of these scaffolds were fully analyzed. The *in vitro* bioactivity was also screened by using simulated body fluid solution. Additionally, cytotoxicity test was performed by culturing the L929 cells with the extraction from the scaffolds.

Chapter V relates to the *in vitro* cytocompatibility assessment of the SF and Silk-NanoCaP scaffolds described in Chapter III and IV. The human adipose tissue derived stromal cells (hASCs) were seeded onto these scaffolds and cell attachment, viability, and proliferation were evaluated. Moreover, the enzymatic degradation profile and biomechanical properties of these scaffolds were also characterized.

Chapter VI describes the *in vivo* bone regeneration ability of the developed SF and Silk-NanoCaP scaffolds. These scaffolds were implanted into rat bone defects for three weeks. The *in vivo* biocompatibility, integration with host tissue, and new bone formation inside the scaffolds were evaluated. Furthermore, the long-term *in vitro* degradation profile and *in vitro* mineralization at different time points have been also investigated.

Chapter VII reports the generation of novel Silk/Silk-NanoCaP bilayered scaffold for osteochondral tissue engineering. The Silk-NanoCaP layer aims to promote a good subchondral bone integration and regeneration. The physicochemical properties of the bilayered scaffolds were comprehensively analyzed. *In vitro* biological performance of these scaffolds was fully evaluated by studying the viability, proliferation, and osteogenic differentiation of rabbit bone marrow mesenchymal stromal cells seeded in the scaffolds. *In vivo* biocompatibility was examined by subcutaneous implantation of these scaffolds in rabbit

for 4 weeks. Furthermore, these scaffolds were implanted in critical size osteochondral defects in the knee of rabbits for 4 weeks. The regeneration of subchondral bone and cartilage layer was evaluated by histological staining and micro-computed tomography analysis.

Section 4

This section comprises two chapters related to enzymatically cross-linked SF hydrogel and based on research work already submitted for publication.

Chapter VIII reports that injectable SF hydrogels can be prepared via peroxidase mediated cross-linking procedure under physiological conditions. The novelty of this work is that these SF hydrogels can be prepared in a few minutes without using external stimulus or harsh preparation conditions. These hydrogels can be used as injectable tissue substitutes, drug delivery systems, and short-term cell culture platform. The physicochemical properties of these hydrogels were studied, such as conformation, gelation time and mechanical properties. Besides, the stimulus responsiveness of the SF hydrogels was also investigated. Cell encapsulation in these hydrogels was performed using ATDC-5 cells, and the cell viability was analyzed. Subcutaneous implantation of these hydrogels in mice was carried out in order to study its *in vivo* biocompatibility.

Chapter IX describes a simple method to prepare SF hydrogels with core-shell structure. This work shows that SF hydrogel of spatially controlled properties can be prepared via modulation of its conformation. The core-shell SF hydrogels can be applied for drug delivery systems or tissue substitutes. The conformation distribution in the developed core-shell hydrogels was characterized. Additionally, other physicochemical properties of these hydrogels were also evaluated. Albumin was used as a model drug and encapsulated into the core-shell hydrogels, and the controlled release performance of the core-shell SF hydrogels was *in vitro* studied up to 7 days.

Section 5

Chapter X presents the summarization and general conclusions related with all the research works performed in the scope of this thesis, as well as some final remarks concerning the future perspectives and proposed future works.

Section 1.

Chapter I

Tissue Engineering Strategies for the Treatment of
Osteochondral Lesions: From Clinical Studies to Preclinical
Challenges

Chapter I

Tissue Engineering Strategies for the Treatment of Osteochondral Lesions: From Clinical Studies to Preclinical Challenges

Abstract

Although several procedures are presently being used in the clinical treatment of osteochondral defects (OCD), the ideal long-term strategies are still far from being accomplished. Tissue engineering opens a new field of opportunities for improving results. In the last decade, a great effort has been made to validate tissue engineering strategies in preclinical studies (*in vitro* and *in vivo*) and furthermore in clinical trials on OCD regeneration. Besides the matrix-associated chondrocyte implantation (MACI) procedure, matrix-induced autologous stem cells implantation (MASI) was also tested at the clinical level. Layered scaffolds have been applied for human implantation, mimicking the stratified nature of osteochondral (OC) tissue. In the preclinical studies (*in vitro* and *in vivo*), one of the main strategies is the development of biomimetic and bioactive scaffolds, using decellularized extracellular matrix scaffolds, bilayered or multilayered scaffolds alone or incorporation with growth factors and/or stem cells. Modulation of stem cells towards OC differentiation constitutes another hot topic. Interface regeneration became a new and attractive field in OCD tissue engineering. Computer-aided design/manufacturing, nanotechnology, and gene transfection technologies may bring new insights for OCD regeneration. This review aims at summarizing the status of research at the clinical trial level and identifies the new challenges at the preclinical stage on OC tissue engineering, while giving some perspectives for the ideal direction towards tissue regeneration.

This chapter is based on the following publication: **Yan LP**, Oliveira JM, Oliveira AL, Reis RL. Tissue Engineering Strategies for the Treatment of Osteochondral Lesions: From Clinical Studies to Preclinical Challenges. 2014, Submitted.

1. Introduction

Articular cartilage is a connective tissue that acts as a shock absorber and facilitates joint's motion in low friction [1]. Many reasons can lead to cartilage lesions, such as traumatic events, chronic repetitive microtrauma, and aging. Cartilage lesions are normally irretrievable due to the typical avascular nature of cartilage and consequently lack of supplementation of potential reparative cells/bioactive factors [2]. As the cartilage lesion progresses, it will extend to underlying subchondral bone and osteochondral defect (OCD) appear. Other diseases originating from the subchondral bone and subsequently reaching the cartilage layer can also induce OCD, such as osteochondritis dissecans and osteonecrosis [3]. Osteochondral (OC) fracture, which is a common injury in children and adolescents, represents another cause for OCD. Besides the OCD in the knee, nowadays an increasing amount of attention is being given to OC lesion of the talus (OLTs) because they primarily affect a young athletic population and often lead to long-term disability [4, 5]. Similarly, OC fractures of the patella represent a major complication following patella instability or dislocation [6]. OCD often leads to the formation of fibrocartilage which only provides poor protection to the subchondral bone. Subsequent degradation of the repaired and adjacent tissues is often observed [2]. Furthermore, OCD is associated with severe pain, impaired joint mobility and low quality of life. It also generates huge amount of health care costs every year. In United States alone, the annual cost for the treatment on OCD is about \$95 billion [7].

There has been increased evidence that without the support from the subchondral bone, any treatment on the cartilage layer is likely to fail [3, 8]. Thus the regeneration of cartilage and subchondral bone should be taken into account as one unit during OCD regeneration, instead of considering separately. Actually, the cartilage layer and subchondral bone are tightly connected. No matter what type the lesion, from either the cartilage or the subchondral bone, the connected and surrounded tissues will always be affected, contributing to negatively change the mechanical homeostasis of the whole joint. Therefore, the main goal for OCD regeneration is to restore the biomechanical properties of OC tissue, together with the regeneration of the top cartilage layer and the bottom subchondral layer.

Currently, there are several methods used in the clinical setting for treating OCD lesions. Arthroscopic debridement is used for relief of pain from small defects. In smaller defects

up to 1-2 cm², microfractures are commonly selected [9]. OC autograft transplantation (mosaicplasty, OATS) constitutes another option if the defect size is between 1 and 2.5 cm² [10, 11]. For lesions of size higher than 2.5 cm², autologous chondrocyte implantation (ACI) technology has been used. This method presents an advantage of possibly achieving regeneration with hyaline cartilage. However it requires two-step surgeries and may induce complications of chondrocyte apoptosis and necrosis, or hypertrophy of the cells [12, 13].

In the recent years, tissue engineering strategy emerged as a promising alternative to regenerate OCD [14]. Tissue engineering is a multi-disciplinary approach, involving the advances in material science, chemical engineering, biology, and medicine [15]. Probably the current most appealing clinical application of tissue engineering for OCD regeneration is the matrix-associated autologous chondrocyte implantation (MACI) technology [16]. In order to achieve ideal OCD regeneration, numerous efforts have been made on scaffold/hydrogel development, stem cells differentiation, growth factors incorporation, and animal models [14, 17-19]. However, few studies have been extended to clinical trials [20-22]. Many important and interesting findings were made, and some new technologies and subjects have emerging during the last few years [23-25].

In this review, the most important breakthroughs in OC tissue engineering in the past 11 years were overviewed. The clinical trials, *in vitro* and *in vivo* studies (preclinical), which are using tissue engineering strategy for OCD regeneration have been summarized herein. Therefore, this review intends to add new insights regarding the current research status and challenges in OCD clinical trials and preclinical studies using tissue engineering strategies. In addition, the future directions and new trends in OC tissue engineering are briefly discussed.

2. Tissue Engineering Strategies in OCD Regeneration

2.1. Clinical studies on OC tissue engineering

MACI is the first application of tissue engineering for OCD regeneration. Collagen and hyaluronic acid scaffolds have been used for delivering autologous chondrocytes in the OCD. Comparing with ACI, MACI is advantageous in minimizing the donor site and getting rid of periosteal harvesting and suturing. Some clinical studies showed that MACI

is an efficient method for OCD treatments, both in ankle and knee lesions [20, 26]. Giannini *et al.* [20] showed that the American Orthopaedic Foot and Ankle Society (AOFAS) mean scores increased dramatically after performing MACI on the ankle of 46 patients for one and three years.

Similar to ACI procedure, MACI also requires the harvesting of chondrocytes from cartilage tissue and this process would induce secondary morbidity and increase the cost. In order to solve this limitation, other cell sources have been exploring as an alternative (Matrix-induced autologous stem cells implantation, MASI), such as bone marrow-derived stem cells (BMSCs) and adipose tissue-derived stem cells (ASCs). This new technology requires only a single operation and minimizes the invasion. Nejadnik *et al.* [27] compared the clinical outcomes of autologous chondrocyte and autologous bone marrow-derived stem cells for cartilage regeneration, and found there were no differences. Very interestingly, they also found that the younger patients showed better outcomes in the ACI group, while the age did not make differences in the BMSCs group. Scaffolds seeded with concentrated bone marrow-derived cells were also investigated for OCD regeneration in clinical trials. Giannini *et al.* [24] investigated the combination of concentrated bone marrow derived cells and scaffolds (collagen powder or hyaluronic acid membrane) for talar OCD in patients. The AOFAS scores were improved significantly in the 2 year follow up in both collagen and hyaluronic acid groups. Magnetic resonance imagines (MRI) showed the restoration of the cartilage layer and the subchondral bone in the two years follow-up. In another study, Kon *et al.* [28] confirmed that the one-step bone marrow derived cell transplantation technology achieved good clinical and radiographic outcomes for patients with Osteochondritis dissecans.

The MACI and MASI approach focus on the regeneration of cartilage layer in OCD, and indeed they have demonstrated effectiveness for this purpose. While recent 5-year MRI follow-up showed that subchondral bone diseases (such as edema, cysts, sclerosis, and granulation) were observed in 50% of the patients underwent MACI [3]. This addresses the need to regeneration of subchondral bone together with the regeneration of cartilage layer in OCD healing. Development of layered scaffold, which mimicking the structure and matrix component of OC tissue, provides a promising option to overcome this problem [29]. The implantation of layered scaffolds only requires one surgery and no need for fixation. Currently, there are three artificial cell-free layered scaffolds available for clinical implantation in OCD: Trufit® CB plug, MaioRegen®, and Chondromimetic®.

Table 1. Clinical studies on OC tissue engineering

Ref	Scaffold/ cell	Patient information	Defect site/follow up time	Method	Outcome
Giannini <i>et al.</i> [20]	Hyalograft C® scaffold with human autologous chondrocyte. (MACI)	46 patients with a mean age of 31.4 years (ranged from 20-47), had posttraumatic talar dome lesions.	Ankle 12 and 36 months	At first, an ankle arthroscopy was performed to harvest cartilage. Chondrocytes were cultured on a Hyalograft C® scaffold. In the second step, the construct was arthroscopically implanted into the lesion. Patients were evaluated clinically with the AOFAS score preoperatively and at 12 and 36 months after surgery.	The mean preoperative AOFAS score was 57.2±14.3. After 12 and 36 months, the scores were 86.8±13.4 and 89.5±13.4, respectively. Clinical results were significantly related to the age of patients and to previous operations for cartilage repair. The histological staining revealed that hyaline-like cartilage was formed.
Giannini <i>et al.</i> [24]	Col powder or hyaluronan membrane loaded with concentrated BMSCs	23 patients treated with Col/BMSCs, and 25 patients treated by hyaluronan/BMSCs.	Ankle 6, 12, 18, and 24 months	Porcine Col powder (Spongostan® Powder), and hyaluronic acid membrane (HYAFF® -11) were used. At first, bone marrow was harvested and concentrated. And then, the Col powder or hyaluronic acid membrane was mixed with bone marrow and platelet-rich fibrin gel. Afterwards, the composites were implanted.	In the Col powder group, the mean AOFAS scores of pre-operation and 24 months post-operation were 62.5±18 and 89.8±9.8, respectively. In the hyaluronic acid group, the scores increased from 66.2±10.5 to 92.8±5.3, 24 months after the surgery. At the 2 years follow up, the MRI images showed the restoration of the cartilage layer and subchondral bone in the patients.
Pietschmann <i>et al.</i> [26]	Biphasic collagenous scaffold (NOVOCART® 3D) with autologous chondrocyte (MACI)	30 patients	Knee 6 and 12 months	Col scaffolds derived from bovine pericardium were used for the MACI procedure. IKDC and MOCART scores were used to evaluate the results.	The IKDC scores increased from 24 (pre-operative) to 44 and 66 after 6 months and 1 year post-operation, respectively. The MOCART score was improved from 11.5 (6 months post-operative) to 13 (1 year post-operative). The morphological abnormal cells were related with poor clinical outcome. Defect aetiology and quality of implanted cells were critical factors for good clinical outcome.
Bedi <i>et al.</i> [30] (B)	Multilayered scaffold contains PLGA, PGA, and calcium sulphate (Trufit BGS® plug)	26 patients with mean age 28.72 years; 25 knee defects, 5 ankle defects.	Knee 6-39 months	The patients underwent OC autologous transplantation for chondral defects. All donor sites were filled with the plugs. Cartilage-sensitive MRI studies and T2-mapping MRI studies were performed postoperatively.	The plug demonstrated flush morphology at early follow-up (≤6 months) and at longer follow-up (≥16 months), but with deteriorated appearance at intermediate follow-up (~12 months). T2 relaxation times of the plug approached those of normal articular cartilage with longer postoperative duration.
Giza <i>et al.</i> [34]	Col I/III bilayered membrane with autologous chondrocyte (MACI)	10 patients with average age of 40.2 years (ranged from 25 to 59).	Ankle 1 and 2 years	The size and location of the defects were analyzed by arthroscopy, and cartilage was harvested from the border of the lesion. Expanded chondrocytes were seeded into the Col membrane. The joint was exposed with a small anterolateral or anteromedial. The graft was cut and placed to the defect on top of a layer of fibrin sealant.	The AOFAS hindfoot scores increased from 61.2 (preoperative, ranged from 42-76) to 74.7 (1 year postoperative, ranged from 46-87) and 73.3 (2 year postoperative, ranged from 42-90). After 19 months postoperation, MRI images showed the regeneration of articular cartilage and subchondral bone.

Table 1. Continued (1)

Ref	Scaffold/ cell	Patient information	Defect site/follow up time	Method	Outcome
Kon <i>et al.</i> [22] (B)	Multilayered nano-composite scaffold containing Col and Col/HA scaffold (MaioRegen®)	13 patients (15 defects) with mean age of 36.7 years (ranged from 27-51 years).	Knee 6 months	The lesions were templated and the templates were used to prepare the grafts. The grafts were implanted using a press-fit method.	4-5 weeks post-operative observation showed 13 of the 15 lesions were of completely attached graft and repair tissue. Complete filling of the cartilage defect and congruency of the articular surface were seen in 10 defects with MRI observation 6 months post-operative. Oedema or sclerosis in subchondral bone was presented in 8 defects. Histological analysis showed the formation of subchondral bone without the presence of materials.
Aurich <i>et al.</i> [35]	Porcine Col I scaffold with autologous chondrocytes (MACI)	18 patients (19 defects) with average age of 29.2±10.9 years.	Ankle Mean follow up 24.5 month	Arthroscopy was used for the evaluation and debridement on the defects, as well as the harvest of cartilage. Cultured chondrocytes were seeded into the Col membrane and implanted in the defects, with fibrin as the glue. MOCART score, the pain and disability module of the FFI, AOFAS score, and the Core Scale of the Foot and Ankle Module of AAOS Lower Limb Outcomes Assessment Instruments were used.	FFI pain before MACI: 5.5 ± 2.0, after MACI: 28 ± 2.2. FFI disability before MACI: 5.0 ± 2.3, after MACI: 2.6 ± 2.2. AOFAS before MACI: 58.6 ± 16.1, after MACI: 80.4 ± 14.1. AAOS standardized mean before MACI: 59.9 ± 16.0, after MACI: 83.5 ± 13.2. According to AOFAS hindfoot score, 64% were rated as excellent and good, whereas 36% were rated fair and poor. The results correlated with the age of the patient and the duration of symptoms, but not with the size of the lesion. Mean MOCART score was 62.4 ± 15.8 points. There was no relation between MOCART score and clinical outcome.
Barber <i>et al.</i> [32] (B)	Trufit BGS® plug	9 patients	Knee 2-63 months	Patients were underwent autologous OC transplantation. The donor sites were filled by Trufit®. And the repair results were evaluated by micro-CT scan.	After the operation, the CT scan showed decrease in the House Units from 84 (4 months) to 19 (13 months) in the donor site. The ossification quality score of the implants was 1 (soft-tissue density) instead of 4 (cancellous bone). The implants showed no evidence of bone ingrowth, osteoconductivity, or ossification. The density of the donor sites declined over time to that of fibrous scar.
Kon <i>et al.</i> [31] (B)	MaioRegen®	30 patients with mean age of 29.3 years. Lesion size ranged from 1.5 to 6.0 cm ² .	Knee 6, 12, and 24 months	The scaffolds were implanted in the knee chondral or OC lesions. The outcomes were evaluated by the IKDC, Tegner scores and MOCART.	The Tegner, IKDC, and subjective scores significantly enhanced from the baseline evaluation to the 6, 12, and 24 months follow-ups. The MRI results demonstrated that complete filling of the cartilage and complete integration of the graft was observed in 70% of the lesions.
Macmull <i>et al.</i> [36]	Type I/III Col membrane with autologous chondrocyte (MACI)	7 patients underwent MACI on osteochondral lesions, with ages between 14-18 years	Knee 1 year	Autologous chondrocytes were seeded in the Col scaffold, and then implanted into the defects with fibrin glue over the defect. Visual analog scale score for pain, Bentley Functional Rating Score, and the Modified Cincinnati Rating System were used to evaluate the patients preoperatively and postoperatively.	Pain reduction and significant improvement in function were observed after the MACI.

Table 1. Continued (2)

Ref	Scaffold/ cell	Patient information	Defect site/follow up time	Method	Outcome
Macmull <i>et al.</i> [36]	Type I/III Col membrane with autologous chondrocyte (MACI)	7 patients underwent MACI on osteochondral lesions, with ages between 14-18 years	Knee 1 year	Autologous chondrocytes were seeded in the Col scaffold, and then implanted into the defects with fibrin glue over the defect. Visual analog scale score for pain, Bentley Functional Rating Score, and the Modified Cincinnati Rating System were used to evaluate the patients preoperatively and postoperatively.	Pain reduction and significant improvement in function were observed after the MACI.
Dhollander <i>et al.</i> [33] (B)	Trufit® plug.	20 patients with mean age of 31.6 (17-53 years) years.	Knee 6 and 12 months	The plugs were implanted into the defects by tamping down with a punch until the surface of the plug was continuous with the adjacent tissue.	The short-term clinical and MRI results were modest. No deterioration of the repaired tissue was observed. 3 of the 15 patients failed and had to undergo autologous OC transplantation.
Joshi <i>et al.</i> [21] (B)	Trufit®	10 patients with mean age of 33.3years (16-49 years).	Patella 6 to 24 months	Plugs were implanted into the OC patella defects. SF-36, KOOS, and visual analog scale results were obtained preoperatively and postoperatively.	After 1 year follow-up, the results were satisfactory in 80% patients. At 18 months follow up, 9 patients suffered pain and knee swelling. Reoperation rate for implant failure reached 70%. MRI screened at final follow up showed a cylindrical cavity of fibrous tissue instead of subchondral bone ingrowth.
Kon <i>et al.</i> [28] (B)	MaioRegen® and HYAFF® -11	MaioRegen®: 8 patients, mean age 27.5 ± 6.4. HYAFF® -11: 7 patients, mean age 25.4 ± 12.6	Knee 2-3 years	For MaioRegen® group, the multilayered scaffolds were implanted into the defects. For HYAFF® -11, the bone marrow was concentrated and seeded into the hyaluronan for implant in the defects. The IKDC score, the EuroQol visual analog scale, radiographs and MRI were used for the outcome evaluation.	When MaioRegen® was used, the median IKDC score significantly improved from around 40 (preoperatively) to around 80 (postoperatively). And the Tegner score also increased significantly after the implantation. In case of HYAFF® -11, IKDC and Tegner scores were also significantly improved.
Chiang <i>et al.</i> [37] (B)	Bilayered PLGA/PLGA-TCP scaffolds with a chamber between the two layers	10 patients (6 male), mean age 27.6 years.	5 located on lateral condyle and 5 in medial condyle. 3, 6, 12, and 24 months.	Autologous cartilage was harvested and minced once, then digested by collagenase for 20 minutes. The dissociated tissue was transferred with a syringe to the chamber in the biphasic implant. OCD of 8.5 mm in diameter and 8.5 mm in depth were created and then implanted the.	No patient experienced serious adverse events. Cancellous bone formed in the osseous phase without pre-seeding of cells. Postoperative mean KOOS in "symptoms" subscale had not changed significantly from pre-operation until 24 months. Whereas those in the other four subscales (Pain, activities of daily living, sports and recreational activities, quality of life) were significantly higher than pre-operation at 12 and 24 months. Arthroscopy showed the grafted sites were completely filled, and regenerated cartilaginous surfaces flushed with surrounding native joint surface. The regenerated cartilage appeared hyaline.

(B)=layered scaffolds/hydrogels; 3D=three dimensional; AAOS=the American Academy of Orthopaedic Surgeons; AOFAS=the American Orthopaedic Foot and Ankle Society; BMSCs=bone marrow mesenchymal stromal cells; Col=collagen; FFI=the Foot Function Index; HA=hydroxyapatite; IKDC=International Knee Documentation Committee; KOOS=Knee injury and Osteoarthritis Outcome Score; MACI=matrix-induced autologous chondrocyte implantation; Micro-CT=micro-computed tomography; MOCART=Magnetic Resonance Observation of Cartilage Repair Tissue; MRI=magnetic resonance imaging; OC=osteochondral; OCD=osteochondral defect(s); PGA=poly(glycolic acid); PLGA=poly(lactic-co-glycolic acid); Ref=reference; SF-36=patient outcome scores; TCP=tricalcium phosphate.

Trufit® CB plug is a cylindrical porous scaffolds containing poly(lactic-co-glycolic acid), poly(glycolic acid), and calcium phosphate. It is a press-fit implant, with tunable length and diameter ranges from 5 to 11 mm [30]. MaioRegen® is a biomimetic three layer scaffold (Figure 1). The composition from the top layer to the bottom layer resembles the contents of collagen and hydroxyapatite in the cartilage, tidemark and subchondral bone [31]. Chondromimetic® is bilayered porous implant contains collagen, glycosaminoglycan, and calcium phosphate. There are no clinical reports on this product yet.

Table 1 summarized the clinical studies on OCD regeneration. In two provided studies, Trufit® was used to fill the donor void after autologous OCD transplantation [30, 32]. It was found that TruFit® did not support bone ingrowth during the follow [32]. When Trufit® was implanted in the knee OCD, the short term clinical outcome was modest [33].

In one study on patella, Trufit® was not able to support subchondral bone ingrowth [21]. All the 3 studies of MaioRegen® presented satisfying clinical outcomes [22, 28, 31]. Kon *et al.* [31] reported the use of MaioRegen® for the treatment of knee lesions (30 patients). The Tegner and International Knee Documentation Committee (IKDC) scores improved significantly after 2 years' follow up. Cartilage defects were completely filled and the integration with the graft was observed in 70% lesions. Kon *et al.* [28] also compared different approaches for the treatment of knee OCD. Results demonstrated that MaioRegen® was able to achieve satisfactory clinical and radiographic outcomes. The different clinical outcomes of the layered scaffolds may be related to the type of injury, site of lesion, and the properties of the biomaterials. More evidences and further comparative studies are required to understand and relate all these aspects.

Overall, tissue engineering approaches have already shown its charm in clinical OCD regeneration. In the future, the development of layered biomimetic scaffolds is still important for the clinical application of tissue engineering in OC regeneration. In order to provide a satisfactory environment for the fast formation of specific tissues, the components in the chondral and the subchondral layers of the scaffold should resemble the ones in the counterpart of the OC tissue. Another crucial issue is related with the mechanical stability and degradation profile of the scaffold *in vivo*. The scaffold should be capable to maintain its structure integrity when implanted and presents a degradation profile matching the growth pace of the *de novo* tissues. It is worthy to develop layered

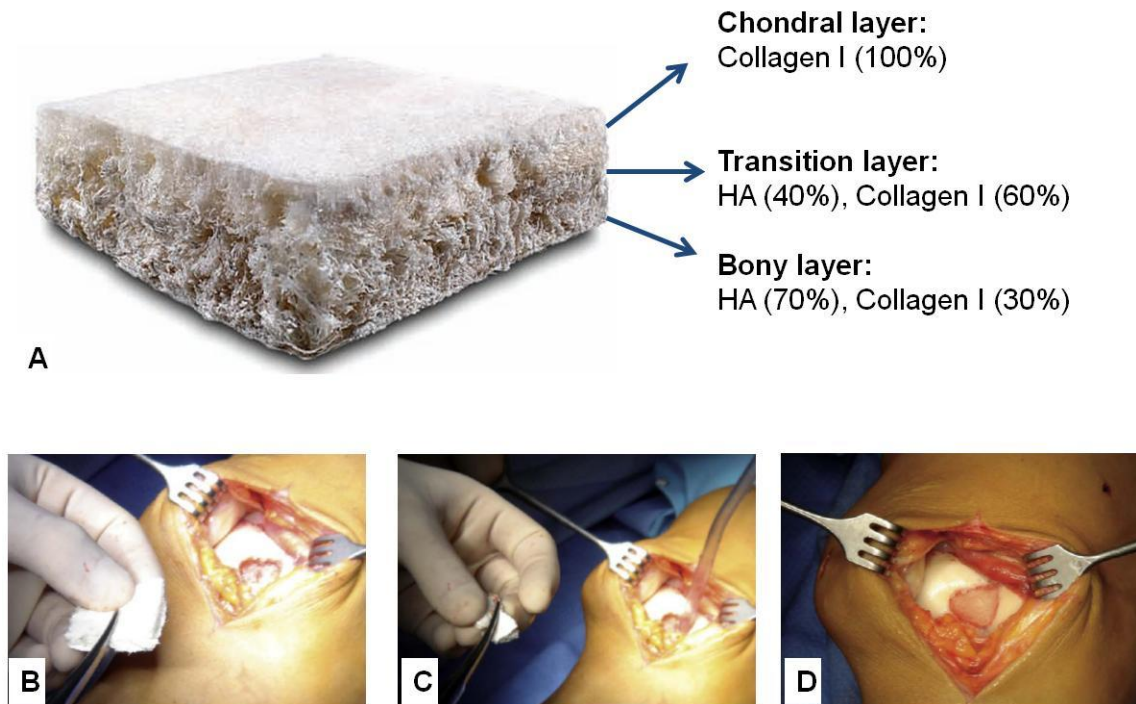


Figure 1. Biomimetic osteochondral scaffold for clinical application (MaioRegen®). (A) Scaffolds morphology and components. (B-D) Images showing the surgical procedure: (B) cutting the scaffold, (C) the scaffold is templated using an aluminium foil to obtain the exact size of the graft needed, (D) implantation of the scaffold using a press-fit technique. Adapted from [31] and [22], with permissions from SAGE and Elsevier, respectively.

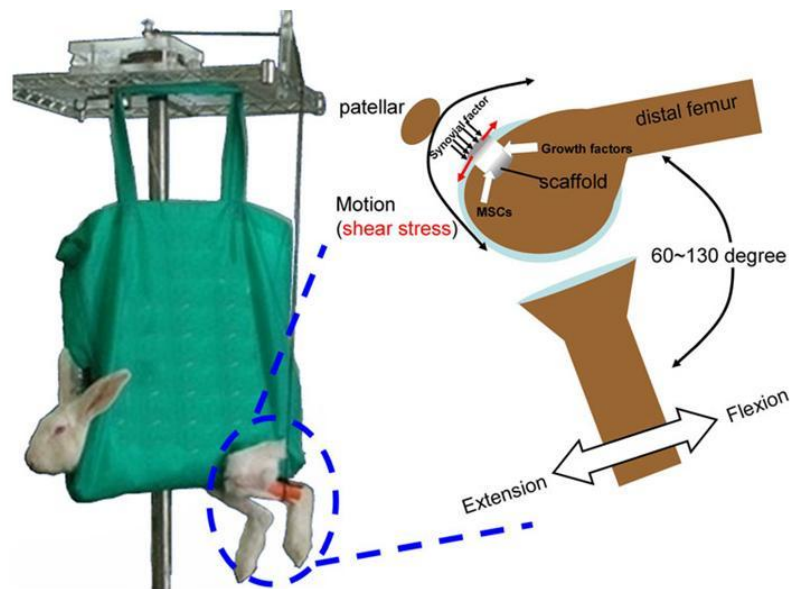


Figure 2. Illustration of continuous positive motion treatment. Adapted from [128], with permission from Springer.

scaffolds incorporating with bioactive agents or introduce stem cells for OCD treatments. Before transforming to the clinics, there are still many aspects should be figured out, such as optimization of the incorporation dose and release profile of the bioactive factors, and harness the fate of stem cells *in vivo* and guiding them towards OC differentiation is still challenging.

2.2 *In vitro* Studies on OC tissue engineering

Biomaterials, bioactive agents, and cells are the three key factors for tissue engineering strategy. A lot of biomaterials have been explored for OCD regeneration *in vitro*. These materials include synthetic or natural polymers, bioceramics, and composites of these materials. The biological performances of these materials were investigated by seeding with cells after processing into different forms and structures, such as porous scaffolds and hydrogels, single layer or bilayered structure (Table 2 and Table 3).

Biomimetic strategies have been introduced to produce scaffolds displaying the adequate chemical cues and/or structure cues for OC tissue [29]. OC tissue is a stratified tissue composed of collagen II and glycosaminoglycan (GAG) in the cartilage layer of hydrogel form, as well as hydroxyapatite and collagen I in the highly porous subchondral bone layer [29]. Based on this, layered constructs which presented a similar microenvironment to the corresponding layer in the OC tissue were studied intensively. Varied combinations have been presented in the top layer and bottom layer of the bilayered structure, such as integrated porous scaffolds or hydrogels or electrospun fiber meshes, hydrogel and porous scaffolds, cell pellet and porous scaffolds, or above systems incorporated with bioactive reagents (Table 2 and Table 3).

Mahmoudifar *et al.* [38] seeded osteoblasts and chondrocytes into two porous poly(glycolic acid) scaffolds and cultured the sutured scaffolds in a bioreactor. They found that only the cartilage layer contains glycosaminoglycan (GAG) and only the bone layer was mineralized. Oliveira *et al.* [29] developed a well-integrated porous hydroxyapatite (HA) and chitosan bilayered scaffold. Osteogenic and chondrogenic differentiation of goat BMSCs were performed on the HA and chitosan layers, respectively. Results showed that the scaffolds supported the growth and differentiations of BMSCs. Human BMSCs (hBMSCs) were seeded into different porous silk scaffolds

Table 2. *In vitro* studies on OC tissue engineering using layered scaffolds/hydrogels

Ref	Scaffold(s) # Cartilage part & Interface part * Bone part	Cell type(s)	Method	Outcome
Cao <i>et al.</i> [39]	# PCL scaffold *PCL scaffold	Human BMSCs and chondrocytes	FDM-fabricated PCL scaffold was partitioned into two halves. BMSCs were seeded in one half. 18 days later, chondrocytes were seeded in another half, and then co-cultured the two compartments.	Both kinds of cells proliferated, and integrated at the interface. Dense and mineralized ECM deposited in the bone compartment, while rich ECM of smooth surface appeared in the cartilage compartment.
Hung <i>et al.</i> [40]	# Agarose * Bovine trabecular bone	Bovine chondrocytes	1. The trabecular bone was penetrated by agarose with chondrocytes encapsulation to form a cylinder bilayered construct, and then the constructs were cultured for 6 weeks. 2. chondrocyte-seeded agarose constructs with human patellar articular shape were integrated into anatomically shaped trabecular bone substrate, and then cultured for 2 weeks.	Chondrocytes remained viable over the experiment time period. Agarose maintained its shape and still firmly integrated with the bony substrate. These constructs presented positive Col type II staining, as well as enhanced GAG content and mechanical properties.
Tuli <i>et al.</i> [41]	# BMSC pellet * PLA	Human BMSCs	BMSCs pellet press-coated on top of PLA scaffold, and maintained in chondrogenic medium for 2 or 5 weeks. Then, osteogenic BMSCs were seeded in the PLA scaffolds. The osteochondral construct was then cultured in cocktail medium.	The construct consisted of cartilage-like layer adherent to a bone-like component. The results revealed the interface between the two layers resembled the native OC junction. All parameters, such as mechanical properties improved with increased culture time.
Lu <i>et al.</i> [42] (I)	# Agarose & Gel within the bony part * PLGA/Bioactive glass microsphere	Bovine chondrocytes and osteoblasts	Chondrocytes were loaded into agarose and cast in a mold. The sintered microsphere scaffold was added to the chondrocyte-agarose suspension prior to setting. Osteoblasts were subsequently seeded onto the microsphere scaffold. The formed osteochondral constructs were co-cultured <i>in vitro</i> .	The agarose gel layer penetrated into the PLGA/bioactive glass layer, the integrity of the bilayered construct was maintained. Chondrocytes and osteoblasts remained viable over time. Chondrocytes maintained the spherical morphology and migrated only to the interface region. GAG-rich matrix deposited in the gel and interface region, and mineralized matrix was observed in the microsphere layer and the interface domain.
Mahmoudifar <i>et al.</i> [38]	# PGA * PGA	Human osteoblasts and chondrocytes	Two PGA scaffolds were seeded with osteoblasts and chondrocytes, respectively. Afterwards, the two constructs were sutured together and cocultured in a bioreactor. Human cartilage fragments or bone pieces were placed between the two layers forming sandwich construct and cultured in bioreactor as control.	The osteochondral construct presented good integration between each layer. Only the cartilage layer contained GAG and only the bone layer was mineralized. The GAG and Col contents in the cartilage part of the construct were higher than the ones of the control cartilage culture.
Malafaya <i>et al.</i> [43]	# Chitosan particles * Chitosan/HA particles	L929 cells and ASCs	Aggregated chitosan/HA particles and the chitosan particles were used to form the bony and chondral layers, respectively. And then, these two layers were combined by fibrin glue or chitosan glue. The osteogenesis and chondrogenesis differentiation of ASCs was screened using chitosan scaffold. L929 cells were used to evaluate the cytotoxicity of the bilayered chitosan/HA scaffold by using the scaffold extractions.	Extensive characterization of the bilayered scaffold was presented. Cytotoxicity tests showed that the chitosan based scaffolds were non-cytotoxic. And no mineralization was observed on chitosan layer during the bioactivity test. Chitosan scaffold was able to support osteogenesis and chondrogenesis of ASCs under osteogenic and chondrogenic conditions, respectively.

Table 2. Continued (1)

Ref	Scaffold(s) # Cartilage part & Interface part * Bone part	Cell type(s)	Method	Outcome
Oliveira <i>et al.</i> [29]	# Chitosan * HA scaffold	Goat BMSCs	Chitosan solution was transferred into the porous HA scaffold to generate a bilayered construct. BMSCs were separately seeded into the non-integrated chitosan or HA layer. Chondrogenesis and osteogenesis studies were performed for the chitosan and HA layers, respectively.	HA layer and chitosan layered in the bilayered scaffolds integrated very well, and they supported the growth and differentiation of BMSCs into osteoblasts and chondrocytes, respectively. The ALP content was increased during the 2 weeks <i>in vitro</i> study.
Augst <i>et al.</i> [44]	# Silk scaffold * Silk scaffold	Human BMSCs	BMSCs were seeded into silk scaffolds and cultured in a rotating bioreactor, with either chondrogenic or osteogenic medium for 3 weeks. Afterwards, the cartilage and bone constructs were sutured together and cultured for another 3 weeks in three kinds of medium: chondrogenic, osteogenic, and normal medium.	BMSCs cultured on silk scaffold in bioreactors presented well-mineralized regions and substantially less cartilage regions, indicating BMSCs had higher capacity for producing engineered bone than engineered cartilage. Chondrogenic factors had significant influence in the integration of the two compartments.
Guo <i>et al.</i> [75] (G)	# OPF hydrogel and TGF- β 1 * OPF hydrogel	Rabbit BMSCs	OPF hydrogel was used to encapsulate BMSCs and form bilayer construct. The cartilage layer contained TGF- β 1 loaded gelatin microparticles and BMSCs, the bony layer contained BMSCs or osteogenically precultured BMSCs. The bilayered constructs were cultured in chondrogenic medium supplemented with β -GP.	Chondrogenesis was observed in the cartilage layer, especially in the presence of TGF- β 1. In the bone layer, osteoblastic phenotype was maintained. Calcium deposition in the bone layer was limited, but this layer promoted chondrogenic differentiation of BMSCs in the cartilage layer.
Malafaya <i>et al.</i> [46]	# Chitosan particles * Chitosan/HA particles	L929 cells	The chitosan/HA particles were prepared by sintered or non-sintered HA particles. Both the chitosan and chitosan/HA particles were cross-linked by glutaraldehyde, and then aggregated in a mold to form the bilayered scaffolds. The cytotoxicity of the scaffolds was tested by culturing the L929 cells with the extraction of the scaffolds.	The scaffolds containing non-sintered HA particles showed cytotoxicity while the scaffolds with sintered HA particles was non-cytotoxic. The scaffolds were mechanically stable in dry and in wet/dynamic state.
Dormer <i>et al.</i> [47] (G)	# PLGA/TGF- β 1 & Gradient transition * PLGA/BMP-2	Human BMSCs and UCMSCs	Microsphere based PLGA scaffolds were produced with opposing gradient of BMP-2 and TGF- β 1. BMSCs or UCMSCs were seeded into these scaffolds and cultured with defined medium for osteochondral differentiation.	Human BMSCs response to the gradient design was well defined within their gene expression, but there was no significant difference compared to UCMSCs in terms of the biochemical performance. The gradient scaffolds produced regionalized ECM, and was superior as compared to the blank control scaffolds in cell number, GAG production, Col content, and ALP activity.
Grayson <i>et al.</i> [48]	# Agarose * Trabecular bone	Human BMSCs	Undifferentiated or pre-differentiated BMSCs were encapsulated or seeded into the agarose and trabecular bone, respectively. The trabecular bone was overlaid with the agarose to generate the biphasic construct. The biphasic constructs were then cultured under chondrogenic or cocktail medium, in static condition or in a bioreactor.	Predifferentiated of BMSCs before seeding in the scaffold/gel only favored bone formation. The perfusion condition and cocktail medium inhibited chondrogenesis of BMSCs. Perfusion improved the integration of the bone-cartilage interface.

Table 2. Continued (2)

Ref	Scaffold(s) # Cartilage part & Interface part * Bone part	Cell type(s)	Method	Outcome
Guo <i>et al.</i> [45] (G)	# OPF/TGF- β 3 * OPF hydrogel	Rabbit BMSCs	Osteogenic differentiated BMSCs were mixed with OPF solution to form the osteogenic layer. Then a mixture of chondrogenic differentiated BMSCs, OPF solution, and TGF- β 3 was injected on top of the osteogenic layer. The formed osteochondral construct was cultured under chondrogenic condition.	In the chondrogenic domain, the GF induced chondrogenic differentiation of BMSCs. Osteogenesis differentiated cells, along with GF, improved the chondrogenic gene expression of the BMSCs. In the osteogenic part, cells maintained ALP activity during coculture, while mineralization was delayed at the presence of GF.
Ho <i>et al.</i> [49]	# PCL/fibrin * PCL/TCP/fibrin	Human BMSCs	PCL and PCL/TCP scaffolds were produced by rapid prototyping. BMSCs loaded fibrin or fibrin/alginate solution was seeded into the PCL based scaffolds. After <i>in vitro</i> chondrogenesis and osteogenesis differentiation, the biphasic construct were integrated by fibrin gel and then co-cultured.	Fibrin promoted the chondrogenic differentiation of BMSCs, while fibrin/alginate declined the expression of Col II and aggrecan gene expression. Mineralized tissue formed in the bone phase of the biphasic construct. Mineralized boundary was observed in the interface of the construct.
Jiang <i>et al.</i> [50] (I)	# Agarose & Gel within the bony part * PLGA/Bioactive glass	Bovine chondrocyte and osteoblast	Chondrocytes were loaded into agarose and cast in a mold. The sintered PLGA or PLGA/bioactive glass microsphere scaffold was added to the chondrocyte-agarose suspension prior to setting. Osteoblasts were subsequently seeded onto the microsphere scaffold. The formed osteochondral constructs were co-cultured <i>in vitro</i> .	The co-culture of chondrocytes and osteoblasts resulted in three distinct yet continuous regions of cartilage, calcified cartilage and bone-like matrices. The PLGA-Bioactive glass phase facilitated the formation of a calcified interface. Higher chondrocytes density led to improved graft mechanical property over time. The PLGA/bioactive glass layer induced chondrocyte mineralization around the interface region and favored the formation of calcified interface.
Scotti <i>et al.</i> [51]	# Col * Devitalized bone	Human chondrocytes	Chondrocyte loaded Col (Chondro-Gide®) construct was combined with the devitalized bone (Tubobone®) by fibrin gel (Tisseel®), after being pre-cultured for 3 or 14 days in chondrogenic medium. Afterwards, the biphasic construct was co-cultured in chondrogenic medium for 5 weeks <i>in vitro</i> .	Pre-culture of the chondral layer for 3 days prior to the generation of the bilayered construct resulted more efficient cartilaginous matrix formation than that of the no pre-culture, also induced superior bonding to the bony part than that of the 14 days of pre-culture. The bony part scaffold induced the cells to secrete osteoblast-related gene-bone sialoprotein.
Cheng <i>et al.</i> [23] (I)	# Col microspheres & Col gel with undifferentiated BMSCs * Col microspheres	Rabbit BMSCs	The BMSCs were loaded into the Col solution and then the cell laden Col microspheres were generated. Afterwards, chondrogenesis and osteogenesis differentiation were performed <i>in vitro</i> , respectively. Following, the chondral and osteogenic layers were combined by Col gel containing undifferentiated BMSCs. Then, the OC constructs were co-cultured in chondrogenic, osteogenic, and normal medium, respectively.	When co-culture was performed in the chondrogenic medium, an intact and continuous calcified cartilage zone was formed separating the upper chondrogenic layer and the underlying osteogenic layer. Cells at the interface region presented hypertrophic phenotype, with Col type II and X, calcium mineral and vertically oriented fibers in the ECM. In the case of osteogenic medium, the upper layer chondrogenic tissue became calcified. In the normal medium, undifferentiated BMSCs were found in the interface, and the pre-differentiated BMSCs were able to maintain their chondrogenic and osteogenic phenotypes.

Table 2. Continued (3)

Ref	Scaffold(s) # Cartilage part & Interface part * Bone part	Cell type(s)	Method	Outcome
Erisken <i>et al.</i> [52] (G)	# Electrospun PCL/insulin * Electrospun PCL/β-GP	Human ASCs	Insulin/PCL/β-GP scaffolds were produced by twin-screw extrusion/electrospinning technology, with opposite gradient concentration of insulin and β-GP. ASCs were seeded onto each side of the PCL mesh and cultured for 1, 4 and 8 weeks.	Chondrogenic differentiation of the stem cells increased at insulin-rich part and mineralization matrix increased at β-GP rich domain.
Wang <i>et al.</i> [53] (I)	# PLLA scaffold & One layer of Undifferentiated UCMSCs * PLLA scaffold	Human UCMSCs	The UCMSCs were seeded into the PLLA scaffolds, and undergone chondrogenic and osteogenic differentiations <i>in vitro</i> , respectively. And then, the two constructs were sutured with one layer of undifferentiated UCMSCs in the interface. The OC constructs were co-cultured in a composite medium <i>in vitro</i> for 3 weeks.	The chondrogenesis and osteogenesis of UCMSCs were confirmed by the expression of type II Col and runt-related transcription factor 2 genes, respectively. Increased ECM secretion was observed during the co-culture. Better integration and transition of the OC constructs were only presented in the group with one layer undifferentiated cells in the interface as compared to the control.
Zhou <i>et al.</i> [54]	# Col & Gradient Col/HA * Col/HA	Human BMSCs	Chondrogenesis and osteogenesis differentiation of the BMSCs were performed after seeding the cells in the Col and Col/HA scaffolds.	The Col layer was more efficient in inducing BMSCs chondrogenesis as compared to Col/HA layer. While the latter possessed the superiority on promoting hMSCs osteogenesis over Col layer or pure HA tablet.
Rodrigues <i>et al.</i> [55]	# Agarose * SPCL scaffold	Human AFSCs	Chondrogenesis and osteogenesis differentiation were performed by seeding or incorporating the AFSCs in the agarose and SPCL, respectively. Afterwards, two compartments were combined by using agarose solution. Then, the OC constructs were co-cultured in an OC defined medium.	Predifferentiated AFSCs seeded into SPCL scaffolds did not need OC medium to maintain the phenotype and they secreted abundant mineralized ECM for up to 2 weeks. While pre-chondrogenic differentiated AFSCs still required further OC medium to maintain their phenotype, but not IGF-1.
Bian <i>et al.</i> [56]	# Col * β-TCP	Rabbit BMSCs	At first, the histological analysis in the transitional structure of human OC tissue was performed. And then the acquired data were used to design the biomimetic biphasic scaffold. The bone and transitional phases were fabricated by β-TCP, and cartilage layer was formed by casting the collagen solution on top of the bone layer. Rabbit MSCs were cultured on the scaffolds.	The ceramic scaffolds were composed of a bone phase with the following properties: 700-900 um pore size, 200-500 um interconnected pore size, 50-60% porosity, fully interconnected, and 12 MPa compressive strength. The biomimetic transitional structure acted as a physical lock for cartilage phase and ceramic phase. Scaffold showed satisfactory cellular results.
Shim <i>et al.</i> [57]	# PCL/Alginate *PCL/Alginate	Human chondrocyte and osteoblasts	A multi-head tissue/organ building system was developed. Alginate hydrogels incorporated with chondrocyte and osteoblasts were infused into the chondral or subchondral layer of the PCL framework to form the 3D construct, respectively.	The line width and dispensing resolution of PCL and alginate hydrogels were readily adjustable. A variety of cells encapsulated in the alginate hydrogel could be accurately dispensed into the pores of a preformed PCL framework. The cells were viable up to 7 days after being dispensed.
Chen <i>et al.</i> [58]	# Silk * Silk	Rabbit BMSCs	BMSCs were seeded into the silk scaffold. Then chondrogenesis and osteogenesis were performed, respectively. After two weeks, the two differentiated constructs were combined using the peptide, and subsequently co-cultured for another two weeks.	A complete OC construct with cartilage-subchondral bone interface was regenerated with only one cell source. In the intermediate region, hypertrophic chondrogenic gene markers Collagen X and MMP-13 were found on both chondrogenic and osteogenic section edges after co-culture.

Table 2. Continued (4)

Ref	Scaffold(s) # Cartilage part & Interface part * Bone part	Cell type(s)	Method	Outcome
Galperin <i>et al.</i> [59]	# Modified PHEMA * PHEMA/HA	Human BMSCs	A PMMA sphere-templating technique was applied to fabricate an integrated bilayered scaffold. Subchondral layer was of 38 μ m pore size and its surface was coated with HA particle. Chondral layer was decorated with hyaluronan and of 200 μ m pore size. Human BMSCs and chondrogenic differentiated BMSCs were sequentially seeded in the bony and chondral layer, respectively. The constructs were co-cultured for 4 weeks in basal medium.	The integrated bilayered scaffold supported simultaneous matrix deposition and adequate cell growth of two distinct cell lineage in each layer during four weeks co-culture <i>in vitro</i> in the absence of soluble growth factor. The bony layer provided a suitable environment for hMSCs differentiated toward osteoblast, and the chondral layer retained the chondrocyte phenotype.
Mahmoudifar <i>et al.</i> [60]	# Non-woven PGAscaffold * Non-woven PGA scaffold	Human ASCs	Scaffolds seeded with ASCs, and then chondrogenic or osteogenic differentiation was performed for 1 week, respectively. Then, the two differentiated constructs were sutured and placed into a two chamber bioreactor. Afterwards, the osteogenic or chondrogenic differentiation was continued in each chamber for two weeks.	After two weeks, total collagen synthesis was 2.1-fold greater in the chondrogenesis induced sections compared with the osteogenesis induced sections. Differentiation markers for cartilage and bone were produced in both sections of the constructs, due to the diffusion and interchange of induction factors.
Nam <i>et al.</i> [61]	# Electrospun PCLscaffold * Electrospun PCL scaffold	Rat chondrocytes and osteoblasts	Electrospun PCL scaffolds were separately seeded with articular chondrocytes and osteoblasts, respectively. After culturing for 3 days, the two constructs were sutured and cultured under cyclic compressive mechanical stimulus for 2 weeks.	The dynamic mechanical stimulus induced the improved compressive modulus of the OC constructs. Also the BMP2, BMP6, and BMP7 were upregulated in the dynamic group. BMP3 was downregulated in a time- specific manner.
Yunos <i>et al.</i> [62]	# Electrospun PDLLA fibers * Bioglass® scaffolds with PDLLA coating	ATDC-5	The porous Bioglass® scaffolds were dipped in the PDLLA solution to prepare the PDLLA/Bogalss® porous scaffolds. Then the PDLLA fibers were electrospun onto the PDLLA/Bioglass® porous scaffolds to form the bilayered scaffolds. ATDC-5 cells were seeded onto the bilayered scaffolds.	The thickness of PDLLA layer increased by prolonging the processing time. ATDC5 cells attached, proliferated and migrated well in these scaffolds.

(G)=growth factors or bioactive reagents incorporated scaffolds/hydrogels; (I)=studies on regeneration of osteochondral interface; 3D=three dimensional; AFSCs=amniotic fluid-derived stem cells; ALP=alkaline phosphatase; ASCs=adipose tissue derived stromal cells; BMP=bone morphogenetic protein; BMSCs=bone marrow mesenchymal stem cells; Col=collagen; ECM=extracellular matrix; FDM=fused deposition modeling; GAG=glycosaminoglycan; GF=growth factor; HA=hydroxyapatite; OC=osteochondral; OPF=poly(ethylene glycol) fumarate hydrogel; PCL=poly(ϵ -caprolactone); PDLLA=poly(DL-lactide); PGA=poly(glycolic acid); PHEMA=poly(hydroxyethyl methacrylate) hydrogel; PLA=poly(lactic acid); PLGA=poly(glycolic-co-lactic acid); PLLA=poly(L-lactic acid); PMMA=poly(methyl methacrylate); Ref=reference; SPCL=starch-PCL composite; TCP=tricalcium phosphate; TGF- β =transforming growth factor β ; UCMSCs=umbilical cord-derived mesenchymal stem cells; β -GP=glycerol phosphate.

Table 3. *In vitro* studies on OC tissue engineering using non-layered scaffolds/hydrogels

Ref	Scaffold(s)	Cell type(s)	Method	Outcome
Allan <i>et al.</i> [63] (I)	CPP	Cow deep zone chondrocytes	Chondrocytes were seeded on top of the CPP scaffolds, and then cultured <i>in vitro</i> . At day 7, β -GP was supplemented in the medium, and the culture was continued period up to 8 weeks.	Cartilage tissue presented two zones, one calcified region adjacent to the CPP scaffold and a hyaline-like zone on the surface. Little or no mineral was observed in the absence of β -GP. The mineral was HA. The formed cartilage tissue possessed significant stiffness and interfacial shear properties compared with the control.
Hu <i>et al.</i> [64]	Porous nanofibrous PLLA scaffold	Human BMSCs	BMSCs were seeded into the 3D highly porous nanofibrous PLLA scaffolds, and then osteogenesis and chondrogenesis differentiation were performed, respectively.	Scaffold supported <i>in vitro</i> bone formation. Cells presented altered shape when culture in the nanofibrous scaffold compared with those on smooth films. And early chondrogenic commitment gene Sox-9 was enhanced in the nanofibrous scaffold.
Wang <i>et al.</i> [65] (G)	Alginate gel or silk fibroin scaffold	Human BMSCs	1. The rhBMP or rhIGF was encapsulated into PLGA or silk microparticles, and then these particles were incorporated in BMSCs loaded alginate gel with gradient distribution. The constructs were cultured in OC medium for 3 weeks. 2. The rhBMP and/or rhIGF encapsulated silk particles were gradually distributed in aqueous silk salt leached scaffold. BMSCs were seeded into these scaffolds and the constructs were cultured in OC medium for 5 weeks.	In the case of alginate gel, silk microspheres were more efficient in rhBMP-2 delivery, and less efficient in delivering rhIGF-1 compared with PLGA microspheres. The growth factor gradients induced non-gradient trends in hMSCs OC differentiation, due to shallow GF gradients. Regarding the silk scaffold, both growth factors formed deep and linear concentration gradients. The cells presented osteogenic and chondrogenic differentiation along the concentration gradients of rhBMP-2 or reverse gradient of rhBMP-2/rhIGF-1, but not the case of rhIGF-1 gradient system.
Abrahamsson <i>et al.</i> [66]	Woven PCL scaffold and Col gel	Human BMSCs	MSCs were loaded in type I Col gel, and then seeded into the PCL scaffold. Then, osteogenesis and/or chondrogenesis differentiation were performed, respectively.	In chondrogenic condition, cartilaginous tissue formed at day 21, and hypertrophic mineralization was observed in the interface by day 45. The formed cartilages like tissue presented comparable mechanical properties to the ones of the native cartilage.
Chen <i>et al.</i> [67] (I)	Silk scaffold	Rabbit chondrogenic BMSCs and osteoblasts	Chondrogenic BMSCs were cultured on the silk scaffold and osteoblasts were cultured in cell culture plates. Subsequently, the 3D constructs and the osteoblasts were co-cultured by contacting them together. Non-co-cultured samples were used as control.	In comparison with the control group, significant moderate downregulation of chondrogenic marker genes, such as Collagen II and Aggrecan, was observed. But the Sox-9 and Collagen I expression increased. And Only the chondrogenic BMSC layer in contact with the osteoblasts expressed OC interface markers, such as collagen X and MMP-13, which were not observed in control group.
Cui <i>et al.</i> [68]	Printed PEGDMA hydrogel	Human chondrocytes	PEGDMA incorporated with human chondrocytes were printed onto the OCD in rabbit OC plugs, and cultured <i>in vitro</i> .	Compressive modulus of printed PEGDMA was 395.73 ± 80.40 KPa, close to the range of native human articular cartilage. Viability of printed chondrocyte increased 26% in simultaneous polymerization than polymerized after printing. Printed cartilage attached firmly with surrounding tissue and greater amount of proteoglycan deposition was observed at the interface of the implants and the native cartilage confirmed by Safranin-O staining. The interface failure strength enhanced as time. With the OC plugs, PEGDMA presented elevated GAG content compared to that without OC plugs.

Table 3. Continued (1)

Ref	Scaffold(s)	Cell type(s)	Method	Outcome
Khanarian <i>et al.</i> [69] (I)	Agarose/HA	Bovine deep zone chondrocytes	Hypertrophic or non-hypertrophic chondrocytes were first mixed with micro or nano-sized HA particles, and then the mixture were combined with agarose.	Hypertrophic chondrocyte presented higher ECM and mineral deposition in the presence of HA. Higher compressive and shear mechanical properties were observed in the constructs as compared with the acellular ones. Cell hypertrophy was independent of ceramic size, while higher ECM deposition was only observed in the micro-sized particles group.
Khanarian <i>et al.</i> [70] (I)	Alginate/HA	Bovine deep Zone chondrocytes	Cellular alginate scaffolds with 1.5 wt/v% HA were prepared and cultured <i>in vitro</i> for 4 weeks.	The HA phase enhanced the formation of GAG and Col II when seeded with deep zone chondrocyte, also increased mechanical properties were observed as compared with non-mineral control. Presence of HA also promoted hypertrophy of the chondrocyte, as well as Col X deposition.
McCanless <i>et al.</i> [71] (G)	Alginate and TCP composite	Rat BMSCs.	Alginate/TCP suspension was mixed with isolated human platelet releasate, and then the mixture was gelled in the culture plate. MSCs were culture on the surface of the gel.	Gene expression profiles indicated MSCs were toward an OC differentiation pathway, more accurate, to the immature nonhypertrophic chondrocyte phenotype.
St-Pierre <i>et al.</i> [72] (I)	CPP	Calf deep zone chondrocytes	One layer of CaP film was coated on the surface of CPP scaffold, and then chondrocytes were seeded on top of the scaffold. The constructs were cultured in non-mineralization or mineralization medium for 4 weeks.	The cartilaginous tissue formed on top of the CaP-coated CPP was comparable to that formed on uncoated CPP. The biphasic constructs presented a 3.3 fold increased interfacial shear strength as compared to the one of the control constructs cultured in the non-mineralization medium.
Elder <i>et al.</i> [73]	Chitosan-CaP microspheres sintered scaffolds	Human BMSCs	The dry scaffolds were immersed in a agarose mold with porcine BMSCs on top or at the bottom of the scaffolds. Then the constructs were cultured in chondrogenic medium for 4 weeks.	40% of the sparsely seeded human BMSCs attached and proliferated rapidly. One of the technique exhibited a layer of cartilaginous tissue partially covered the scaffolds surfaces.
Miyagi <i>et al.</i> [74]	TCP	Human BMSCs	BMSCs were cultured in a culture insert under chondrogenic medium for 3 weeks, during which β -TCP block was placed on the cell sheet at day 1 or at day 14. Then the constructs were placed on another cell sheet prepared one day before, and cultivated for 3 weeks.	The addition of β -TCP resulted in a combined OC like construct and comparable staining intensity by Alcian blue, while the expression levels of the aggrecan and type II collagen genes decreased a little.

(G)=growth factors or bioactive reagents incorporated scaffolds/hydrogels; (I)=studies on regeneration of osteochondral interface; 3D=three dimensional; BMSCs=bone marrow mesenchymal stem cells; CaP=calcium phosphate; Col=collagen; CPP=calcium polyphosphate; ECM=extracellular matrix; GAG=glycosaminoglycan; GF=growth factor; HA=hydroxyapatite; OC=osteochondral; OCD=osteochondral defect(s); PCL=poly(ϵ -caprolactone); PEGDAM=poly(ethylene glycol) dimethacrylate; PLGA=poly(glycolic-co-lactic acid); PLLA=poly(L-lactic acid); Ref=reference; rhBMP=recombinant human bone morphogenetic protein; rhIGF=recombinant human insulin-like growth factor; TCP=tricalcium phosphate; β -GP=glycerol phosphate.

and sutured after chondrogenic and osteogenic differentiation [44]. The result showed that hBMSCs had higher capacity for producing engineered bone than engineered cartilage.

Mimicking the microenvironment of OC tissue, hydrogels and porous scaffolds were combined to form bilayered scaffolds. In one study from Hung *et al.* [40], chondrocytes were incorporated with agarose and then integrated with trabecular bone to form the bilayered construct. Chondrocytes were viable after culturing for 6 weeks and the agarose still firmly integrated with the bony substrate. In a following study, undifferentiated or pre-differentiated human BMSCs were incorporated with agarose and trabecular bone bilayered scaffolds, followed by culturing in different conditions [48]. It was found that pre-differentiated BMSCs only favored bone formation and perfusion bioreactor was helpful to improve the integration of bone-cartilage interface.

Bioactive reagents, such as growth factors and some drugs, were incorporated into the bilayered structure for cells differentiation. Guo *et al.* [45, 75] incorporated TGF- β 1 or TGF- β 3 in poly(ethylene glycol) fumarate bilayered hydrogels (OPF), and successful chondrogenesis of BMSCs was achieved. Dormer *et al.* [47] produced gradient TGF- β 1 and BMP-2 incorporated scaffolds and found the human BMSCs responded well to the gradient design and regionalized extracellular matrix (ECM). Further developments on scaffolds/hydrogels loaded with clinical relevant doses of growth factors would be of great interest in the future.

Besides primary cells (osteoblasts and chondrocytes), increasing attention has been shifted to stem cells for OCD regeneration, such as BMSCs, ASCs, umbilical cord mesenchymal stromal cells (UCMSCs), and amniotic fluid-derived stem cells (AFSC). The differentiation of these stem cells in scaffolds incorporated with bioactive factors was studied intensively [45, 47, 52, 65, 71, 75]. Every kind of stem cells has their own advantage and disadvantage. BMSCs and ASCs are more abundant than other somatic stem cells. AFSC and UCMSCs are of higher proliferation and chondrogenic differentiation capacity compared to other adult stem cells. Embryonic stem cells (ESCs) are pluripotent and have been used for cartilage regeneration, but their application is still under ethical argument [76]. Recently, induced pluripotent stem cells (iPS), as an alternative to ESCs, have been investigated intensively [77]. Different to the ESCs, iPS can be created from

somatic cells and thus circumvent the ethical controversy of ESCs. The effectiveness of these cells for OCD regeneration needs to be further studied.

During OCD regeneration, most of the attention has been given to the generation of the integrated cartilage layer and the subchondral layer. Only recently, there is increased awareness that the regeneration of the interface between chondral and subchondral bone also plays an important role in OCD tissue engineering [23]. The OCD interface is a calcified cartilage layer locating between the hyaline cartilage and the subchondral bone [23, 69, 70]. The hypertrophic chondrocytes and extracellular matrix of collagen I, II, and X, are the unique properties of this layer. The OCD interface acts as a barrier minimizing the diffusions between cartilage layer and subchondral layer, and therefore prevents the invasion of vascular from bone. Khanarian *et al.* [69, 70] studied the influence of HA on the hypertrophy of chondrocytes by mixing deep zone chondrocytes into the agarose/HA or alginate/HA composite hydrogel. It was found that the addition of HA promoted the formation of GAG, collagen II, and collagen X, as well as facilitate the chondrocyte hypertrophy. Chondrocytes were also seeded into calcium polyphosphate scaffolds by Allan *et al.* [63], zonal cartilaginous tissue composed of hyaline-like cartilage and calcified cartilage was formed. Cheng *et al.* [23] produced a tri-layered collagen microsphere scaffold, which contained BMSCs of several differentiated status in specific layers. When co-cultured in chondrogenic medium, an intact and continuous calcified cartilage zone was formed. As these studies bring new insights in OCD regeneration, further work on controlling the thickness of the interface and improving the integrated strength of each layer should be considered.

2.3. *In vivo* studies on OC tissue engineering

The *in vivo* studies under tissue engineering category for OCD regeneration have been summarized in Table 4 to 6.

Compared with the simplified and optimized culture conditions for *in vitro* study, the *in vivo* study provides more complex conditions and thus closely mimics the real scenario of OCD. The influences of materials intrinsic properties and structure characteristics on OCD regeneration can be reflected by *in vivo* studies. By using this approach, we can select the most suitable component and the best structure for further study. Igarashi *et al.*

Table 4. *In vivo* studies on OC tissue engineering using bioactive agent(s) incorporated scaffolds/hydrogels or biologically derived scaffolds

Ref	Scaffold(s) # Cartilage part & Interface part * Bone part	Animal model/implantation time	Cell type(s)	Method	Outcome
Emans <i>et al.</i> [81]	Ectopically produced cartilage in periosteum	Rabbit, 6 months old, OCD in the medial condyle (3 mm in diameter and 2.05±0.39 mm in depth). 3 weeks and 3 months.	Cells from injured periosteum	Defects were made in the periosteum. Cartilage defects were also generated. After 14 days, the cartilage defects were extended to subchondral bone. The ectopically formed cartilages were implanted. Empty defects and hyaluronan filled defects served as controls.	Ectopic cartilage filled defects were repaired to the level of the surrounding cartilage after 3 weeks. Three months later, ectopic cartilage filled defects contained mixture of fibrocartilaginous and hyaline cartilage, with tidemarks in some of them. Subchondral bone repair was excellent.
Holland <i>et al.</i> [82] (B)	# OPF/TGF-β1 * OPF	Rabbit, 4 months old, OCD in the medial femoral condyle (3 mm in diameter and 3 mm in depth). 4 and 14 weeks.		The GF encapsulated gelatin microspheres were combined with the OPF hydrogel. Following, the bilayered hydrogels were prepared, and then implanted.	The chondral region was filled with hyaline cartilage whose quality improved over time. The subchondral region was filled with trabecular and compact bone, and the underlying subchondral bone was completely integrated with surrounding bone after 14 weeks. No bone upgrowth into the chondral region. TGF- β1 loading in the top layer appeared to exert some effect on cartilage quality in the defect area.
Huang <i>et al.</i> [83](2007)	PLLA/ACP scaffolds with bFGF or PLLA scaffolds with bFGF	Rabbit, 3.0 kg/each, OCD in the femoral condyles (4 mm in diameter and 5 mm in depth). 4 and 12 weeks.		The ACP/PLLA/bFGF and PLLA/bFGF composite scaffolds were implanted into the OCD in rabbits.	In PLLA/bFGF group, the mainly formed tissue was fibrocartilage and limited bone formation was observed. Only a little amount of Col II and no aggrecan genes were measured. In the case of ACP/PLLA/bFGF, most defects were filled with well-established cartilage tissue with large amount cartilaginous ECM, also positive Col II staining was observed. High level of Col II and aggrecan genes were detected.
Yagihashi <i>et al.</i> [84]	DDM from bovine	Rabbit, 13 weeks old, 2.5-3.0 kg/each, OCD in the patellar fossa (5mm in diameter and 10 mm in depth). 1, 3, 6 and 9 weeks.		Different amount of DDM powder (50 and 100 mg) were filled into the defects. Untreated defects used as control.	At 3 weeks, the 100 mg group had higher new bone formation compared with other groups, but the difference decreased with time. The 100-mg group showed better cartilage regeneration compared with the other groups, with hyaline cartilage in the peripheral area at 6 weeks and hyaline cartilage with similar thickness to the normal cartilage after 9 weeks.
Guo <i>et al.</i> [85]	OPF loaded with TGF-β1 incorporated gelatin microspheres	Rabbit, 6 months old, OCD in the femoral condyles (3 mm in diameter and 3 mm in depth). 12 weeks.	Rabbit BMSCs	OPF hydrogel/blank microspheres, OPF/BMSCs, and OPF/MSCs/microspheres loaded with GF were implanted.	In scaffold alone group, new formed chondral tissue presented hyaline cartilage with zonal organization and intensive GAG, while hypertrophic cartilage with some extent bone formation was observed. With MSCs in the scaffold, specifically with growth factor incorporated, subchondral bone formation was enhanced. But the incorporation of MSCs with or without growth factor did not improve cartilage morphology.

Table 4. Continued (1)

Ref	Scaffold(s) # Cartilage part & Interface part * Bone part	Animal model/implantation time	Cell type(s)	Method	Outcome
Maehara <i>et al.</i> [86]	* HA/Col scaffolds with bFGF	Rabbit, 2.7-3.5kg/each, OCD in trochlear groove (5 mm in diameter and 4 mm in depth). 6, 12, 24 weeks.		HA/Col scaffolds with two bFGF concentrations or PBS impregnation were implanted into the defects, with no implantation as control. The scaffolds were 3 mm in height and inserted 2 mm beneath the cartilage surface.	Large amount of bone formation was observed in the HA/Col group as compared to the no implants group. The lower amount BFGF group displayed the most abundant new bone formation, as well as satisfactory hyaline-like cartilage regeneration.
Sun <i>et al.</i> [87]	PLGA scaffolds with PRP	Rabbit, 2.8-3.2 kg/each, OCD in the patellar groove (5 mm in diameter and 4 mm in depth). 4 and 12 weeks.		PRP incorporated PLGA scaffolds, and PLGA scaffolds alone were implanted in the defects. Untreated defects served as control.	At 4 weeks, PLGA/RPR group presented a higher amount of GAG and chondrogenesis than the other groups. At 12 week, in the PLGA group, the defects were filled with fibrocartilage tissue with clear boundary. The PRP/PLGA group presented hyaline cartilage tissue integrated with host tissue and abundant new formed subchondral bone.
Chen <i>et al.</i> [88] (B)	# Chitosan/gelatin scaffolds loaded with plasmid TGF-1 gene * Chitosan/gelatin/HA scaffold loaded with plasmid BMP-2 gene.	Rabbit, 4 months old, OCD in the patellar groove (4 mm in diameter and 5 mm in depth). 4, 8 and 12 weeks.		The bilayered scaffolds were combined by fibrin gel. And then bilayered scaffolds with or without genes, mono layer scaffolds with BMP-2 gene or TGF gene were used for the implantation. Non-treated defects were used as control.	The <i>in vivo</i> studies showed that, the monolayer scaffolds with BMP-2 gene presented complete trabecular bone ingrowth within subchondral region and good integration with native bone tissue, but with abundant Col I in the cartilage part. While the monolayer scaffolds with TGF- β 1 gene showed similar cartilage surface with native cartilage, however the regeneration of subchondral bone was insufficient. The bilayered gene incorporated scaffolds showed successful reconstitution of cartilage and subchondral bone.
Jin <i>et al.</i> [25]	Chondrocyte derived ECM scaffolds	Rabbit, 3 months old, 3.0-3.5 kg, defects in the patella groove (5 mm in diameter and 3 mm in depth). 1 and 3 months.	Rabbit Chondrocytes	Scaffolds were generated by lyophilization of the ECM from <i>in vitro</i> culture of chondrocytes. Chondrocytes were seeded into the scaffolds and cultured for 2 day (Group 2), 2 weeks (Group 3), and 4 weeks (Group 4) <i>in vitro</i> before the implantation. The constructs were implanted. Untreated defects served as control (Group 1).	After 1 month, group 3 and 4 repaired with hyaline cartilage like tissue, while fibrocartilage tissues were observed in Group 1 and 2. After 3 months, group 4 presented striking features of hyaline cartilage, with a mature matrix and a columnar arrangement of chondrocytes and prominently zonal distribution of Col II. Subchondral bone was well restored.
Mohan <i>et al.</i> [89] (B)	# PLGA microspheres loaded with TGF- β 1 & Gradient transition of the two layers. * PLGA or PLGA/nano-HA microspheres loaded with BMP-2	Rabbit, 6 months old, OCD in the medial condyle (3.5 mm in diameter and 3 mm in depth). 6 and 12 weeks.		Blank PLGA scaffold, PLGA scaffold with gradient growth factors loading, PLGA and PLGA/HA gradient scaffold without growth factors, and PLGA and PLGA/HA with gradient growth factors loading scaffolds were used for the implantation.	The gross morphology, MRI, histology data showed that the greatest extent of regeneration of cartilage and subchondral bone were achieved by the PLGA and PLGA/HA scaffolds with gradient growth factors loading. This group presented similar GAG content and cartilage thickness to native cartilage, as well as higher bone filling and better edge integration with host bone compared to other groups.

Table 4. Continued (2)

Ref	Scaffold(s) # Cartilage part & Interface part * Bone part	Animal model/implantation time	Cell type(s)	Method	Outcome
Yang <i>et al.</i> [90] (B)	# DCM * DCBM	Canine, 2 years old and 20-25 kg/each, OCD in the bilateral femoral condyles (4.2 mm in diameter and 6 mm in depth). 3 and 6 months.	BMSCs	BMSCs were isolated and expanded under chondrogenesis conditions. And then the cells were seeded into the cartilage layer of the scaffolds before implanted. Scaffolds without cells used as control.	The macroscopic and histologic grading scores of the experimental group were always higher than those of the control group. The scores for experimental group showed higher values at 6 months than those of 3 months. The stiffness of the neocartilage and subchondral bone in experimental group were 70.77% and 74.95% of normal tissues, respectively. Regular subchondral bone formed at both time points, in the two groups.
Dormer <i>et al.</i> [91] (B)	# PLGA microspheres with TGF- β 1 & Gradient transition of the two layers. * PLGA microspheres loaded with BMP-2	Rabbit, 6 months old, OCD in the medial condyle (3 mm in diameter and 3 mm in depth). 6 and 12 weeks.	Rabbit UCMSCs	Blank PLGA scaffold, PLGA scaffold with gradient growth factors loading, UCMSCs seeded PLGA scaffolds with gradient growth factors were used for the implantation. Defects without any treatment acted as control.	After 12 weeks, gradient-only and gradient group with UCMSCs group presented almost identical appearance to the untreated group in cartilage regeneration. Untreated-group had complete filling in the defect with mineralization. The blank group had more new bone formation than the one from the 6 th week but inferior to untreated group. Gradient group and UCMSC seeded gradient group presented similar repair outcome but were inferior to the untreated group.
Jung <i>et al.</i> [92]	PLGA/TCP scaffolds	Rabbit, male, 4 months old, 3.0-3.5 kg/each, OCD in the femoral groove (2 mm in diameter and 3 mm in depth). 4 and 12 weeks.		PLGA/TCP scaffolds were immersed in the BMP-7 solution, and then implanted into the OCD of knee. Constructs without BMP-7 were used as control.	At 12 weeks, histological analysis revealed that neo-cartilage completely regenerated, and integrated well with surrounding normal cartilage and subchondral bone. Partial degradation of the PLGA during the repair period guided neo-cartilage formation. Adjacent BMP-7-untreated defects were also repaired with cartilage regeneration suggesting the effect of local BMP-7 release in the synovial fluid. Control only presented fibrous tissue filtration. Defects with BMP-7 exhibited an architecture characteristic of mature hyaline cartilage and trabecular bone, with some remodeling compact bone.
Marmotti <i>et al.</i> [93]	# Hyaluronicacid/PRP & Cartilage fragments loaded into fibrin glue * Hyaluronic acid/PRP	Rabbit, mature, 16 weeks old, OCD in the center of the trochlea of the right knee (4.5 mm in diameter and 4 mm in depth). 1, 3, 6 months.		For the implantation, 5 treatment groups were used: autologous cartilage fragments with fibrin glue were loaded onto Hyaluronic acid/PRP scaffolds (G1), without fibrin (G2), membrane along with fibrin (G3) or without fibrin (G4), empty defect (G 5).	At 6 months, cartilage fragment-loaded scaffolds induced significantly better repair tissue than the scaffold alone using histological scoring. G2 was superior to that in any of the control groups. Autologous cartilage fragment loaded hyaluronic acid/fibrin/PRP scaffold improved the repair process. Human fibrin hampered the rabbit healing process.

Table 4. Continued (3)

Ref	Scaffold(s) # Cartilage part & Interface part * Bone part	Animal model/implantation time	Cell type(s)	Method	Outcome
Reyes <i>et al.</i> [98]	# Lyophilized alginate & Lyophilized alginate with BMP-2 loaded PLGA microspheres * Gas formed PLGA scaffold	Rabbit, mature, 6 months old, 3-4 kg/each, OCD in medial femoral condyle (4.5 mm in diameter, 4 mm in depth). 12 weeks.	Rabbit allogenic chondrocytes and BMSCs	PLGA and BMP-2 incorporated alginate bilayered scaffold was prepared, and then implanted in the defect. Alginate layer (with or without cells) was placed on top of the bilayered scaffold.	After 6 weeks, cartilage-like tissue formed in BMP-2 (with or without cells) groups. With cells, repaired tissue showed higher histological scores. Similar observation was observed until 12 weeks. Combination of cells did not result additive or synergistic effect. After 6 weeks, the subchondral bone regeneration was completed. Equally efficient OCD repair was achieved with chondrocytes, BMSCs, and BMP-2 treatment.

(B)=layered scaffolds/hydrogels; ACP=amorphous calcium phosphate; ADSCs=adipose tissue derived stem cells; bFGF=basic fibroblast growth factor; BMP=bone morphogenetic protein; BMSCs=bone marrow mesenchymal stromal cells; Col=collagen; DCBM=decellularized cancellous bone matrix; DCM=decellularized cartilage matrix; DDM=demineralized dentin matrix; ECM=extracellular matrix; GAG=glycosaminoglycan; GF=growth factor(s); HA=hydroxyapatite; IGF=insulin-like growth factor; MRI=magnetic resonance imaging; OC=osteochondral; OCD=osteochondral defect(s); OPF=poly(ethylene glycol) fumarate hydrogel; PBS=phosphate buffered saline; PLGA=poly(lactic-co-glycolic acid); PLLA=poly(lactic acid); PRP=platelet-rich plasma; PU=polyurethane; Ref=reference; TCP=tricalcium phosphate; TGF- β =transforming growth factor β ; UCMSCs=umbilical cord mesenchymal stromal cells.

Table 5. *In vivo* studies on OC tissue engineering using non-layered scaffolds/hydrogels

Ref	Scaffold(s)	Animal model/implantation time	Cell type(s)	Method	Outcome
Case <i>et al.</i> [99]	PGA disk	Rabbit, 8-12 months old, OCD in the distal femoral metaphyses (6.3 mm in diameter and 6.3 mm in depth) 4 weeks.	Chondrocytes	Chondrocytes were seeded into the scaffolds and cultured for 4 weeks. And then the constructs were transferred into an empty bone chamber which previously implanted in the defects. <i>In vivo</i> mechanical loading on bone formation was tested. Scaffolds without cells served as control.	The bone volume fraction of the implants was nearly doubled of that in the control. The application of an intermittent cyclic mechanical load was found to increase the bone volume. Tissue-engineered cartilage constructs implanted into a bone defect would support directly appositional bone formation.
Guo <i>et al.</i> [100]	TCP scaffolds	Sheep, 10 months old, 34 ± 4.8 kg/each, OCD in the shoulders (10 mm in diameter and 4 mm in depth). 2 and 24 weeks.	Chondrocytes	Chondrocytes were harvested and seeded into the scaffolds. Constructs were implanted into the defects. Defects without scaffolds used as control.	After 24 weeks, the surface of the cartilage defects was completely regenerated. The TCP degradation was observed. Ceramic particles and macrophages were presented at the ceramic-tissue interface. But no macrophages in the neocartilage tissue.
Ito <i>et al.</i> [101]	Col sponge surrounded by PLLA mesh	Rabbit, 12 weeks old, 2.0 kg/each, OCD in the patellar groove (4 mm in diameter and in depth). 4 and 12 weeks.	Autologous chondrocytes	Autologous chondrocytes were seeded into the Col sponge/PLLA mesh composite and cultured for 2 weeks. The constructs were implanted in the defects. Defects treated with the plugs without cells as control.	At both time points, experimental group showed significant higher histological scores than those of control group. At 12 weeks, this group showed hyaline cartilage like tissue and well-organized subchondral bone formation. The control group only showed soft fibrous tissue at both time points.
Oshima <i>et al.</i> [102]	Fibrin gel	Wild type rat, OCD in the medial femoral condyle (1.5 mm in diameter and 3 mm in depth). 24 weeks.	MSCs from GFP transgenic rats	MSCs masses were introduced into the defects. Fibrin glue was used to fix the cells.	After 24 weeks, the defects were repaired with hyaline-like cartilage and subchondral bone. The GFP positive cells were observed in the new formed tissues for 24 weeks, with decreased numbers with time.
Solchaga <i>et al.</i> [103]	Hyaluronan or PLLA or PGA	Rabbit, 4 month old, 2.6-3.0 kg/each, OCD in the center of the medial femoral condyle (3 mm in diameter and 1.5 mm in depth). 4, 12 and 20 weeks		The regeneration performance of hyaluronan-based scaffolds (ACP™ and HYAFF®-11) and polyester-based scaffolds (PLGA and PLLA) were compared. The scaffolds were implanted in the defects.	ACP™ group showed bone regeneration at the base of the defect at the 4 th week. After 12 weeks, only ACP™ and PLGA (fast dissolving implants) presented bone restoration consistently. After 20 weeks, the PLGA group showed fibrillation more frequently than ACP™ group. The other two groups presented more cracks and fissures.
Tatebe <i>et al.</i> [104]	Non woven PGA mesh	Rabbit, 6 months old, 2-3 kg/each, OCD in the femoral trochlear (10 mm in length, 5 mm in width and 2 mm in depth). 2, 8 and 42 weeks.	BMSCs	The PGA scaffolds were seeded with the PKH26 labeled cells, and then implanted in the defects.	After 2 weeks, immature cartilage formed. After 8 weeks, neocartilage became thinner and Col type II disappeared from the basal region which became positive of Col type I. The thickness of the neocartilage remained stable up to 42 weeks.

Table 5. Continued (1)

Ref	Scaffold(s)	Animal model/implantation time	Cell type(s)	Method	Outcome
Willers <i>et al.</i> [105]	Col I/III (MACI)	Rabbit, 12-20 weeks old, 3-4.2 kg/each for males, 2.3-4 kg/each for females, OCD in the medial femoral condyle (3 mm in diameter and in depth) 6 and 12 weeks.	Autologous chondrocytes	The defects were created first. Chondrocytes were harvested and expanded for 3-5 days. And then the cells were seeded into the scaffolds and subsequently implanted. Scaffolds without cells and empty defects were used as control.	All untreated defects showed inferior fibrocartilage and/or fibrous tissue repair. The experiment group presented neocartilage and healthy OC architecture at the 6 th week. At the 12 th week, cartilage restoration was maintained with reduced thickness and proteoglycan compared with adjacent cartilage. The histology outcome was not affected by the cell density, but was significantly better than that of scaffolds without cells.
Chang <i>et al.</i> [106]	Gelatin/chondroitin sulfate /hyaluronic acid tri-copolymer scaffold	Porcine, experiment group (7-8 months old and 42-52 kg/each), control group (4.6-7 months old and 27-30 kg/each), OCD in medial or lateral femoral condyle (8 mm in diameter and 5 mm in depth) 18, 24, 36 weeks.	Allogeneous chondrocyte	Allogeneous chondrocytes were seeded into the scaffolds and cultured for 14 days before implantation. Autogeneus OC transplantation was also performed. Scaffolds without cells and empty defects used as control.	The best results were obtained with autogeneus transplantation. Scaffolds with cells group showed satisfied results, with the formation of hyaline cartilage and/or fibrocartilage. But the subchondral bone was not restored.
Zhou <i>et al.</i> [107]	PLA coated PGA fiber mesh	Porcine, 8 weeks old and 10-15 kg/each. Defects in femur trochlea (8 mm in diameter and 6 mm in depth). 3 and 6 months.	Autologous BMSCs	BMSCs were treated with Dex or with Dex and TGF- β 1. And then the cells were seeded into the scaffolds for implantation. Scaffolds alone and empty defects used as control.	The gross view and histology showed that scaffolds seeded with cells presented better reparative results compared with controls. At 6 months, TGF- β 1 treated group showed 70% defects were completely repaired with hyaline cartilage and cancellous bone. Dex treated groups showed 30% defects repaired with hyaline cartilage and cancellous bone. The TGF and Dex treated groups also presented compressive moduli of 80.27% and 62.69% to normal cartilage, respectively.
Hoemann <i>et al.</i> [108]	chitosan/GP/blood composite	Rabbit, 9-15 months old, 4.6 kg/each, OCD in the trochlear groove (3.5 mm in diameter and 4 mm in depth). 8 weeks.		Generated bilateral trochlear defects debried into the calcified cartilage, with 4 micro-drilled holes reached the subchondral bone. The defects were filled by chitosan/GP/blood. Drilling alone as control.	Drilled defects with chitosan/GP/blood led to the formation of a more integrated and hyaline repair tissue on top of a more porous and vascularized subchondral bone plate, compared to drilling alone.
Kasahara <i>et al.</i> [109]	Chitosan/hyaluronan fiber mesh scaffold	Rabbit, 8 weeks old, 2.6-3.1 kg/each, OCD in the patellar groove (5 mm in diameter and 2 mm in depth). 12 weeks.	Allogenic chondrocyte	Chondrocytes were isolated and seeded into the scaffolds for 8 weeks before implantation. Cushion and cylinder scaffolds were used. Empty defects used as control.	After 12 weeks, the reparative tissue consisted of hyaline-like cartilage integrated to the native cartilage, and normal reconstitution of subchondral bone was observed. Compression modulus of reparative tissue showed comparable value to the one of normal cartilage.
Ikeda <i>et al.</i> [110]	PLGA scaffolds of different porosities	Rabbit, 3.1 kg/each, OCD in the patellar groove (5 mm in diameter and in depth). 6 and 12 weeks.		PLGA scaffolds of 80% (I), 85% (II) and 92% (III) porosities were implanted into the defects.	At both time points, group II and III presented significant higher histological scores than that of group I. The better results came from the higher porosity which allowed better migration of MSCs.

Table 5. Continued (2)

Ref	Scaffold(s)	Animal model/implantation time	Cell type(s)	Method	Outcome
Jansen <i>et al.</i> [80]	(PEOT/PBT) with varied ratio of each monomer.	Rabbit, 6 months old, 3.2-4.4 kg/each, OCD in the medial femoral condyle (4 mm in diameter and in depth). 12 weeks.		Scaffolds of 70% PEOT (70/30) and 55% (55/45) PEOT were implanted in the defects. Empty defects used as control.	The 70/30 scaffolds consisted of cartilage-like tissue on top of the trabecular bone, while the 55/45 scaffolds consisted mainly of trabecular bone. O'Driscoll scores were higher in 70/30 group compared with control and 55/45 group. Scaffolds with low mechanical properties were superior in cartilage repair.
Abarrategi <i>et al.</i> [79]	Chitosan scaffolds with different properties	Rabbit, 3-3.5 kg/each, OCD in the medial femoral condyle (4 mm in diameter and 5 mm in depth). 3 months.		Freeze-dried chitosan scaffolds were prepared from chitosan powder of different properties (such as deacetylation degree, molecular weight), and then implanted.	Chitosan scaffolds of intact mineral content (17.9%), lowest deacetylation degree (83%), lowest molecular weight (11.49 KDa) presented a well-structured subchondral bone and noticeable cartilaginous tissue regeneration.
Igarashi <i>et al.</i> [78]	Different grades alginate	Rabbit, 2.6-2.9 kg/each, OCD in the patellar groove (5 mm in diameter and 3 mm in depth). 4 and 12 weeks.	Autologous BMSCs	Ultra-purified alginate was compared with commercial grade alginate for repairing the OCD loaded with BMSCs.	The ultra-purified alginate group presented improved histological and mechanical results for OCD.
Pei <i>et al.</i> [111]	PGA scaffold	Rabbit, 8 months old, 3.5-4.0 kg/each, OCD in the medial femoral condyle (4 mm in diameter and 5 mm in depth). 3 weeks and 6 months.	Xenogenic SDSCs from porcine	Porcine SDSCs from porcine were isolated and seeded into PGA scaffolds. The constructs were cultured in a bioreactor for 1 month before implantation. Fibrin gel saturated collagraft® as a bone substitute. Empty defects used as control.	After 3 weeks, the xenoinplantation group showed a smooth, whitish surface while untreated defects remained empty. But after 6 months, the experimental group showed chronic inflammation in synovial tissue. The histological score was much worse than the one of the control.
Xue <i>et al.</i> [112]	PLGA/NHA	Rat, 12-13 weeks old, 250-300 g/each, OCD in the trochlear groove (1.5 mm in diameter and 3 mm in depth). 12 weeks.	Allogenic BMSCs	Scaffolds seeded with undifferentiated BMSCs were implanted after 12 days <i>in vitro</i> culture. PLGA scaffolds with MSCs and empty defects were used as control.	12 weeks later, the histological results revealed that the defects in the PLGA/NHA-BMSCs group were filled with smooth and hyaline-like cartilage. Abundant GAG and Col II were presented, but deficient in Col I. A continue layer of new bone was found. PLGA group showed fibrocartilage in the defects and much smaller amount new bone that of PLGA/NHA-MSCs group.
Zscharnack <i>et al.</i> [113]	Col gel	Sheep, 2-2.5 years old, 68 kg/each, OCD in the medial femoral condyles (7 mm in diameter and 2 mm in depth). 6 months.	BMSCs	BMSCs were isolated and underwent chondrogenesis. The defects were created 6 weeks before the implantation, in order to generate chronic OCD. Col gel without cells, with chondrogenesis BMSCs or undifferentiated BMSCs were implanted. Empty defects were used as control.	After 6 months, the groups with pre-differentiated MSCs showed significant better histologic scores with morphologic characteristics of hyaline cartilage, such as columnarization and presence of Col type II.

Table 5. Continued (3)

Ref	Scaffold(s)	Animal model/implantation time	Cell type(s)	Method	Outcome
Abedi <i>et al.</i> [114]	Electrospun Col/PVA mesh	Rabbit, 6 weeks old, 2.5-3 kg/each, defects in the patellar groove (4 mm in diameter and 3 mm in depth). 12 weeks.	Autologous MSCs	The MSCs were seeded into the scaffold and cultured for 21 days in chondrogenic medium before implantation. Empty defects used as control.	Histology observation showed that the experimental group had better chondrocyte morphology, continuous subchondral bone and much thicker neocartilage as compared to control group.
Chang <i>et al.</i> [115]	Col gels	Pig, 3 months old, 22 kg/each, OCD in medial condyle (6.5 mm in diameter and 3 mm in depth). 6 months	Autologous BMSCs	BMSCs were loaded in Col gels and cultured <i>in vitro</i> , with or without the treatment of TGF- β . Then, the constructs were implanted. Col gel without cells and empty defects were used as control.	After 6 months, Col gel with undifferentiated BMSCs or BMSCs pre-treated by TGF- β 1 induced similar repair outcome in the defects. Regarding the Pineda score grading, the group with undifferentiated MSCs presented the best result.
Chung <i>et al.</i> [116]	POC and HA (nano or micro in size) composite	Rabbit, defects in the bilateral medial femoral condyles (2.7 mm in diameter and 4 mm in depth). 6 weeks.		POC was mixed with nano- or micro-sized HA to prepare composite for the implantation. POC and PLA were also tested for the implantation.	After 6 weeks, all implants integrated well with the surrounding bone and cartilage, no inflammation was observed. Nanocomposites induced more trabecular bone formation at the interface. A thin cartilaginous tissue layer was observed in the POC nano- and microcomposites.
Chung <i>et al.</i> [117]	POC/HA nanocomposite	Rabbit, 2.3-2.7 kg/each, OCD in the medial condyles (2.7 mm in diameter and 4 mm in depth). 26 weeks.		POC was mixed with nano-sized HA to prepare composite for the implantation. POC and PLA were also tested for the implantation.	After 26 weeks, histological results showed that both POC-HA, POC implants were biocompatible. PLLA scaffolds were surrounded by leukocytes. All the implants showed a continuous cartilaginous layer on their surface. As POC-HA degraded, tissue ingrowth was observed.
Sun <i>et al.</i> [118]	Porous TCP scaffold	Dog, 10-12 months old, OCD in the femoral trochlear (5 mm in diameter and depth). 16 weeks.	Allogenic BMSCs and osteoblasts	BMSCs were isolated and underwent chondrogenesis and osteogenesis <i>in vitro</i> . The differentiated cells were seeded in different ends of the TCP scaffolds, respectively. Then, the constructs were co-cultured in a bioreactor for 21 days. Afterwards, the constructs with chondrocytes and osteoblasts (Group A) or only chondrocytes (Group B) were implanted in the defects. Scaffold without cells used as control (Group C).	After 16 weeks, group A showed good integration with host cartilage, with smooth and translucent tissue. Group B showed less integration with host cartilage compared with group A, and presented poor glossy surface. Group C showed ragged margin and soft new formed tissue.
Chang <i>et al.</i> [119]	PLGA scaffolds	Rabbit, 4-5 months old, 2-3 kg/each, OCD in the medial condyle (3 mm in diameter and in depth). 4 and 12 weeks.		Porous PLGA scaffolds were implanted in the defects. CPM treatment was performed and compared with Imm and IAM treatments, in the PI (PLGA implanted) and ED (empty defect) models.	At 12 weeks, the PI-CPM group presented the best outcome with normal articular surfaces, no contracture in the joint and no inflammation. While Imm and IAM groups showed degenerated joints, abrasion cartilage surfaces and synovitis. The CPM group showed significantly higher bone volume compared with the Imm, IAM and ED-CPM groups.

Table 5. Continued (4)

Ref	Scaffold(s)	Animal model/implantation time	Cell type(s)	Method	Outcome
Coburn <i>et al.</i> [120]	PVA-MA and PVA-MA/CS-MA nanofibrous hydrogels	Rat, 6 weeks old, OCD in trochlear groove (1.5 mm in diameter and 1.4-1.5 mm in depth). 6 weeks.		Low density 3D nanofiber network was prepared by electrospinning the polymer into ethanol bath. The prepared PVA-MA or PVA-MA/CS-MA scaffolds were implanted.	When implanted in OCD, the acellular nanofiber scaffolds support chondrogenesis confirming by proteoglycan production which rarely present in defect control. Chondroitin sulphate in the fiber enhanced collagen II synthesis <i>in vitro</i> and <i>in vivo</i> . Nanofiber implanted defects did not have dense subchondral bone surrounding the cartilaginous tissue compared with the control defect. The trabecular bone in nanofiber implanted defects contained numerous cell-rich spaces resembling bone marrow cavities that were immediately adjacent to the proteoglycan-containing areas.
Hannink <i>et al.</i> [121]	PCL/PU scaffold	Rabbit, mature, female, 3.2-4.9 kg/each, OCD in trochlear groove (4 mm in diameter, 3 mm in depth). 8 and 14 weeks.		Defects were filled with PCL-PU scaffold. Scaffolds of 4 or 3 mm in height were used. The scaffolds of 4 mm in height were 1 mm above the surrounding cartilage surface, in order to study the mechanical stimulus on OCD regeneration.	After 8 weeks, both the 3 mm and 4 mm scaffolds were flushed with the native cartilage. Center region had less matrix compared with edge region, no difference in the two groups. After 14 weeks, more cartilaginous tissue presented in the 4 mm scaffolds compared with the 3-mm scaffolds. In the 4 mm scaffold group, progression of cartilaginous tissue from the surface toward the center of the scaffold was observed over time. But the 3 mm scaffold group showed no difference in the central zone compared to the situation at the 8 th week. The results suggested the mechanical forces may not have to be applied over long period of time.
Igarashi <i>et al.</i> [122]	Ultra-purified alginate gel	Dogs, 20 kg/each, OCD in the patellar groove (5 mm in diameter and in depth). 16 weeks.	Autologous BMSCs	The alginate glues was mixed with BMSCs, and subsequently injected into the defects. Alginate gels without cells and empty defects were used as control.	The reparative tissues of BMSCs group were substituted with firm and smooth hyaline-like cartilage tissue and integrated well with host cartilage. Also enhanced subchondral bone and superior compressive modulus of the new formed tissue were observed in this group.
Lee <i>et al.</i> [123]	Col/hyaluronic acid/fibrinogen hydrogel	Rabbit, 8 months old, 3.5-4.0 kg/each, OCD in patellar groove of the distal femur (4 mm in diameter and 3 mm in depth). 4 and 24 weeks.	Synovium-derived MSCs (SDSCs).	SDSCs were encapsulated in the hydrogel and then implanted. Untreated defects and hydrogels without cells served as controls.	The SDSCs were able to differentiate toward the chondrogenic lineage when cultured in the hydrogel under chondrogenic medium <i>in vitro</i> . When implanted for 24 weeks, the SDSCs encapsulated construct group repaired with hyaline cartilage-like tissue which was densely stained by safranin-O and immunostained by Collagen II. The subchondral bone was well-reconstituted.
Miot <i>et al.</i> [124]	Hyaluronic acid scaffold	Goat, female, over 18 months old, OCD in trochlea groove (6 mm in diameter and 5 mm in depth). 8 weeks and 8 months.	Goat autologous chondrocyte	The goat chondrocyte were cultured in hyaluronic acid scaffold for 2 days, 2 weeks, or 6 weeks, respectively. And then the constructs were implanted with HA/hyaluronic acid sponge acted as subchondral support. Three experiment groups (cells cultured in scaffolds for different time) were studied. Empty defect and scaffolds without cells acted as controls.	Increasing pre-culture time resulted in progressive maturation of the grafts <i>in vitro</i> . After 8 weeks <i>in vivo</i> , the quality of the repair was not improved by any treatment. After 8 months, O'Driscoll histology scores indicated poor cartilage architecture was observed in untreated and cell-free treated groups. Scores were improved when cellular grafts were implanted, with best scores observed for grafts with pre-cultured for 2 weeks.

Table 5. Continued (5)

Ref	Scaffold(s)	Animal model/implantation time	Cell type(s)	Method	Outcome
Qi <i>et al.</i> [125]	PLGA scaffold	Rabbit, male, 2.5-3.0 kg/each, OCD in (4 mm in diameter and 3 mm in depth). 6 and 12 weeks.	Rabbit BMSCs	BMSCs were cultured in the PLGA scaffolds, also MSCs cell sheet were formed after culture for 2 weeks <i>in vitro</i> . The scaffolds were encapsulated by the cell sheet and then implanted. Scaffold alone, scaffold with cells but without cell sheet as controls.	Cell sheet encapsulated constructs showed higher amount of hyaline cartilage and histological scores than those in PLGA/BMSCs and PLGA groups. Cell sheet group showed the best integration between the repaired cartilage and surrounding normal cartilage or subchondral bone. After 6 weeks, scaffold alone group presented no subchondral bone formation, while subchondral bone was observed in the scaffold/cell group. About half of the subchondral bone was modelled for cell sheet group. After 12 weeks, completely reconstructed subchondral bone was presented in cell sheet group. Scaffold/cell group showed around 50% subchondral bone were modeled.
Bernstain <i>et al.</i> [126]	TCP scaffold	Sheep, female, 2-4 years old, 74.54±16.55 kg/each, OCD in medial femoral condyle (7 mm in diameter, 25 mm in depth). 12, 26, 52 weeks.	Sheep autologous chondrocyte	The scaffolds were seeded with chondrocytes <i>in vitro</i> and cultured for 4 weeks. Then, the constructs were implanted. Untreated defect served as control.	After 26 and 52 weeks, collagen II positive hyaline cartilage was detected. The biomechanical stable cartilage formed at the outer edge and proceeded to the middle of the defect. After 1 year, this process was still not completed. The TCP scaffolds showed around 81% degradation after 52 weeks, with concomitant bone formation. The original structure of cancellous bone was almost completely restored. O'Driscoll score did not showed healthy cartilage after 1 year. Integration of the newly formed cartilage tissue with the surrounding native cartilage was found.
Chang <i>et al.</i> [127]	PLGA scaffold	Rabbit, 4-5 months old, 2-3 kg/each, OCD in medial femoral condyle (3 mm in diameter and 3 mm in depth). 4 and 12 weeks.	Autologous endothelial progenitor cells (EPC)	The EPC were seeded into PLGA scaffold, and implanted into the OCD. Empty defect and scaffold only groups acted as controls.	Only the EPC-PLGA group showed the neo-cartilage tissue with a smooth, transparent and integrated articular surface. At the 4 th week, the EPC-PLGA showed considerably higher TGF-beta2 and TGF-beta3 expression, a greater amount of GAG, a higher degree of OC angiogenesis in repaired tissues, compared to other groups. At the 12 th week, EPC-PLGA showed enhanced hyaline cartilage regeneration with normal columnar chondrocyte arrangement, higher SOX9 expression, and greater GAG collagen II content. Moreover, this group showed organized OC integration, the formation of vessel-rich trabecular bone and significantly higher bone volume per tissue volume.
Chang <i>et al.</i> [128]	PLGA scaffold	Rabbit, male, 4-5 months old, 2-3 kg/each, OCD in the low-weight bearing zone of the femoral tracheal groove (3 mm in diameter and in depth). 4 and 12 weeks.		PLGA scaffolds were implanted into the OCD, and then performed the intermittent active motion (IAM) or continuous passive motion (CPM), with or without scaffolds.	CPM/scaffold group showed more promising than all other groups. This group showed smooth cartilage surfaces with hyaline cartilaginous tissue composite. Also, good collagen alignment with positive collagen II expression was observed. The GAG content was higher than other groups. Mature bone regeneration and clear tidemark formation were achieved.

Table 5. Continued (6)

Ref	Scaffold(s)	Animal model/implantation time	Cell type(s)	Method	Outcome
Deplaine <i>et al.</i> [129]	PLLA/HA scaffold	Sheep, 3 months old, OCD in medial femoral condyle (6 mm in diameter and 6 mm in depth). 6 weeks.		Scaffolds were subjected to air plasma treatment first, and then immersed in SBF for 21 days. Three groups scaffolds (PLLA, PLLA/HA, and PLLA/HA/SBF) were implanted.	The formation of biomimetic hydroxyapatite on the pore's surface of the PLLA/HA scaffold produced a better integration with the subchondral bone in comparison to bare PLLA scaffolds group.
Hui <i>et al.</i> [130]	Freeze-dried OPF hydrogel	Micropigs, female, mature, 20 kg/each, 8 months old, OCD in the weight-bearing region of the lateral and medial condyles (6 mm in diameter and 1 mm in depth). 2 and 4 months.		The rehydrated hydrogels were implanted into the porcine defects.	The scaffold induced neotissue filling at both 2 and 4 months to 58% and 54%, respectively. Hyaline cartilage made up 39% of neotissue at 4 months, without inducing subchondral bone regeneration.
Jurgens <i>et al.</i> [131]	Col I/III scaffold	Goat, female, mature, 82.4±11.7 kg/each, OCD in medial condyle or trochlear grooves (5 mm in diameter and 3.5 mm in depth). 1 and 4 months.	Freshly SVF or cultured ASCs	Collagen scaffolds were seeded with freshly isolated SVF or cultured ASCs (passage 3), and then implanted. Acellular scaffolds were used as control.	After 1 month, no adverse effects were observed. Cell loaded constructs showed more regeneration. After 4 months, acellular scaffold showed increased regeneration, but less than that from the cell incorporated constructs. The cellular constructs displayed extensive collagen II, hyaline-like cartilage, and higher elastic moduli. The GAG content approached the value of the native tissue. The defects with cellular scaffold contained higher levels of regenerated mature subchondral bone, evidencing by intensive collagen I staining. SVF group tended to perform the best in all parameters.
Lafantaisie <i>et al.</i> [132]	Chitosan/blood hybrid	Rabbit, 2.5 years, 4.68±0.44 kg/each, OCD in femoral trochlea (1.4 mm in diameter and 2 mm in depth). 1 day and 21 days.		Chitosan solutions of three different molecular weights but of the same degree of deacetylation were mixed with rabbit peripheral whole blood and sodium chloride to form a solid. The solid was implanted. Empty defect as control.	All the implants filled the top of the defects at day 1 and were partly degraded <i>in situ</i> at day 21. All implants attracted neutrophils, osteoclasts, and abundant bone marrow derived stromal cells to the bone plate, delaying deposition of collagen and GAG. This procedure stimulated bone resorption, new woven bone repair, and promoted tissue-bone integration. The 150 kDa chitosan implant was less degraded and elicited more apoptotic neutrophils and bone resorption than 10 kDa chitosan implant.
Lim <i>et al.</i> [133]	Freeze-dried OPF hydrogel	Micropigs, 8 months old, mature, female, 20 kg/each, OCD in lateral and medial condyles (6 mm in diameter and 1 mm in depth). 4 months.		Rehydrated freeze-dried OPF hydrogels were seeded with BMSCs, and then implanted. Scaffolds without cells as control.	The scaffold with cells led to 99% tissue filling in the defects and 84% hyaline-like cartilage. And the scaffolds without cells induced higher than 54% neotissue filling and 39% hyaline-like cartilage. But there was no regeneration of subchondral bone.

Table 5. Continued (7)

Ref	Scaffold(s)	Animal model/implantation time	Cell type(s)	Method	Outcome
Mayr <i>et al.</i> [134]	Microporous TCP scaffold	Ovine, female, 2-4 years old, 74.54±16.55 kg/each, OCD in left medial femoral condyle (7 mm in diameter and 25 mm in depth). 6, 12, 26, and 52 weeks.	Ovine autologous chondrocytes	The chondrocytes were seeded in the scaffolds and cultured for 4 weeks <i>in vitro</i> . Then the constructs were implanted and covered by synovial membrane. Empty defect served as control.	The indentation loading values, the contact stiffness, and the absorbed energy increased significantly from 6 to 52 weeks. At the 52 th week, the ICRS scores for the central area of the transplanted area and untreated defects were comparable. While the score for the area from the edge to the centre in the transplanted area was significantly higher than the control. After the 52 th week, the central area of the implants had a lower ICRS score than that of healthy cartilage.
Pulkkinen <i>et al.</i> [135]	Recombinant human Col II scaffold	Rabbit, mature, 9 months old, OCD in patellar groove (4 mm in diameter and 3 mm in depth). 6 months.	Rabbit autologous chondrocytes	The chondrocytes were harvested and cultured with the collagen gel for two weeks before implantation. Untreated lesion used as control.	After 6 months, the defects from both groups were filled by repair tissue. The filling in the scaffold group was more completed. Both groups had high GAG and Col II content, O'Driscoll scores showed no significant differences between the scaffold group and the control groups, representing low quality than intact cartilage. No dramatic changes were detected in the subchondral bone structure.
Rajzer <i>et al.</i> [136]	Hyaluronic acid modified nonwoven carbon fibres	Rabbit, OCD in the trochlear grooves of the knee (2 mm in diameter and 5 mm in depth). 2 and 6 months.		The hyaluronic acid was physically immobilized into the non-woven carbon fibres, and the hybrid was dried at room temperature. Then the scaffolds were implanted. Non-modified fibre as control.	The incorporation of hyaluronic acid resulted in the improvement of cell proliferation. A faster process of tissue regeneration was observed in the HA modified carbon nonwovens.
Sotoudeh <i>et al.</i> [137]	Nano-structured HA/zirconia stabilized yttria	Rabbit, OCD in the patellar groove (4 mm in diameter and 3 mm in depth). 12 weeks.		The composite was prepared by a sol-gel method. Then, the scaffolds were implanted into the defect. Empty defect served as control.	With implantation, the defect was filled with a white translucent cartilage tissue. Defect without treatment remained empty. Experiment group stained positively with collagen II.

3D=three dimensional; ASCs=adipose tissue derived stem cells; BMSCs=bone marrow mesenchymal stromal cells; Col=collagen; CPM=continued passive motion; CS-MA=chondroitin sulfate-methacrylate; Dex=dexamethasone; EPC=endothelial progenitor cells; GAG=glycosaminoglycan; GFP=green fluorescent protein; GP=glycerol phosphate; HA=hydroxyapatite; IAM=intermittent active motion; ICRS=International Cartilage Repair Society; Imm=immobilization; MACI=matrix-induced autologous chondrocyte implantation; OC=osteocondral; OCD=osteocondral defect(s); PCL=poly(ϵ -caprolactone); PEOT/PBT=poly(ethylene oxide terephthalate) and poly(butylenes terephthalate) copolymer; PGA=poly(glycolic acid); PLLA=poly(L-lactic acid); POC=poly(1,8-octanediol-co-citrate); PU=polyurethane; PVA=poly(vinyl alcohol); PVA-MA=poly(vinyl alcohol)-methacrylate; Ref=reference; SBF=simulated body fluid; SDSCs=synovium-derived stem cells; SDSCs=synovium-derived stem cells; SVF=stromal vascular fraction; TCP=tricalcium phosphate.

[78] studied the regeneration potential of alginates of different grades combined with BMSCs for OCD regeneration. It was found that the ultra-purified alginate group demonstrated better histological and mechanical outcomes compared to other groups. Abarrategi *et al.* [79] investigated chitosan scaffolds of different molecular weight and deacetylation degree for OCD tissue engineering. Better subchondral bone regeneration and noticeable cartilaginous tissue regeneration was observed in chitosan scaffolds of intact mineral content, lowest deacetylation degree and molecular weight. Jansen *et al.* [80] compared scaffolds prepared from poly(ethylene oxide terephthalate) (PEOT) and poly(butylene terephthalate) (PBT) copolymer but of varied monomer ratio for OCD regeneration. When PEOT/PBT was 70/30, the scaffolds mainly consisted of cartilage-like tissue on top of the trabecular bone. While decreasing the PEOT/PBT ratio to 55/45, the scaffolds consisted mainly of cancellous bone. Ikeda *et al.* [110] also found that PLGA scaffolds of higher porosity showed better histological scores compared with the scaffolds of lower porosity. Hui *et al.* [130] found that the freeze-dried OPF hydrogels promoted neo-cartilaginous tissue formation but cannot induce the subchondral bone formation.

As the development of tissue engineering, many efforts have been made trying to improve the traditional MACI strategy. Currently, only collagen and hyaluronic acid are used for MACI. Other biomaterials or composites were also explored aiming to provide more options for clinic applications. Guo *et al.* [100] cultured chondrocytes onto the surface of tricalcium phosphate (TCP) scaffolds aimed at finding application in OCD regeneration. After 24 weeks, the surface of the cartilage defect was completely regenerated and TCP was integrated well with the new formed bone, in a sheep model. Ito *et al.* [101] developed poly(L-lactic acid) mesh surrounding collagen sponge and seeded chondrocytes in this scaffold for rabbit OCD regeneration. At the 12th week, hyaline cartilage like tissue and good subchondral bone formation were observed. In addition to autologous chondrocytes, stem cells were also seeded into scaffolds for OCD regeneration. Zscharnack *et al.* [113] incorporated the chondrogenic differentiated BMSCs into collagen gels and implanted in OCD in sheep. After 6 month, the defects were filled with hyaline cartilage composed of collagen II. Zhou *et al.* [107] cultured the BMSCs with dexamethasone or dexamethasone and TGF- β 1 and then seeded the cells into poly(lactic acid) coated poly(glycolic acid) fiber mesh scaffold. They found that scaffolds combined with cells presented better results than scaffold alone. After 6

Table 6. *In vivo* studies on OC tissue engineering using layered scaffolds/hydrogels

Ref	Scaffold(s) # Cartilage part & Interface part * Bone part	Animal model/implantation time	Cell type(s)	Method	Outcome
Alhadlaq <i>et al.</i> [138]	# PEGDA * PEGDA	SCID mice, subcutaneous implantation. 4 weeks.	Rat BMSCs	PEGDA solution was mixed with chondrogenic or osteogenic MSCs, and then the mixture were used to generate a bilayered human condyle like constructs. Constructs were subcutaneously implanted.	After 4 weeks, the implants resembled the macroscopic shape and dimensions of the cell-hydrogel construct. Only the chondrogenic layer showed positive reaction to safranin O, and only the osteogenic layer showed positive staining of von Kossa.
Frenkel <i>et al.</i> [139]	# Chitosan/hyaluronan (PEC), or Col * PLLA/hyaluronan	Rabbit, 9 months old, OCD in the medial femoral condyle (3 mm in diameter and 2.8 mm in depth). 24 weeks.		Two kinds of bilayered scaffolds were implanted. Empty defects were used as control.	PEC and Col groups presented similar repair results, but both were significantly better than untreated defects. The percentage of hyaline-like cartilage was the highest with Col group. PEC group showed the best outcomes in bonding to the host, structural integrity of the neocartilage, and reconstitution of the subchondral bone.
Masuda <i>et al.</i> [140]	# CMCh * CMCh/TCP, or CMCh/HA	Rabbit, 12 weeks old, ~2.0 kg/each, OCD in femoropatellar groove (5 mm in diameter and depth). 2, 4, 8, and 32 weeks.		Freeze-dried Scaffolds were implanted into the defects. Defects filled with TCP granules and defects without treatment were used as control.	TCP/CMCh was completely absorbed after 4 weeks postoperatively. Regeneration of articular cartilage was seen in TCP/CMCh and HA/CMCh bilayered groups, but not in the TCP granules group. The regenerated cartilage maintained after 32 weeks.
Tanaka <i>et al.</i> [141]	# Chondrocyte in Col gel * TCP	Rabbit, 3-3.3 kg/each, OCD in the intercondylar groove (4.2 mm in diameter and 6 mm in depth). 8, 12, and 30 weeks.	Allogenic chondrocytes	Allogenic chondrocytes were harvested and cultured for 2-3 weeks. And then the cells were mixed with Col and the mixture was spotted on top of the TCP scaffolds until it gelled. The biphasic constructs were implanted. Defects filled with TCP alone served as control.	After 8 weeks, biphasic construct showed hyaline-like cartilage. After 12 weeks, most of the TCP was replaced by new bone. The middle and deep domain in the neocartilage showed positive staining to safranin O, but not the case of superficial layer. After 30 weeks, TCP was completely resorbed, 24% defects showed hyaline-like cartilage. Control showed no cartilage formation but induced subchondral bone formation.
Chen <i>et al.</i> [142]	# Col sponge * PLGA/Col	Beagle, 1 year old, OCD in the femoral condyle (4.5 mm in diameter, reached subchondral bone). 4 months.	Autologous BMSCs	MSCs were seeded in the scaffolds and cultured for 1 week before implantation. Scaffolds without cells were used as control.	After 4 months, gross appearance displayed that the scaffold/cells group presented smoother surface and better integration with surrounding tissue compared with scaffold alone group. Histological examination showed cartilage like and underlying bone-like tissues were regenerated in the experimental group. The scaffold alone group showed no evidence of hyaline cartilage.

Table 6. Continued (1)

Ref	Scaffold(s) # Cartilage part & Interface part * Bone part	Animal model/implantation time	Cell type(s)	Method	Outcome
Chen <i>et al.</i> [143]	# Laminated PLGA/Col hybrid mesh * Laminated PLGA/Col hybrid mesh	Nude mice, subcutaneous implantation. 3 and 9 weeks.	Canine BMSCs and chondrocytes	The BMSCs were seeded into the scaffold and undergone osteogenesis differentiation. Chondrocytes were also seeded into the scaffolds. And then the two layers were sutured to form biphasic constructs before implantation.	The shape of the biphasic constructs were maintained during the implantation. In the chondral layer, spherical chondrocytes were surrounded by abundant cartilaginous ECM, and presented positive staining of safranin O, toluidine blue, as well as Col II and aggrecan gene expression. The “bone-like” layer consisted of spindle morphology cells, and Col I and osteocalcin gene expression were detected.
Kandel <i>et al.</i> [144]	# Chondrocytes on top of the CPP scaffold * Porous CPP scaffold	Sheep, 6-9 months old, OCD in the distal aspect of the trochlear groove of the femur (4 mm in diameter and 6 mm in depth). 3 and 9 months.	Autologous chondrocytes	The isolated autologous chondrocytes were seeded on top of the CPP scaffolds. After 8 weeks, the bilayered constructs were implanted. CPP plugs without cells served as control.	The implants can withstand the bear <i>in vivo</i> up to 9 months. Fusion to adjacent cartilage and fixation by new bone ingrowth were observed. The cellularity and proteoglycan content were stable during implantation. The implanted cartilage showed increased thickness and elastic equilibrium modulus with time. Bone ingrowth at both time points were observed. Fibrous tissue was observed in the control after 3 months.
Shao <i>et al.</i> [145]	# Fibrin gel/BMSCs * PCL/fibrin/BMSCs	Rabbit, 3-3.5 kg/each, OCD in the medial femoral condyle (4 mm diameter and 5 mm in depth). 3 and 6 months.	Allogenic BMSCs	BMSCs/fibrin was loaded into the PCL scaffold, and then the constructs were implanted. The cartilage defect was filled by BMSCs/fibrin. Scaffolds and fibrin gel without cells served as control.	The transplanted cells were still viable after 5 weeks. Progressive mineralization from the host-tissue interface towards the inner region of the grafts was presented. At 3 months, the samples showed good cartilage repair. While at 6 months, only one third samples maintained good cartilage appearance.
Shao <i>et al.</i> [146]	# PCL * PCL/TCP	Rabbit, 3-3.5 kg/each, OCD in the medial femoral condyle (4 mm in diameter and 5 mm in depth). 3 and 6 months	Allogenic BMSCs	BMSCs were loaded into PCL and PCL/TCP scaffolds by fibrin gel, respectively. Then, the two constructs were implanted by press-fit method. Scaffolds with no cells served as controls.	The experiment group displayed superior repair results as compared with the control group. Bone regeneration was good and consistent at both time points. At 3 months, all samples presented cartilage tissues mixed with the materials. At 6 months, some samples showed degradation while others presented good appearance. Implanted cells were viable for 6 weeks after implantation.
Jiang <i>et al.</i> [147]	# PDLA * PDLA/TCP	Porcine, 7-9 months old, OCD in the medial/lateral femoral condyle (8 mm in diameter and depth). 6 months.	Autologous chondrocyte	Autologous chondrocytes were seeded into PDLA scaffold before implantation. The biphasic constructs with or without cells were implanted by press-fit method.	In the osseous layer, the scaffold retained in the center and cancellous bone formed in the periphery. In the chondral phase, positive Col II and Safranin O staining showed hyaline cartilage regeneration. Only fibrous tissue formed in the scaffold alone group. Both groups supported subchondral bone regeneration and mineralization.

Table 6. Continued (2)

Ref	Scaffold(s) # Cartilage part & Interface part * Bone part	Animal model/implantation time	Cell type(s)	Method	Outcome
Pilliar <i>et al.</i> [148]	# Chondrocytes on top of the CPP scaffold * Porous CPP scaffold	Sheep, 6-9 months old, OCD in the trochlear groove (4 mm in diameter and 6 mm in depth). 3 and 9 months.	Autologous chondrocytes	The isolated autologous chondrocytes were seeded on top of the CPP scaffolds. After 8 weeks, the bilayered constructs were implanted. CPP plugs without cells served as control.	Implant fixation within the condyle sites was achieved via bone ingrowth. The improvements in structure and mechanical properties were observed during the implantation time period.
Ito <i>et al.</i> [149]	# Col gel * HA scaffolds infiltrated by Col gel and chondrocyte	Rabbit, 12 weeks old, ~2 kg/each, OCD in the patellar groove (4 mm in diameter and 6 mm in depth). 4 and 12 weeks.	Autologous chondrocyte	Chondrocytes were mixed with Col gel and then infiltrated into the porous HA scaffolds. The engineered biphasic constructs were cultured <i>in vitro</i> before the implantation. Scaffolds without cells implanted as control.	At the 12 th week, the defects were repaired with cartilage-like tissue with good subchondral bone regeneration. Histological scores were significantly higher than the ones of the control group.
Moroni <i>et al.</i> [150]	# 300PEOT55PBT45 scaffold * 1000PEOT70PBT30, demineralized bone matrix, and TCP composite scaffold	Nude mice, 6 weeks old, subcutaneous implantation. 25 days.	Goat BMSCs	Undifferentiated MSCs were seeded into the bone component, and chondrogenic BMSCs were seeded into the chondral component. And then the biphasic constructs were intramuscularly implanted.	In the chondral part, cells exhibited round morphology. Mineralized matrix within the chondral part was presented. In the osseous part, osseous tissue was composed of a mineralized matrix. Osteocytes could be detected in the matrix.
Petersen <i>et al.</i> [151]	# Expanded chondrocytes & One layer of chondrocytes * CaP carriers (2 mm in height)	Pig, about 27 months, ~40 kg/each, OCD in the medial femoral condyle (4.5 mm in diameter and 3.0±0.5 mm in depth). 26, 52 weeks.	Autologous chondrocytes	One layer of chondrocytes were first coated and cultured on top of the calcium phosphate carrier. Chondrocytes were also expanded in alginate beads. Expanded chondrocytes were sedimented onto the calcium phosphate carriers. The biphasic constructs were implanted after culturing for 3 weeks.	At both time points, the defects were resurfaced with hyaline-like cartilage. The engineered cartilage was integrated with the adjacent cartilage. The ICRS scores increased from 26 to 52 weeks. Partial reconstruction of the subchondral bone was observed.
Tampieri <i>et al.</i> [152]	# Hyaluronan/Col & Biom mineralized Col with lower content of mineral * Biom mineralized Col with higher content mineral	Nude mice, 1 month old, subcutaneous implantation. 8 weeks.	Ewe BMSCs	BMSCs were isolated and expanded. Cells were loaded onto the multilayered scaffolds and then implanted.	After 8 weeks, a nicely structured bone tissue presented in the bone layer, and a loose connective tissue in the chondral layer. Chondral layer allowed the chondrocyte differentiated and cartilaginous matrix deposition. Bone tissue only formed within the subchondral layer.

Table 6. Continued (3)

Ref	Scaffold(s) # Cartilage part & Interface part * Bone part	Animal model/implantation time	Cell type(s)	Method	Outcome
Zhou <i>et al.</i> [153]	# PGA * HA	Rabbit, 9-10 months old, ~3.5 kg/each, OCD in the trochlear groove (3.2 mm in diameter and 6 mm in depth). 16, 32 weeks.	Autologous BMSCs	BMSCs were seeded into the PGA and HA scaffolds. Chondrogenesis and osteogenesis was carried out in the PGA and HA scaffolds, respectively. The two layers were integrated by fibrin gel before implantation. Scaffolds without cells or empty defects as control.	At both time points, scaffolds without cells showed irregular opaque tissue in the defects. Experimental group showed improved quality in the reparative tissue which presented structural organization similar to native tissue, with well-defined subchondral bone under the hyaline cartilage.
Liu <i>et al.</i> [154]	# Porous PLGA & Dense PLGA/TCP * Porous PLGA/TCP	Rabbit, 8 weeks old, ~2 kg/each, OCD in the patellar groove (4 mm in diameter, reached the marrow cavity). 6 weeks.	BMSCs	Scaffolds were prepared by multi-nozzle low temperature deposition technology. Isolated and expanded BMSCs were seeded into the scaffolds. Scaffolds with cells were implanted. Empty defects were used as control.	After 6 weeks, gross examination showed only soft fibrous tissues formed in the control. While firm cartilage-like tissue, with similar color and texture to the native cartilage surface, was presented in the experimental group. Histological results showed no bone-like or cartilage-like tissue regeneration in control group. But the treated group presented cartilage-like tissues and some bone-like tissues in the bone region.
Bal <i>et al.</i> [155]	# PEG gel/BMSCs * Porous Tantalum, or allograft bone, or bioactive glass scaffold	Rabbit, above 2 kg/each, OCD in trochlear groove and medial condyle (3.2 mm in diameter and 4 mm in depth). 6 and 12 weeks.	Allogenic BMSCs	BMSCs were cultured under chondrogenesis condition and then loaded into PEG solution. Afterward, the BMSCs/PEG gels were integrated with tantalum, allograft bone, or bioactive glass scaffolds, respectively. The bilayered constructs were implanted.	Bioactive glass and porous tantalum were superior to bone allograft regarding the integration to adjacent host tissue, regeneration of the hyaline-like tissue at the top surface, and the secretion of Col type II.
Chiang <i>et al.</i> [156]	# PDLA * PDLA/TCP	Pig, 10-11 months old, OCD in the femoral condyle (8 mm in diameter and in depth). 6 months.	Autologous chondrocyte	Pre-cultured or freshly isolated chondrocytes were seeded in the PDLA layer of the bilayered scaffold, and then the constructs were implanted. Scaffolds without cells or empty defects served as control.	The two experimental groups were repaired with hyaline cartilage with Col II. Scaffold alone group showed mainly the fibrocartilage. In null group no regeneration was observed. Experimental groups showed comparable results with control in subchondral bone regeneration. No significant differences between two experimental groups in all categories.
Ho <i>et al.</i> [157]	# PCL scaffold, or PCL scaffold with a layer of electrospun PCL/Col mesh * PCL/TCP	Pig, 6 months old, OCD in the medial condyle and patellar groove (8 mm in diameter and in depth). 6 months.	Autologous BMSCs	PCL scaffolds were seeded with BMSCs, and then covered with a PCL/Col mesh before implantation (Group A). Constructs without mesh (Group B) or scaffold without cells but with mesh (Group C) were also implanted.	After 6 month, explants from medial condyle showed that Group A and B had higher amount of GAG in the surface of the reparative cartilage compared with Group C. Group A presented the least fibrocartilage compared with Group B and C. In the case of patellar groove, a mixture of hyaline, fibrocartilage and fibrous tissue was observed in all the groups. Bone ingrowth and remodeling occurred in all the samples.

Table 6. Continued (4)

Ref	Scaffold(s) # Cartilage part & Interface part * Bone part	Animal model/implantation time	Cell type(s)	Method	Outcome
Im <i>et al.</i> [158]	# Hyaluronant/Col * HA/TCP	Pig, 23-25 months old, ~50.5 kg, OCD in the medial and lateral condyle (6 mm in diameter and 7 mm in depth). 5 months.	Autologous chondrocytes	Five treatments were performed for the defects: (I) scaffolds with cells, (II) scaffolds alone, (IIIa) autologous OC transplantation, (IIIb) chondrocytes implantation, (IV) empty defects.	ICRS macroscopic scores for each group: I (9), II (9.1), IIIa (9.1), IIIb (7.4), IV (6.2). And ICRS histological scores: I (11.6), II (13.6), IIIa (11.4), IIIb (12.8), IV (10.1). All the defects were filled with cartilaginous or fibrous tissue, except most of the defects in IV.
MarqUSA <i>s et al.</i> [159]	# Col gel & Autologous plasma * β -TCP	Ovine, 2-2.5 years old, ~65 kg, OCD in the medial femoral condyles (6.6 mm in diameter and 12 mm in depth). 6 and 12 months.	Autologous BMSCs	BMSCs were cultured in the Col gels under chondrogenesis condition. BMSCs were also mixed with autologous plasma and seeded onto β -TCP scaffold. The two constructs were successively implanted in the defects. OC autografts were used as control.	There were no significant differences between the histological scores of both groups. O'Driscoll scores showed superior cartilage bonding in 6 month compared with control. But at 12 month, the control group showed better cartilage matrix morphology compared with triphasic group.
Cui <i>et al.</i> [160]	# PLGA * TCP	Pig, 7-8 months old, OCD in the medial condyle (8 mm in diameter and in depth). 6 months.	Autologous chondrocytes and osteoblasts	Chondrocyts and osteoblasts were seeded into the PLGA and TCP scaffolds, respectively. The biphasic constructs were formed by suture before implantation. PLGA scaffolds with chondrocytes and empty defects were used as control.	After 6 months, the ICRS macroscopic scores of each group: biphasic (14.25), PLGA (9.13), control (2.5). And ICRS histological scores for each group: biphasic (14.5), PLGA (9.54), control (4.13). The compressive properties and GAG contents results revealed that better repair results were showed in the biphasic group.
Qu <i>et al.</i> [161]	# PVA * n-HA/PA6	Rabbit, implanted in the muscle pouch. 4 and 8 weeks.	BMSCs	BMSCs were undergone osteogenesis and chondrogenesis, respectively. The chondrogenic cells were seeded in the PVA layer, and the osteogenic cells were seeded in the n-HA/PA6 layer. The bilayered constructs were implanted. Scaffolds without cells were used as control.	After 8 weeks, ectopic neocartilage formation was observed in the PVA layer, and reconstruction of subchondral bone was presented in the n-HA/PA6 layer. During the implantation, the two layers of the biphasic constructs were well integrated.
Deng <i>et al.</i> [162]	# Gelatin/chondroitin sulphate/sodium hyaluronate * Gelatin /ceramic bovine bone	Rabbit, 12 weeks old, 2.5-3.0 kg/each, large OCD on the patella of the right distal femur (15x10x5 mm) 6, 12, 24 weeks.	Rabbit chondrocyte and BMSCs	Distal femur shape bilayered scaffolds were prepared. Chondrocytes and osteogenic differentiated BMSCs were injected into the chondral and bony layer, respectively. The constructs with cells (G-A) or without cells (G-B) were kept in osteogenic medium for 48 hours before implantation. Empty defects as control (G-C).	After 6 and 12 weeks, hyaline-like cartilage formation was observed in G-A with repaired tissue stained for collagen II. This group also presented higher collagen II expression detected by RT-PCR compared with other groups. G-A showed most of the bony scaffold was replaced by bone, and little remained in the underlying cartilage. After 24 weeks, bony layer in G-A was completely resorted and a tidemark was observed in some areas. G-B and G-C showed no cartilage formation but a great amount of fibrous tissue, with only a little bone formation.

Table 6. Continued (5)

Ref	Scaffold(s) # Cartilage part & Interface part * Bone part	Animal model/implantation time	Cell type(s)	Method	Outcome
Fedoroyich <i>et al.</i> [163]	# Alginate hydrogels * Alginate hydrogels/BCP	Female nude mice, 6 weeks old, subcutaneous implantation. 6 weeks.	Human chondrocytes and BMSCs	Bilayered constructs were prepared by a 3D fiber deposition technique using alginate/chondrocytes and alginate/BCP/BMSCs hydrogels. Then, the constructs were implanted.	After 6 weeks, constructs exhibited heterogeneous tissue formation corresponding to the deposited cell type. Osteocalcin and Col I positive matrixes were presented around the BCP particles, indicating onset of osteogenic differentiation of the MSCs. Positive cartilage-specific markers-Col II and Col VI were presented.
Giannoni <i>et al.</i> [164]	# PCL * PCL/HA	Nude mice, 1 month old, subcutaneous implantation. 9 weeks.	Bovine chondrocytes and BMSCs	The chondrocytes and BMSCs were seeded onto the chondral and subchondral layers, respectively. Then the constructs were implanted.	The structural integrity of the graft was successfully validated by tension fracture tests. Ceramic granules within the bony layer were surrounded by thick mature bone. A cartilaginous matrix appeared in the chondral layer. Vascularization was mostly observed in the bony layer, with a statistically significant higher blood vessel density and mean area than those in chondral layer.
Da <i>et al.</i> [165]	# Bovine decellularized articular cartilage ECM & Compact PLGA/ β -TCP layer * PLGA/ β -TCP skeleton wrapped with Col I scaffold	Rabbit, more than 24 months old, OCD in the patellofemoral groove (5 mm in diameter and 6 mm in depth). 3 and 6 months.	Rabbit BMSCs	The osteogenic differentiated cells were seeded onto the bony layer, and the chondrogenic differentiated cells were seeded onto the chondral layer. After 3 days, the constructs were implanted into OCD. Bilayered constructs without compact layer as control.	The anti-tensile and anti-shear properties of the compact layer-containing biphasic scaffold were significantly higher than those of the compact layer-free biphasic scaffold <i>in vitro</i> . <i>In vivo</i> studies revealed superior macroscopic scores, GAG, collagen content, micro tomography imaging results, and histological properties of regenerated tissue in compact layer-containing biphasic scaffold compared to the control.
Ding <i>et al.</i> [166]	# PLA coated PGA scaffold * PCL/HA	Nude mice, subcutaneous implantation. 10 weeks.	Goat chondrocytes and BMSCs	CAD/CAM technology was employed to prepare PGA/PLA scaffold in the shape of the cartilage, and PCL/HA scaffolds in the shape of femoral head without cartilage. Chondrocytes were cultured on the chondral layer in chondrogenic medium and BMSCs were cultured on the bony layer in osteogenic medium, for 2-3 weeks. Then the two constructs were assembled and implanted.	After 10 weeks, the goat femoral heads were successfully regenerated by the cell-scaffold constructs. The regenerated femoral heads presented a precise appearance in shape and size similar to that of native goat femoral heads, with a smooth, continuous, avascular, and homogeneous cartilage layer on the surface and stiff bone-like tissue in the microchannels of PCL/HA scaffold. Histological analysis showed well-integrated OC interface.
Duan <i>et al.</i> [167]	# PLGA * PLGA	Rabbit, 5-6 months old, 3.0-3.3 kg/each, OCD in femoral condyles (4 mm in diameter and 5 mm in depth) 6 and 12 weeks.	Allogenic rabbit BMSCs	Bilayered PLGA scaffolds of different pore size in the chondral or subchondral layers were prepared. The scaffolds were seeded with allogenic BMSCs and cultured for 7 days, then implanted.	The cell/scaffold supported the simultaneous regeneration of articular cartilage and subchondral bone. The best results were observed in scaffold with 100-200 μ m pores in the chondral layer and 300-450 μ m pores in the osseous layer.

Table 6. Continued (6)

Ref	Scaffold(s) # Cartilage part & Interface part * Bone part	Animal model/implantation time	Cell type(s)	Method	Outcome
Jiang <i>et al.</i> [168]	# Col * Col	Rabbit, male, 2.5-3 kg/each, OCD in patellar groove (4 mm in diameter and 3 mm in depth). 6 and 12 weeks.		The PVP-I loaded bilayered collagen scaffold were implanted in rabbit OCD. Scaffolds alone and untreated defect were used as controls.	Implantation of PVP-I treated scaffold enhanced subchondral bone formation at the 6 th week compared with scaffold alone. There were no significant differences in cartilage regeneration by the scaffolds with PVP-I or scaffold alone.
Sheehy <i>et al.</i> [169]	# Agarose hydrogel with chondrocyte * Agarose hydrogel with BMSCs	Nude mice 28 days.	Porcine chondrocytes and BMSCs	Chondrocytes and BMSCs were incorporated in agarose hydrogels, and the bilayered constructs were prepared. Non-layered hydrogels also prepared with only one kind of cells. Constructs were cultured in chondrogenic medium for 3 weeks, and then implanted subcutaneously, or subjected to hypertrophic medium for 4 weeks.	The co-cultured bilayered constructs showed enhanced chondrogenesis in the chondral layer, maintaining the chondrogenic phenotype of chondrocyte. This system suppressed hypertrophy and mineralization in the osseous layer. The hypertrophic culture induced mineralization of the osseous layer. <i>In vivo</i> , endochondral ossification was restricted to the osseous layer, leading to the form of an OC tissue.
Zhang <i>et al.</i> [170]	# Porous Col layer * Dense Col layer Or # PLLA nanofibrous layer * Porous Col layer	Rabbit, male, 2.5-3.0 kg/each, OCD in patellar groove (4 mm in diameter and 3.5-4 mm in depth). 6 and 12 weeks.		Bilayered Col or bilayered Col/PLLA nanofiber scaffolds were implanted into the OCD of rabbit. Untreated defects as control.	Implantation of COL-nanofiber scaffold induced more rapid subchondral bone emergence and better cartilage formation compared to the control, based on the histological staining, biomechanical test, and micro-CT data.
Zhang <i>et al.</i> [171]	# Col * Silk/HA	Rabbit, 2.5-3.0 kg/each, OCD in patellar groove (4 mm in diameter and 3.5 mm in depth). 16 weeks.		Scaffolds were implanted in the defects. And intra-articular injection of the defects with PTHrP or PBS (control) was carried out at the 4 th -6 th weeks, 7 th -9 th weeks, and 10 th -12 th weeks with time windows every 7 days.	Defects treated with PTHrP at the 4 th -6 th weeks time window exhibited better regeneration (reconstitution of cartilage and subchondral bone) with minimal terminal differentiation (hypertrophy, ossification and matrix degradation), as well as enhanced chondrogenesis (cell shape, collagen II, and GAG content), compared with treatment at other time windows. The timing also influenced PTHrP receptor expression.

3D=three dimensional; BCP=biphasic calcium phosphate (TCP/HA); BMSCs=bone marrow mesenchymal stem cells; CAD/CAM=computer-aided design and computer-aided manufacturing; CaP=calcium phosphate; CMCh=carboxymethyl chitin; Col=collagen; CPP=calcium polyphosphate; EDTA=ethylenediamine tetraacetic acid; HA=hydroxyapatite; ICRS=International Cartilage Repair Society; n-HA=nano-Hydroxyapatite; OC=osteochondral; OCD=osteochondral defect(s); PA6=polyamide 6; PBS=phosphate buffered saline; PBT=poly(butylenes terephthalate); PCL=poly(ϵ -caprolactone); PDLA=poly(D-lactic acid); PEC=polyelectrolyte composite; PEGDA=poly(ethylene glycol) diacrylate; PEOT=poly(ethylene oxide terephthalate); PLGA=poly(glycolic-co-lactic acid); PLLA=poly(L-lactic acid); PVA=poly(vinyl alcohol); PVP-I=polyvinylpyrrolidone-iodine; Ref=reference; SCID=severe combined immunodeficiency; TCP=tricalcium phosphate.

months, dexamethasone and TGF- β 1 treated group showed 70% of the defects were repaired with hyaline cartilage and cancellous bone.

Similar to the *in vitro* studies, layered scaffolds were investigated intensively for OCD regeneration *in vivo* using acellular or cellular strategy (Table 6). Alhadlaq *et al.* [138] mixed the poly(ethylene glycol) diacrylate solution with chondrogenic and osteogenic MSCs respectively, and then generated a bilayered human condyle like structure which was implanted subcutaneously in mice. The results showed only the chondrogenic layer presented GAG and the osteogenic layer demonstrated calcium. Shao *et al.* [145] loaded the allogenic BMSCs into polycaprolactone (PCL) scaffolds with fibrin gel and implanted the constructs into rabbit OCD as subchondral bone part. The cartilage layer was filled with fibrin gel with MSCs. It was found that the transplanted cells were viable after 5 weeks. At 3 months, the samples showed good cartilage repair, but only around 30% samples maintained good cartilage appearance after 6 months. They also loaded allogenic BMSCs into PCL and PCL/tricalcium phosphate scaffolds by fibrin gel, and then implanted the scaffolds sequentially by press-fit method [146]. The results showed good and consistent bone regeneration. After 3 months, the explants showed regenerated cartilage with the scaffolds. At 6 months, both degraded samples and good appearance samples were observed. Tampieri *et al.* [152] developed a graded biomimetic scaffold composed hyaluronan/collagen corresponding to the cartilage layer, biomineralized collagen with lower content mineral mimicking the tidemark, and biomineralized collagen with higher content mineral resembling the subchondral bone. These scaffolds were seeded with BMSCs and subcutaneously implanted in nude mice. After 8 weeks, cartilaginous matrix deposition was observed only in the loose chondral layer, and bone tissue was only formed in the subchondral layer.

Biological cues have been introduced into the scaffolds for the purpose of enhancing chondrocyte proliferation, or guiding the progenitor cells to mature and subsequently form healthy OC tissues. A representative method is the incorporation of growth factors in the scaffolds. Holland *et al.* [82] incorporated TGF- β 1 loaded gelatin microspheres into the chondral part of bilayered OPF hydrogels for OCD implantation in rabbit. They found that the growth factor had some therapeutic effect on the quality of the repaired tissue. Huang *et al.* [83] combined the basic fibroblast growth factor (bFGF) in the poly(L-lactic acid)/amorphous calcium phosphate (ACP/PLLA) hybrid scaffolds and implanted these scaffolds in rabbit OCD. After 12 weeks, the defects were filled with well generated

cartilage tissues which were positively stained with collagen II. Nevertheless, pure PLLA scaffolds with bFGF only showed fibrocartilage tissue filling in the defect and inferior bone formation ability as compared to the ACP/PLLA group. Gene delivery was also employed for OCD regeneration. Chen *et al.* [88] generated a bilayered gene-activated scaffold which contained plasmid TGF- β 1 activated chitosan-gelatin scaffolds as chondral part and plasmid BMP-2 activated HA/chitosan-gelatin scaffolds for subchondral part. When BMSCs were cultured in these scaffolds, high level of TGF- β 1 and BMP-2 protein secretions were observed in the chondral layer and subchondral layer, respectively. Further implantation in OCD in rabbits showed that these scaffolds can simultaneously support articular cartilage and subchondral bone regeneration under a spatial controlled manner. In the further, incorporation of clinical relevance dose of growth factors and the control of the release manner should be further investigated.

Development of bioactive scaffolds without growth factors is an attractive approach for OCD regeneration. Extracellular matrix (ECM) based scaffold could be a good choice for this purpose. ECM is produced by the host cells and contains bioactive molecules for driving tissue regeneration as well as biomimetic microenvironments for cells homing. There were successful reports on ECM application in clinics, such as cardiovascular and muscle [172, 173]. The ECM based scaffolds can be vitalized or devitalized tissues. Emans *et al.* [81] explored the ectopically generated cartilage tissue in periosteum for OCD regeneration in rabbit, and the defects were repaired with cartilage of similar quality to the normal one after 3 weeks implantation. Chondrocytes-derived ECM scaffolds were studied by Jin *et al.* [25]. The lyophilized ECM scaffolds were used for chondrocytes culture and then implanted in OCD in rabbit. They found that the longer the chondrocytes cultured in the ECM scaffolds, the better results were achieved regarding hyaline cartilage regeneration and subchondral bone restored. Decellularized OC tissue and non-osteochondral tissues were also used for OCD regeneration. Yagihashi *et al.* [84] filled the rabbit OCD with decellularized dentin matrix and found that cartilage with similar thickness to the normal one were achieved after 9 weeks. Yang *et al.* [90] produced a bilayered decellularized cartilage matrix and decellularized cancellous bone matrix scaffold and combined with chondrogenic differentiated BMSCs in chondral part for canine OCD regeneration. Both scaffold only group and the scaffolds with cells group showed regular subchondral bone regeneration after 3 and 6 month implantation. With

cells, the defects exhibited higher histologic scores than those of the pure scaffolds group, and repaired with cartilage-like tissues.

Besides the scaffolds, cells, and biological cues, the external stimulus (such as mechanical stimulus) is also important for the healing of OC tissues. Chang *et al.* [128] found that the continuous passive motion (CPM) treatment on the rabbits which undergone OCD implantation can promote better OCD regeneration compared with rabbits subjected to the immobilization or the intermittent active motion treatment (Figure 2). These results inspired that the post-surgery physical stimulus synergistically contribute to the healing of OCD.

3. Future Perspectives in OC Tissue Engineering

In the future, the development of bioactive and biomimetic scaffolds for OCD regeneration will remain a major issue. The ideal scaffold should be easily integrated with host tissue and include signals to guide the proliferation and differentiation of specific cells to form normal stratified OC tissues. During the regeneration, the scaffolds should be capable of preventing the invasion of synovial fluid to the subchondral bone and the vascularization in the chondral layer. All these requirements depend on the advances of materials science, processing technology, and drug/gene delivery systems.

The defect conditions of the patient should always be taken into account when design the scaffolds. Since OCD in every patient is different, the customized design of the scaffolds for patients is of great demand. Nowadays, the computer-aided design and computer aided manufacture (CAD/CAM) technologies provide possibility to built scaffolds for specific individuals, no matter the shape and size of the defects [166]. In the following, efforts should be addressed to use these techniques for preparation of scaffolds with various materials, or even including bioactive factors. Also, the application technique of the scaffold should be taken into consideration, since non-invasive techniques (preferred by surgeons nowadays) imply many times dimensional constrains to the materials.

In OC tissue engineering, nanotechnology presents an enormous potential. For instance, scaffolds exhibiting nanofibrous structure can enhance cells' attachment and thus promote tissue regeneration [52, 64, 157]. Hu *et al.* [64] showed that the expression of early chondrogenic gene marker was enhanced on the nanofibrous matrix. Erisken *et al.*

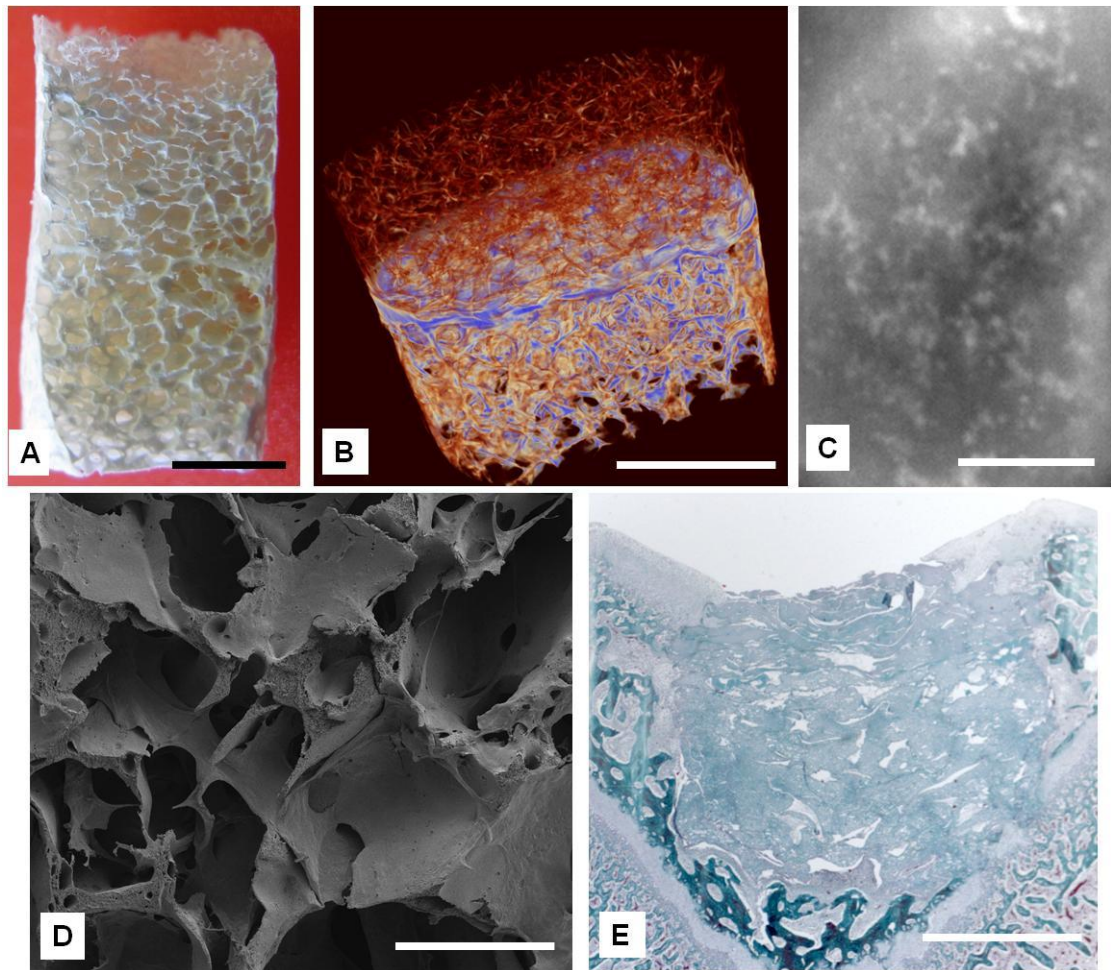


Figure 3. Silk based bilayered scaffold for OCD regeneration. (A) Macroscopic image of the bilayered scaffold. Top layer composed of silk fibroin, bottom layer constituted by silk and nano-calcium phosphate particles (Scale bar: 4 mm). (B) Three dimensional micro-computed tomography image. Brown area indicated silk matrix, and the blue domain was corresponding to the calcium phosphate phase (Scale bar: 4 mm). (C) Nano-calcium phosphate particles distribution in the bottom layer of the scaffold. The white particles indicated the nano-sized CaP particles and the gray region was silk matrix (Scale bar: 2 μ m). (D) Rabbit BMSCs attachment on the bilayered scaffolds after 7 days culture *in vitro* in basal medium (Scale bar: 500 μ m). (E) Masson's trichrome staining of the explants after implantation of the bilayered scaffolds in rabbit OCD for 3 weeks (Scale bar: 2 mm).

[52] evaluated electrospun nanofibrous meshes in OCD regeneration and good reparative outcome was achieved. Coburn *et al.* [120] developed nanofibrous hydrogels which presented better chondral regeneration outcomes compared with the non-nanofibrous hydrogels. The use of ceramic based nanoparticles to generate a nanocomposite matrix is another very interesting approach. Nano-sized ceramic particles

not only provide the scaffold with osteoconductive properties but also are able to modulate the degradation rate of the composite due to their easy dissolution. Such nanocomposites have been applied for OCD regeneration in preclinical or clinical studies, or *in vitro* for OCD interface regeneration [22, 31, 69, 89, 112, 116, 117]. Promising results were achieved from these studies. Recently, we have produced silk/nano-sized CaP (silk/nano-CaP) porous scaffolds [174]. These scaffolds promoted new bone ingrowth in a preliminary *in vivo* study in a rat model [175]. Based on this work, we have specifically designed a bilayered scaffold consisting of a porous silk matrix as the top layer and a silk/nano-CaP as the bottom layer for OCD tissue engineering (Figure 3). The results of subcutaneous and OCD implantation in rabbit showed that these scaffolds induced no inflammation *in vivo*, allowed tissue ingrowth, integrated well with the host tissue, and promoted cartilage and subchondral regeneration [176]. In the future, it would be also interesting to explore the application of nanoparticles as drug carriers or as cell targeting bullets in OCD tissue engineering. Previously, our group has developed dexamethasone incorporated nano-sized dendrimer which could induce the osteogenic differentiation of MSCs [177]. Besides its application in the tissue engineering scaffolding, nanotechnologies can also be applied in cell therapy approaches. Nano-sized magnetic particles have also been successfully used to label cells and the accumulation of labeled cells was achieved in the OCD defects under external magnetic force [178, 179]. These studies open a door to better monitor the cells behavior *in vivo*.

Another promising topic for OCD regeneration is the utility of reprogrammed cells. The gene transfer technology has been used for OCD regeneration [180-187]. Grande *et al.* [180] transduced rabbit periosteal stem cells with either bone morphogenetic protein-7 (BMP-7) or sonic hedgehog (Shh) gene and then the transduced cells were seeded into poly(glycolic acid) (PGA) scaffolds as implantation in rabbit OCD. The scaffolds with BMP-7 or Shh gene significantly enhanced the quality of the repaired tissue resulting in a smoother surface and more hyaline cartilage compared with control group. The BMP-7 group remodeled the subchondral bone much faster than the Shh gene group. Schek *et al.* [181] prepared biphasic composite scaffolds and seeded with fibroblasts transduced with BMP-7 in the ceramic phase and differentiated chondrocytes in the polymeric phase. The subcutaneous implantation results showed the scaffolds promoted simultaneous growth of bone, cartilage, and a mineralized interface tissue. Ueblicher *et al.* [187] performed the transfection of rabbit chondrocytes with lacZ gene which fused with

tetracycline-responsible element (TRE). The gene expression of the transfected cells was controlled by the non-toxic drug, such as tetracycline or doxycycline. The transfected cells were seeded into collagen sponges and implanted into OCD in rabbit. The lacZ-gene-expression can be detected for 3 weeks with doxycycline treatment. The implants were integrated well with the host tissue. This study provided new insights for regulation of gene expression for OCD treatment. Other gene transferred primary or stem cells, such as iPS, are also promising cell sources for OCD regeneration. In the future, more investigations on the optimization of introduced genes, improvement of transfection efficiency, and modulation of the transfected gene expression should be performed.

OC Tissue Engineering		
	Strategies	Challenges
Clinical strategies	MACI	Two operations; fixation problem; donor site morbidity; non-sufficient subchondral bone regeneration.
	MASI	Multi-steps on blood and bone marrow collection, platelet gel formation, and bone marrow concentration; Fixation problem; non-sufficient subchondral bone regeneration.
	Layered scaffold without cells	Clinical results are preliminary; the influence of scaffold properties on long-term regeneration outcomes needs to be validated .
	Combination of MACI and layered scaffold	Donor site morbidity; clinical results are preliminary; the long-term regeneration outcomes and influence of scaffold properties need to be validated.
Pre-clinical strategies	Single layer or layered scaffold alone	Interaction between OCD regeneration outcomes and scaffolds properties (morphology, component, degradation...) or external stimulus (mechanical, chemical...) still needs to be elucidated.
	Single layer or layered scaffold with cells	The influence of scaffolds properties (morphology, chemical components...) on the cells phenotype or differentiation is not fully understood; controlling the cell fate is still a big hinder.
	Single layer or layered scaffold with GF/Bioactive agents alone or GF/Bioactive agents and cells	The incorporation dose and release profile of GF/Bioactive agents need to be optimized; The long-term cells fate and regeneration outcome need to be validated.

Scheme 1. Current tissue engineering strategies and challenges for OCD regeneration. For clinical strategies, MACI: Matrix-induced autologous chondrocyte implantation; MASI: matrix-induced autologous stem cells implantation. For pre-clinical strategies, “scaffolds” indicated porous scaffold or hydrogels with single layer or layered structure, “cells” indicated primary cells or stem cells, “GF” indicated growth factor(s).

Based on the amount of information gathered over the last decades while searching for effective strategies for OCD regeneration, it is possible to comprehend the degree of complexity of this task and its limitations (Scheme 1).

4. Conclusions

Tissue engineering strategies, despite introducing more complexity and detail to the current treatments, present a high potential for OCD regeneration. During the last decade, numerous advances have been achieved in OCD tissue engineering. MACI is one of the standard procedures in clinical treatment of OCD. By its turn, MASI has been successfully applied in a few clinics trials, but only limited to BMSCs. A few layered scaffolds have showed successes for OCD regeneration in clinics with acellular strategy. Preclinical studies have showed promising achievements in biomimetic and bioactive layered scaffolds, scaffolds combined with growth factors or stem cells or reprogrammed cells, and interface regeneration. In fact, OCD regeneration is a systematic engineering, integrative strategy should be employed. The future challenges include the development and application of bioactive and biomimetic scaffolds in clinical trials, for example using decellularized ECM scaffolds, or scaffolds incorporation of growth factors and/or stem cells. Besides, the production of customized scaffolds for patients using the computer-aided design and prototyping technologies is promising. The influence of the post-operation treatments on the defect sites, including mechanical or other stimuli, are worthy to explore. Although there are still many critical problems to be solved, tissue engineering still represents the most promising alternative for OCD regeneration.

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Section 2.

Chapter II

Materials and Methods

Chapter II

Materials and Methods

This chapter intends to provide in detail the experimental work and protocols related to the obtained results presented in Section 3 and 4. Furthermore, the rationale of this thesis will be introduced, namely the aspects regarding the selection of the materials, the scaffolds processing methods, the hydrogels preparation approaches, and the physicochemical and biological evaluation techniques.

1. Materials

1.1. Silk fibroin (SF)

SF studied in this thesis was produced by the *Bombyx mori* silkworm (Figure 1). SF is synthesized by the epithelial cells of the silkworm, followed by storage in the silkworm glands (up to 30 %, wt/vol.) before spinning into fibers [1, 2]. Spun SF fibers in the cocoons are normally 10-25 μm in diameter. It contains two fibroin proteins-a light chain (around 26 kDa) protein and a heavy chain (about 390 kDa) protein conjugated by a single disulfide bond [3]. The surface of the SF fibers is coated by a glue-like protein named sericin (20-310 kDa) [3]. It has been reported that sericin can induce inflammatory response *in vivo*, thus it is necessary to extract sericin from the cocoons before processing SF for biomedical applications [1]. Sericin can be extracted by degumming process (Figure 1), namely boiling the cocoon in alkaline solution, such as sodium carbonate solution or soap solution [4]. After degumming, the obtained SF is around 70% of the original cocoon mass (not including the worm mass) [3].

SF fiber is well-known for its extreme strength, which comes from its secondary structure [1]. In the heavy chain of SF, the repetitive amino acid sequence glycine-alanine-glycine-alanine-glycine-serine (GAGAGS) self-assemble into an anti-parallel β -sheet structure (Silk-II). The stacked β -sheets are highly crystalline and the protein molecules among these area present strongly interaction through intra- and inter-molecular hydrogen

bonds, and van der Waals forces between the chains. These interactions endow the SF fiber with robust mechanical properties.

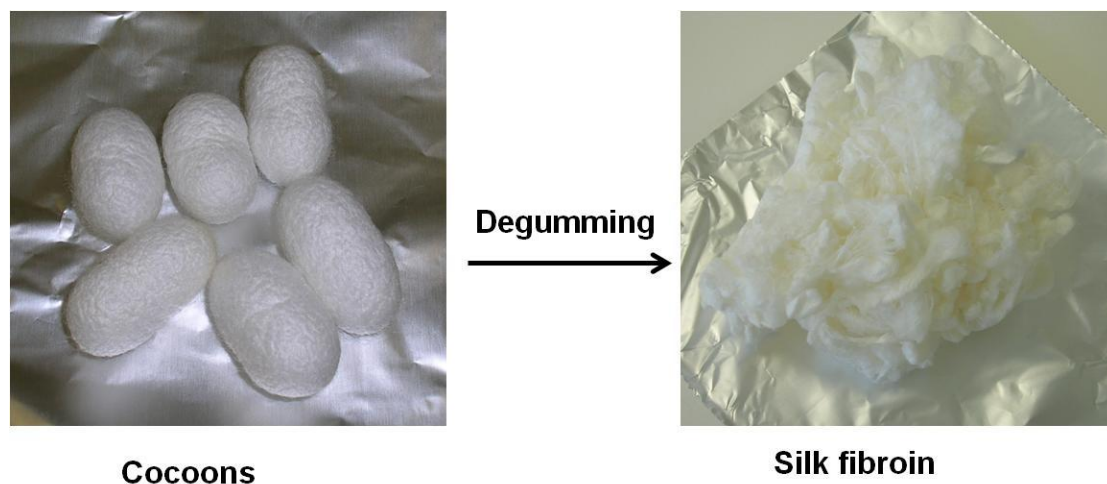


Figure 1. *Bombyx mori* cocoons and purified silk fibroin.

Degummed SF fibers can be used as sutures [1], or dissolved and subsequently processed into different formats, such as scaffold [5], hydrogel [6], membrane [7], and microspheres [8]. Organic solvent hexafluoroisopropanol (HFIP) has been used to dissolve SF [5]. SF also can be dissolved in concentrated lithium bromide solution [5], ionic liquid [9], lithium thioisocyanate [4], and calcium chloride/ethanol/water system (in a 1:2:8 molar ratio) [10]. The constructs prepared from the regenerated SF solution (Organic, aqueous, or ionic liquid system) would form β -sheet structure when subjected to chemical or physical stimulus, such as addition of salt [11], immersion in alcohol solution [5], increase of the temperature [5], decrease of pH [12], ultrasonication [13], and vortex [14]. The crystalline structure gives the SF constructs with superior mechanical properties, water insoluble ability, and slow degradation profile [11]. Additionally, the β -sheet content in SF can be controlled by the method used, thus SF constructs with a broad tuning window in the properties can be prepared [15].

SF is a biodegradable biopolymer and has been extensively studied for tissue engineering application [16, 17]. Many studies showed that SF constructs are cytocompatible and did not induce severe *in vivo* inflammatory response [12, 18, 19].

Regarding the research work within this thesis, the SF was first isolated from *Bombyx mori* cocoons and then used for preparation of SF based scaffolds and hydrogels. The cocoons were supplied by the Portuguese Association of Parents and Friends of Mentally Disabled Citizens (APPACDM, Castelo Branco, Portugal).

1.2. Calcium phosphate (CaP)

CaP is the most important inorganic component in the hard tissues of human body [20]. It can be found in bone and teeth [21]. The human bone consists of 50-60% CaP which provides the bone hardness and stability [20]. In bone, the CaP presents in the form of low crystalline carbonated hydroxyapatite- $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$.

Bone defects induced by trauma or diseases are common problems in orthopedic [22]. These problems bring pain and morbidity to the patients. Autologous or allogenic implantation is disadvantageous in lack of sufficient donors or risk of diseases [23]. Artificial bone implants developed in the early period had problems to bind to the natural bone [21, 24]. Later on, these problems were overcome by the finding of bioactive materials which can bond to the bone in the living body [24]. Those bioactive materials includes Bioactive glass®, sintered CaP (such as hydroxyapatite, β -tricalcium phosphate, biphasic hydroxyapatite/ β -tricalcium phosphate), and glass-ceramic A-W system [24]. These bioactive materials had been applied in clinics as bone substitute [20, 24]. However, these materials degrade very slowly or non-degradable, and their mechanical properties did not match the one in the natural bone [21].

Recently, tissue engineering strategy has been introduced for bone regeneration by using three-dimensional (3D) scaffolds implantation in the bone defects [22, 25]. With this strategy, it is possible to regenerate natural bone by using 3D scaffolds consisting of bioactive materials. The pure CaP based scaffolds, such as hydroxyapatite (HA) or β -tricalcium phosphate (β -TCP) were able to promote new bone formation *in vivo* [21]. The sintered CaP porous scaffolds are of intrinsic brittle nature and slow degradation profile [21]. Development of inorganic/organic bioactive composite scaffolds is a promising approach for bone tissue engineering [26, 27]. The preparation of low crystalline CaP particles is helpful to decrease the degradation time of these materials and meanwhile maintain their bioactivity in bone regeneration [28].

In this thesis, low crystalline CaP nano-particles were synthesized via an *in-situ* method aiming for bone regeneration. The details of the synthesis of nano-CaP particles will be described in the Scaffold Preparation section.

1.3. Reagents

Unless addressed otherwise, all the reagents used in this thesis were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2. Scaffold Preparation

2.1. Methodologies for scaffold processing: Overview

Many efforts have been paid to develop methods to generate porous structure in scaffolds with adequate pore size and porosity, as well as specific morphology for biological application [22]. Nowadays, there are several common methods for scaffolding, for instance, salt-leaching [11], fiber-bonding [29], microsphere sintering [30], freeze-drying [19], and rapid-prototyping [31]. Among all these methods, salt-leaching is an efficient and low cost approach to prepare scaffolds with controlled pore size, high interconnectivity, and homogeneity. This approach is normally applied for water-insoluble polymers. In practice, the polymer will be dissolved organic solvent, and then transferred into a mould. In the following, salt particles of specific size are added into the mould. After drying, the salt in the constructs are leached out in water, thus the porous polymer scaffolds can be formed. In this approach, the pore size is controlled by the size of the salt particles.

Regarding the aqueous polymer, this method is not feasible, since the polymer would dissolve in water during salt-leaching. But SF is an exception. After dissolving in concentrated salt solution and dialyzing, the SF aqueous solution can be formed. Kim *et al.* [11] found that the addition of sodium chloride particles into the SF aqueous solution would induce the β -sheet formation in SF, by this way the aqueous-stable porous SF scaffolds is developed. Compared to the preparation of salt-leached SF scaffolds from the HFIP system [5], the aqueous derived salt-leaching approach is more friendly for environment protection and low cost.

However, Kim *et al.* [11] were only able to prepare salt-leached SF scaffolds with no more than 10 wt.% aqueous SF solution. And the mechanical properties from these scaffolds were much lower than the ones from the SF scaffolds prepared in HFIP system. It is necessary to improve the mechanical properties of the aqueous-derived salt-leached SF scaffolds to better fulfill the requirement of tissue engineering application.

In order to prepare a bioactive scaffold for bone tissue engineering, various composite scaffolds combining CaP particles and polymers have been developed [27, 28, 32, 33]. However, the physical blending of the particles with polymeric materials would induce the aggregation of the particles and compromise the homogeneity of the system. To increase the affinity between the CaP particles and organic phase and to achieve homogeneous distribution of the CaP particles in the polymeric phase without aggregation are still big challenges. The *in-situ* synthesis of CaP particles in the polymer phase is a good way to solve this problem [34]. This approach was able to prepare the nano-sized CaP particles with homogeneous distribution in the polymeric phase [35].

In this thesis, it was attempted to overcome the above mentioned technology limitation and prepare mechanical robust SF based scaffolds for cartilage, bone, and osteochondral regeneration, by using salt-leaching approach and highly concentrated aqueous SF solution. Three kinds of SF based scaffolds were successfully developed: (i) Pure salt-leached SF scaffolds were prepared by using up to 16 wt.% aqueous SF solution, aiming for cartilage or meniscus regeneration; (ii) nano CaP particles were introduced into the SF scaffolds by an *in-situ* synthesis method, and then salt-leached silk/nano CaP (Silk-NanoCaP) scaffolds were generated for bone tissue engineering; (iii) based on the previous works, a salt-leached bilayered Silk/Silk-NanoCaP scaffolds were created for osteochondral regeneration.

2.2. Salt-leached aqueous-derived SF scaffolds

At first, SF was purified from the cocoons. For this purpose, each *Bombyx mori* cocoon was cut into several pieces, and the worms and impurities inside the cocoon were removed. In the following, 5 g cocoons were boiled for 1 hour in 2 L sodium carbonate solution (0.02 M) in order to extract the glue-like protein sericin and wax [36]. Afterwards,

the purified SF was washed by distilled water several times, and dried in a clean place. The dried SF should be stored in dark at room temperature.



Figure 2. Concentrated aqueous silk fibroin solution.

In order to obtain aqueous SF solution, 5 g purified SF was dissolved in 25 mL of 9.3 M lithium bromide solution at 70°C for 1 hour, yielding a solution around 16% (wt./vol.) [7]. The solution was dialyzed in distilled water using a benzoylated dialysis tubing (MWCO: 2 kDa), for two days. During the dialysis, the water should be changed at least three times per day. Afterwards, the SF aqueous solution was dialyzed against a 20 wt.% poly(ethylene glycol) solution (20,000 g/mol) for around 6 hours [2]. Finally, the dialysis tubing was carefully washed in distilled water, and SF solution was collected to a flask (Figure 2). For determination of the concentration of the SF solution, around 0.5 mL SF solution (weight measured) was dried in an oven overnight at 70°C, and then the concentration was obtained through dividing the dried weight by the wet weight. The prepared SF solution was stored at 4°C until further use.

Granular sodium chloride was prepared by sieving the sodium chloride in an analytical sieve shaker (Retsch, Haan, Germany) in the range of 500-1000 μm . The prepared concentrated SF solution was diluted into 8%, 10%, 12% and 16% (wt.%), respectively. The scaffolds were prepared by transferring 1 mL of SF solution (8-16%) into a silicon tubing (inner diameter: 9 mm), followed by addition of 2 g of granular sodium chloride (500-1000 μm) [11]. In the case of the preparation of scaffolds from SF solutions of 12% and 16%, the sodium chloride particles were slowly added into the silicon tubing, with

gentle tapping in the tubing wall to facilitate the precipitation of the salt particles. Afterwards, the silicon tubing was placed in a Petri dish and dried at room temperature for 48 hours. In order to extract the sodium chloride, the tubing was immersed in distilled water for 3 days. Finally, the scaffolds were obtained by using a stainless steel punch (inner diameter: 6 mm) in order to remove the outer skin, followed by freezing at -80°C more than 6 hours and freeze-drying (CRYODOS-80; Telstar, Barcelona, Spain) for 3 days. The prepared SF scaffolds are herein designated as silk-8, silk-10, silk-12 and silk-16, according to their initial concentrations, respectively (Figure 3).

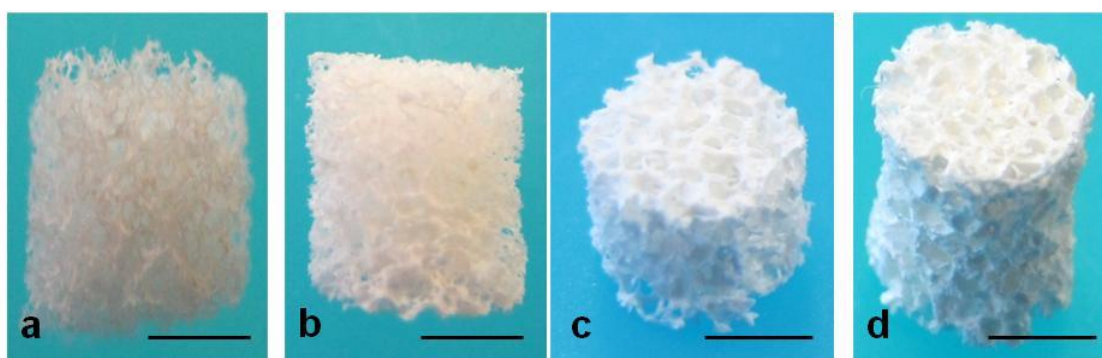


Figure 3. Macroscopic images of the silk fibroin scaffolds prepared by salt-leaching/freeze-drying approach. (a-d) scaffolds derived from 8, 10, 12 and 16 wt.% aqueous silk fibroin solutions, respectively (Scale bar: 3 mm).

2.3. Salt-leached aqueous-derived Silk-NanoCaP scaffolds

The purification of SF from cocoon, the dialysis of SF, and the concentration of SF solution were performed as mentioned above (Section 2.2. in this Chapter).

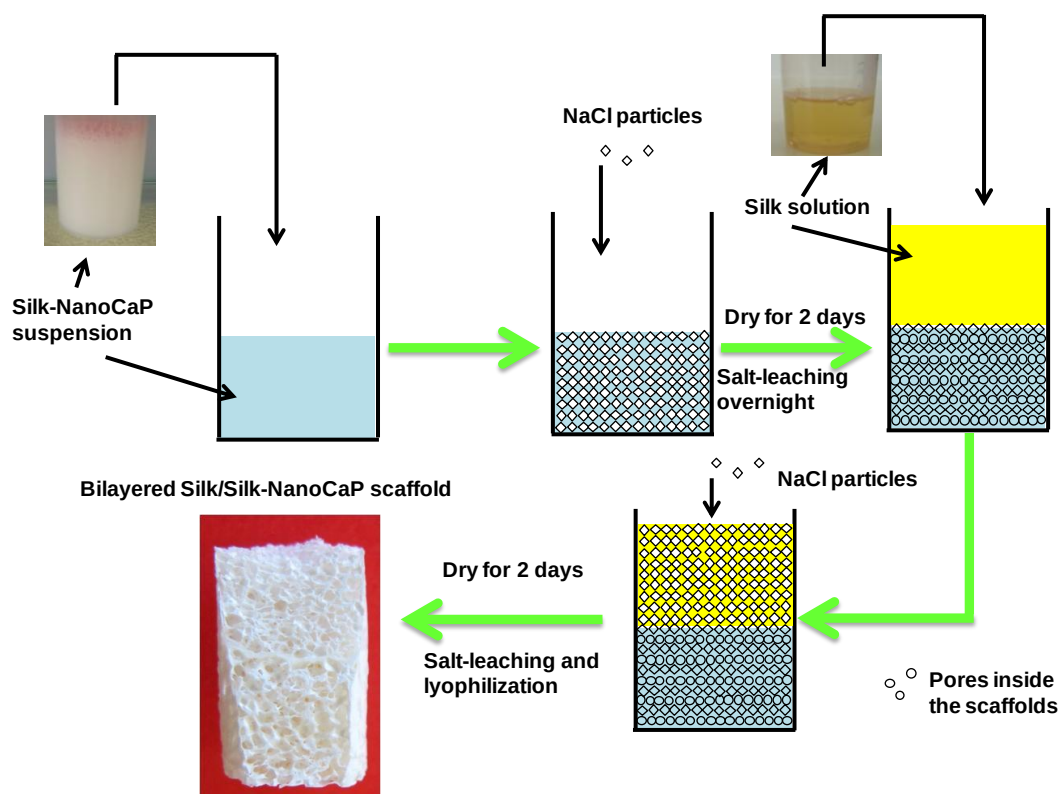
Silk-NanoCaP composite was prepared via an *in-situ* synthesis method [37]. At first, the concentrated SF aqueous solution was diluted to 16 wt.%. Different amounts of a calcium chloride solution (6 mol/L) were mixed with the SF solution for 5 minutes, followed by the addition of different amounts of an ammonia dibasic phosphate solution (3.6 mol/L). The theoretical calcium to phosphate atomic ratio was maintained at 1.67 in each group. The pH value of the system was adjusted to around 8.5 by the addition of ammonia (30%). The suspension was stirred for 30 minutes and subsequently aged for 24 hours at room temperature (Figure 4). The theoretical content of the CaP formed in the SF solution was determined based on the hypothesis that the calcium and phosphate species would react

completely to form stoichiometric hydroxyapatite- $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ [38]. Silk-NanoCaP composites possessing a theoretical CaP content (theoretical CaP mass divided by the total mass of SF) of 4, 8, 16 and 25 wt.% were prepared. Fraction of sodium chloride particles having a size in the range of 500-1000 μm were obtained by using an analytical sieve shaker (Retsch, Haan, Germany). The Silk-NanoCaP scaffolds were prepared by addition of 2.0 g of sodium chloride granule (500-1000 μm) to 1 mL Silk-NanoCaP suspension, in a silicone tubing of 9 mm inner diameter; followed by drying the material inside the silicone tubing at room temperature, for 2 days. Sodium chloride and the by-products were removed by immersion in distilled water for 2 days. The skin of the Silk-NanoCaP scaffolds was removed by a stainless steel punch of 6 mm inner diameter. Finally, the scaffolds were frozen at -80°C for at least 6 hours followed by lyophilization in a freeze-drier (CRYODOS-80; Telstar, Barcelona, Spain). The prepared Silk-NanoCaP scaffolds were designated as silk/CaP-4, silk/CaP-8, silk/CaP-16, silk/CaP-25, according to their initially incorporated amount of CaP, respectively. The SF scaffolds (control) without CaP were also prepared from a 16 wt.% aqueous solution following above mentioned procedure (Section 2.2. in this Chapter).



Figure 4. Representative image of the prepared silk/nano calcium phosphate suspension.

2.4. Salt-leached aqueous-derived bilayered Silk/Silk-NanoCaP scaffolds



Scheme 1. Procedure for the preparation of bilayered Silk/Silk-NanoCaP scaffolds.

Regarding the preparation of the bilayered scaffolds (Scheme 1), Silk-NanoCaP scaffolds were prepared firstly as mentioned above (Section 2.3. in this Chapter) until the addition of sodium chloride particles. The amount of theoretically introduced CaP was fixed at 16 wt.% (CaP:Silk). After addition of sodium chloride particles into the Silk-NanoCaP suspension in the mould, the mould was dried for 2 days, and then immersed in distilled water overnight. In the following day, the Silk-NanoCaP scaffolds were cut into pieces after removal from the moulds. Each piece of the scaffolds was placed into the bottom of a new silicon mould and 300 μL of 16 wt.% silk solution was added onto the top of Silk-NanoCaP scaffolds. Then, 600 mg of sodium chloride particles (500-1000 μm) were added to the suspension in the mould. After drying for 2 days, the scaffolds were extracted in distilled water to remove the sodium chloride and by-products. Afterwards, the length of the bilayered scaffold was tailored to achieve specific lengths for the Silk-NanoCaP layer and the silk layer. The skin of the scaffold was removed by a stainless steel punch (diameter: 6 mm). The final scaffolds were obtained by lyophilization in a

freeze drier (CRYODOS-80; Telstar, Barcelona, Spain) after freezing the scaffolds at -80°C for at least 3 hours. As controls, pure silk scaffolds and Silk-NanoCaP scaffolds were also prepared by using 16 wt.% silk solution and introducing 16 wt.% CaP content, respectively. The pure SF scaffolds, the Silk-NanoCaP scaffolds, and the bilayered scaffolds were abbreviated as S16, SC16 and Bilayered, respectively.

3. SF Hydrogels Production

3.1. Methodologies for hydrogel preparation: Overview

Since their similarity to the extracellular matrix and easily tuned physicochemical properties, hydrogels have been studied widely in cell encapsulation, drug delivery, and tissue regenerations [39-41]. Particularly, the injectable hydrogels are attracting increasing interests for tissue engineering and regenerative medicine [42, 43]. For instance, these systems can repair the tissue of any shape by minimal invasive surgery, and it is possible to combine cells or bioactive agents. Various methods have been used to prepare injectable hydrogels, such as photo-polymerization, Michael addition, click reaction, enzymatic reaction, and ionic gelation, thermal gelation [42, 43]. Among these methods, the enzyme mediated gelation presents several advantages [44]. It can be performed in physiologic condition without external stimulus. This kind of reaction can be finished in a few minutes and requires very few amount of enzyme. Recently, several enzyme mediated cross-linked hydrogels have been developed and applied for tissue engineering [45-48]. It has been reported that tyrosine or tyramine groups containing water-soluble polymers can be cross-linked by horseradish peroxidase/hydrogen peroxide system [48].

Previously, SF hydrogels have been prepared mainly by physical approaches, such as increasing the temperature [12, 49], decreasing the pH [12], ultrasonication [13], and vortex [14]. These approaches were disadvantageous in the long gelation time or the harsh conditions, and not suitable to use as injectable system.

SF also contains certain amount of tyrosine groups (around 5 %) [4], which could be cross-linked via peroxidase mediating. Another goal of this thesis was to develop injectable SF hydrogels via peroxidase mediated cross-linking, for tissue engineering or drug delivery.

On the other hand, the traditional prepared hydrogels are of homogeneous property. However, tissues and organs are not homogenous matrix. They are normally of heterogeneous or stratified structure. This requires the scaffold or hydrogels involved should possess spatial or temporal tunable properties [50-52]. Based on the peroxidase mediated cross-linked SF hydrogels, this thesis also developed core-shell SF hydrogels of spatially controlled conformation and properties.

3.2. Peroxidase mediated cross-linked SF hydrogels

SF was purified according to the protocol mentioned above (Section 2.2. in this Chapter). The purified SF was dissolved in 9.3 M lithium bromide solution in an oven at 70°C for 60 minutes, followed by dialysis against distilled water for 48 hours in a benzoylated dialysis tubing (MWCO: 2 kDa). And then the SF solution was dialyzed in 0.2 M phosphate buffered saline (PBS, without calcium and magnesium ions) solution for 12 hours before concentration by 20 wt.% poly(ethylene glycol) solution [53]. The final concentration of the SF was determined by drying the concentrated SF solution in the oven at 70°C overnight. The saline content in the SF was 1.73 ± 0.03 wt.% tested by thermal gravimetric analysis (TGA Q500, TA Instruments, DE, USA). The prepared SF solutions were stored in a room at temperature between 4-8°C before use. Horseradish peroxidase (HRP) solution (0.84 mg/mL) and hydrogen peroxide solution (H_2O_2 , 0.36 wt.%) were prepared respectively in PBS solution. The SF solutions (pH 7.0-7.1) were diluted into 10, 12 and 16 wt.% by PBS solution and used for the hydrogel preparation. SF hydrogels were prepared by mixing 1 mL SF solution with varied amount of HRP and H_2O_2 solutions in a 1.5 mL centrifuge tube (Eppendorf, Hamburg, Germany), and then the mixture were warmed in a water bath of 37°C. Micropipettes (M100 and M1000, Gilson, Middleton, WI, USA) and corresponding tips were used for SF hydrogel preparation. The gelation time was determined by inverting the vial, and no flow within 60 seconds was considered as the gel status. SF hydrogel discs were also prepared by the addition of 200 μL the mixture solutions (SF/HRP/ H_2O_2) in a polypropylene mould (8 mm in diameter, 5 mm in height), followed by placing the mould in the oven at 37°C. These discs hydrogels were used for the test unless otherwise mentioned. The SF hydrogels prepared from 10, 12 and 16 wt.% SF solutions were denoted as Silk-10, Silk-12 and Silk-16, respectively. The SF hydrogels can also be prepared using SF solutions without dialysis in PBS solution.

Dialysis the SF against PBS solution aimed to maintain the pH close to the biological pH value for further cell encapsulation.

3.3. Core-shell SF hydrogels

The SF solution was prepared following the same procedure for the preparation of peroxidase mediated cross-linked SF hydrogels. The SF solution was diluted into 16 wt.% by the addition of PBS solution. The SF solution was first gelled via HRP mediated cross-linking. Briefly, 1 mL SF solution was mixing with 100 μ L of HRP solution (0.84 mg/mL) and 65 μ L hydrogen peroxide solution (0.36 wt.%), followed by transferring 200 μ L the mixture into a polypropylene mould (diameter: 8 mm) and placing the moulds into the 37°C oven until the gel formed. Micropipettes (M100 and M1000, Gilson, Middleton, WI, USA) and corresponding tips were used for the SF solution transferring. The SF hydrogel discs were removed from the moulds and used for the preparation of the core-shell SF hydrogels.

The core-shell SF hydrogels were prepared by immersion the prepared gel discs in methanol for 1, 3, 5 and 10 minutes, respectively [53]. At the end of each time point, the hydrogel discs were removed from the methanol and washed in PBS solution for three times to eliminate the organic solvent. These hydrogel discs after methanol treatment formed a core-shell structure, with a stiff outer shell layer and a soft core layer.

3.4. Albumin incorporated core-shell SF hydrogel

The albumin-fluorescein isothiocyanate conjugate (Albumin-FITC) was used as a model drug to study the drug release profile of the core-shell SF hydrogels. Before methanol treatment, the hydrogel discs prepared above (Section 3.3. in this Chapter) were first hydrated in PBS solution for 1 hour after prepared, followed by immersion in 100 μ g/mL Albumin-FITC solution at room temperature overnight (1.5 mL/disc). Afterwards, the hydrogel discs were removed from the Albumin-FITC solution and rinsed in PBS solution. Some of these hydrogels discs were used to prepare the core-shell hydrogels by immersion in methanol for 3, 5 and 10 minutes. Core-shell hydrogels without Albumin-FITC were prepared as control.

4. Physicochemical Characterization Methodologies

4.1. Morphological and microstructural characterization

4.1.1. Scanning electron microscopy (SEM)

SEM provides surface images of a sample by scanning it with a beam of electrons. The electrons contact with the surface atoms in the sample and their interactions can be transferred into detected signals. Thus the sample surface tomography or elemental information can be obtained. In Chapter III, the cross-sectional morphology of the prepared SF scaffolds was observed under the scanning electron microscope (Leica Cambridge S-360; Leica Manufacturer, Cambridge, UK). Prior to the analysis, specimens were coated with gold using a Fisons Instruments Coater (Polaron SC 502; Fisons plc, Ipswich, UK). The cross-sectional morphology of scaffolds after 30 days of degradation was also observed under the SEM (Nova NanoSEM 200; FEI, Hillsboro, OR, USA). The specimens were coated with Au/Pd SC502-314B using a high vacuum evaporator coater (E6700; Quorum Technologies, East Grinstead, UK). Three samples were tested for each condition.

In Chapter IV, V and VI, the cross-sectional morphology of the control silk scaffold and Silk-NanoCaP scaffolds were observed under SEM (Nova NanoSEM 200; FEI, Hillsboro, OR, USA). Prior to the analysis, the specimens were coated with Au/Pd SC502-314B in a high vacuum evaporator coater (E6700; Quorum Technologies, East Grinstead, UK). The size and the microscopic distribution of the CaP particle in the Silk-NanoCaP scaffolds were determined. For this purpose, Silk-NanoCaP scaffolds were milled into powder followed by observation of the CaP particles in the composite powder via Backscattered SEM (NanoSEM-FEI Nova 200) without any coating. The calcium and phosphate content in the powder was investigated by EDX during the SEM observation. For the determination of the Ca/P atomic ratio in the scaffold, the Silk-NanoCaP scaffolds were burned at 700°C for 40 minutes in a furnace (Fornoceramica, Leiria, Portugal) to remove the SF. The obtained residual CaP was adhered in a cooper support for the analysis of the Ca/P atomic ratio by EDX (NanoSEM-FEI Nova 200). In each condition, 5 independent areas (200 μm x 200 μm) of the residual CaP were selected. The ashes obtained after the TGA analysis could be used for the EDX assay. But some formulations (silk/CaP-4 and silk/CaP-8) had low amount of CaP and the ashes from these groups

were not enough for the assay. Thus, burning more scaffolds in the furnace was performed to get enough ashes (CaP).

In Chapter VII, the morphology of the bilayered Silk/Silk-NanoCaP scaffold was observed by SEM, using the one for the morphology observation in Silk-NanoCaP scaffolds. Elemental analysis was performed in four zones around the interface area by EDX affiliated in the SEM. Three independent areas were selected in each zone, and each scanned area was 100 μm x 100 μm . The CaP content in the Silk-NanoCaP layer was evaluated by a thermal gravimetric analysis (TGA). The Ca/P atomic ratio of the ash obtained after the TGA assay was studied by EDX. At least three specimens were used for both assays.

In Chapter IV, VI and VII, the surfaces of the specimen undergone *in vivo* mineralization were analyzed by SEM. The SEM observation was using the one for the morphology observation in Silk-NanoCaP scaffolds. In Chapter IV and VI, the samples were coated with carbon. In Chapter VII, the samples were coated with Au/Pd. For the EDX (NanoSEM-FEI Nova 200) analysis in Chapter IV and VI, the data were collected by scanning three independent areas (5 μm x 5 μm) in each carbon coated specimen for 90 seconds. Three specimens were analyzed for each time point for each group of scaffolds. For the EDX analysis in Chapter VII, Samples without Au/Pd coating were used for elemental analysis by EDX. Three independent areas were selected in each layer, and each scanned area was 100 μm x 100 μm . A minimum of three specimens were analyzed for each time point.

In Chapter VII, the surfaces of the specimen undergone *in vivo* mineralization were analyzed by SEM. The SEM observation was using the one for the morphology observation in Silk-NanoCaP scaffolds. For the EDX analysis, the data were collected by scanning three independent areas (5 μm x 5 μm) in each specimen for 90 seconds. Three specimens were analyzed for each time point for each group of scaffolds.

In Chapter IX, the morphology of the core-shell or non-treated lyophilized SF hydrogels was analyzed by SEM, using the one for the morphology observation in Silk-NanoCaP scaffolds.

4.1.2. Micro-computed tomography (Micro-CT or μ -CT)

The pore size, porosity, interconnectivity, and phase distribution are important aspects for scaffolds. Micro-CT provides a powerful and invasive method to obtain these results for scaffolds [31]. Micro-CT uses X-ray to scan the 3D object and obtain the cross-section information of the object. Following, the obtained data set can be processed by the software, and the quantitative microstructure (pore size, porosity, interconnectivity, trabecular size and so on) and 3D visual image of the object were achieved. In Chapter III, IV, V, VI, the architecture of the SF and the Silk-NanoCaP scaffolds were evaluated using a high-resolution μ -CT Skyscan 1072 scanner (Skyscan, Kontich, Belgium) possessing a resolution of pixel size of $\sim 6.7 \mu\text{m}$ and integration time of 1.3 second. The X-ray source was set at 40 keV and 248 μA for SF scaffolds, and 61 keV and 163 μA for the Silk-NanoCaP scaffolds, respectively. Approximately 300 projections were acquired over a rotation range of 180° with a rotation step of 0.45° . Data sets were reconstructed using standardized cone-beam reconstruction software (NRecon v1.4.3, SkyScan). The output format for each sample was 300 serial 1024×1024 bitmap images. Representative data set of the slices was segmented into binary images with a dynamic threshold of 40-255 (grey values). Then, the binary images were used for morphometric analysis (CT Analyser, v1.5, SkyScan), and to build the 3D models (CT Vol, v2.4, SkyScan). For determination of the CaP content (Vol.%) and distribution in the Silk-NanoCaP scaffolds, representative data set of the slices was segmented into binary images with the dynamic threshold set between 120 and 255 (grey values). At least three samples were tested for each condition.

In Chapter VII, the same micro-CT instrument was used for qualitatively and quantitatively evaluation the porosity and the CaP distribution profile in the bilayered scaffolds. The scanning of the scaffolds was conducted under 61 keV and 163 μA in the micro-CT. Both the diameter and the height of the scaffolds were 8 mm (Silk layer: 3 mm in height; Silk-NanoCaP layer: 5 mm in height). The integration time was fixed at 1.3 seconds and the pixel resolution was $9.4 \mu\text{m}$. For each scanning, around 400 projections were achieved after a rotation of 180° with 0.45° step width. The data sets were processed in a cone-beam model using a standard software (NRecon v1.4.3, Skyscan), and subsequently around 750 serials bitmap images with 1024×1024 pixels was generated for each specimen. The qualitative visualization of the three dimensional morphology and the different phase in the bilayered scaffolds were performed by using

the CTvox software (Skyscan). In order to achieve the porosity and CaP content distribution profiles in the bilayered scaffolds, the generated bitmap images were processed in standardized software (CT Analyser, version 1.5., Skyscan). The images in each dataset were firstly transferred into binary images by using grey values (dynamic threshold). For the porosity calculation and the CaP content determination, dynamic threshold was set from 45 to 255 and 120 to 255, respectively. Five scaffolds were used for the qualitative and quantitative microstructure evaluation.

4.2. X-ray diffraction (XRD)

XRD is a reliable technique to detect the structure of the materials, such as amorphous or crystalline. Under X-ray radiation, the crystalline atoms or molecules of materials can induce the diffraction of the X-ray into specific direction. By measuring the intensity and angles of these signals, it is possible to know the crystallinity and the arrangement of the atoms in the crystals. In Chapter III, IV and VI, the X-ray diffractometer (Philips PW 1710; Philips, Amsterdam, Netherlands) employing Cu-K α radiation ($\lambda=0.154056$ nm) was used to analyze the crystallinity of the SF and Silk/Silk-NanoCaP scaffolds on powder, respectively. Data was collected from 0 to 60° 2 θ values, with a step width of 0.02° and a counting time of 2 second/step. The test was repeated three times for each condition.

4.3. Fourier transform infra-red spectroscopy (FTIR)

When a material is under infrared radiation (wavelength ranged from 700 nm to 1 mm), some of the signal is absorbed and the rest is passed through (transmitted). The intensity and position of the absorbance and transmittance can be recorded and reflected in the spectrum. Acting as a fingerprint, the FTIR spectrum can be used to identify the chemical groups in unknown materials, screen the consistency of products, and evaluate the ratio of components in the composite. In Chapter III and IV, the infrared spectra of the silk fibroin powders were recorded by (Perkin-Elmer 1600 series equipment; Perkin-Elmer, MA, USA). Prior to the analysis, the dried silk fibroin powders were mixed with potassium bromide in a ratio of 1:100 (by wt.) followed by uniaxially pressing into a disk. All spectra were obtained between 4000 to 400 cm⁻¹ at a 4 cm⁻¹ resolution with 32 scans. Each condition was examined for at least three times.

In Chapter VI, the SF conformation and scaffolds composition information were evaluated by attenuated total reflectance (ATR) model in a FTIR instrument (IRPrestige-21; Shimadzu, Kyoto, Japan) equipped with a Germanium crystal. Each specimen was scanned 48 times with a resolution of 4 cm^{-1} . Triplicate samples were used for each group scaffold in this assay.

In Chapter VII, the chemical composition and structural conformation of the bilayered scaffolds were analyzed by ATR using the same FTIR instrument as for ATR analysis in Chapter VI. Each layer of the bilayered scaffolds was respectively scanned by contacting the sample with the germanium crystal. The scanning number was fixed at 48 times with a resolution of 4 cm^{-1} . The spectrum of the atmosphere was used as the background for all the specimens. A minimum three specimens were used for each layer.

In Chapter VIII, the SF solution, the mixture of SF/HRP/H₂O₂ solution, and the formed SF hydrogels were analyzed by ATR, using the same equipment as for ATR analysis in Chapter VI. Each specimen was scanned 48 times from $600\text{-}2000\text{ cm}^{-1}$ with a resolution of 4 cm^{-1} in wet state. PBS solution was scanned and used as background in the ATR.

In Chapter IX, The conformations of different domains in the core-shell SF hydrogels were characterized by ATR, using the same equipment as for ATR analysis in Chapter VI. The samples after methanol treatment were washed and immediately tested by ATR-FTIR. The tested domains were the external surface of the shell layer, the inner surface of the shell layer, the interface area between the shell and the core hydrogel, and the core hydrogel. Each specimen was scanned 48 times from $500\text{-}4000\text{ cm}^{-1}$ with a resolution of 4 cm^{-1} and in wet state. Silk solution and hydrogels without methanol treatment were used as controls. PBS solution was scanned as background. Three specimens were analyzed in each group.

4.4. Ultraviolet-Visible (UV-VIS) spectrophotometry

UV-Vis spectrum reflects the absorbance a solution under UV (10-380 nm) and VIS wavelength (380-700 nm). The absorbance intensity is related with the molecules and their concentration in the solution. Different materials absorb radiation at different wavelengths. Therefore, the UV-VIS spectrophotometry is useful tool in analytical chemistry for quantitative measurement of different chemical components or monitoring

chemical reactions. In Chapter VIII, the optical absorption of the SF hydrogels was analyzed in UV and VIS wavelength ranges. SF solution of 16 wt.% was selected to prepare samples for this characterization. HRP/SF and H₂O₂/SF were fixed at 0.26‰ and 1.1‰ (by wt.), respectively. The optical absorbance of the SF before and after gelation was recorded by a microplate reader (Synergy HT; Bio-Tek, VT, USA). A mixture of the SF, HRP and H₂O₂ solutions (100 µL) was placed in a home-made 96-well quartz plate (well diameter 7 mm) and read from 280-370 nm before and after gelation. Then, 50 µL of the same mixture was also placed into the quartz plate and read from 450-800 nm before and after gelation. The resolution of the UV and VIS absorbance was set at 1 nm. The gelation of the mixture was performed by sealing the quartz plate with paraffin film (Parafilm; Pechiney Plastic Packaging Company, IL, USA), followed by placing the quartz plate in the oven at 37°C.

4.5. Thermal gravimetric analysis (TGA)

TGA measures physical and chemical changes in materials as a function of constant temperature or increasing temperature with constant heating rate. It can quantitatively determine the mass changes in materials induced by dehydration, decomposition, or oxidation with temperature and time. From the TGA curve, it is possible to know the amount of different components, the stability of the materials, and the degradation profiles of products. In Chapter IV and VII, the CaP content in the Silk-NanoCaP scaffolds or in the Silk-NanoCaP layer of the bilayered Silk/Silk-NanoCaP scaffolds was determined by TGA (TGA Q500; TA Instruments, DE, USA). Each specimen was placed in a platinum pan and equilibrated at 50°C for 2 minutes, followed by increasing the temperature to 700°C at a rate of 20°C/minute in air atmosphere. The CaP content in the scaffolds (CaP mass divided by the mass of SF) and the CaP incorporation efficiency were determined following equation 1 (Eq.1) and equation 2 (Eq.2), respectively.

$$CaP\ content = \left(\frac{m_r}{m_i - m_r} \right) \times 100\% \quad (1)$$

$$CaP\ incorporation\ efficiency = \frac{CaP\ content}{Theoretical\ CaP\ content} \times 100\% \quad (2)$$

In Eq.1, m_r is the weight of the residual, and the m_i is the initial dried weight of the material. In Chapter IV, the theoretical contents for silk/CaP-4, silk/CaP-8, silk/CaP-16 and silk/CaP-25 are 4, 8, 16 and 25 wt.%, respectively. Three specimens were evaluated for each formulation.

In Chapter VII, the Ca/P atomic ratio of the ash obtained after the TGA assay was studied by EDX.

In Chapter VIII, the SF solution was dialyzed in 0.2 time PBS solution. The saline content in the concentrated SF solution was tested by TGA, using Eq. 1 above.

4.6. Compression test

Compression test measures the deformation of a material under various compressive forces. This test gives the compression modulus and compression strength of a material. Regarding bone and cartilage tissue engineering, the implanted scaffolds would undergo compressive forces *in vivo*. Therefore, the *in vitro* compression data are critical for selection suitable implants for tissue engineering. In Chapter III, IV and VI, the compressive tests (dry state) of the SF scaffolds, the Silk-NanoCaP scaffolds, and the bilayered Silk/Silk-NanoCaP scaffolds were performed by using a Universal Testing Machine (Instron 4505; Instron, Norwood, MA, USA) with a 1kN load cell at room temperature. The size of the tested specimens was measured with a micrometer. The diameter and the length of the SF scaffolds and the Silk-NanoCaP scaffolds were both around 6 mm. The diameter and the height of the bilayered scaffolds were 6 and 5 mm, respectively (Silk layer: 2 mm in height; Silk-NanoCaP layer: 3 mm in height). The cross-head speed was set at 2 mm/minute and until 60% reduction in specimen height. The elastic modulus (E) was defined by the slope of the initial linear section of the stress-strain curve. A minimum number of 6 specimens were tested.

In Chapter VII, the bilayered samples were also tested in wet state. For this test, the samples were first hydrated in PBS solution overnight at 37°C. Before the test, the absorbed liquid in the specimen was removed by a tissue, and subsequently the compressive test was performed immediately. The cross-head speed was set compressive rate of 2 mm/minute until reaching 60% strain. The modulus was obtained

using procedure mentioned above. S16 and SC16 were used as controls (5 mm in height, 6 mm in diameter). For each test, six specimens of each group were screened.

In Chapter IX, the core-shell SF hydrogels was tested the compressive rate was set at 2 mm/minute until reaching 50% strain. The modulus was determined from the slope of the initial linear domain in the compressive curve. At least six specimens were examined for each group.

4.7. Dynamic mechanical analysis (DMA)

When a scaffold is implanted *in vivo* (such as in cartilage or bone), it will be undergone cyclic loads with different frequency. Besides the static mechanical properties, it is important to know the performance of the scaffolds under dynamic loading *in vitro*. DMA is a helpful tool to reveal this information from scaffolds. In Chapter III, IV and VI, the SF scaffolds, the Silk-NanoCaP scaffolds, and the bilayered scaffolds were analyzed by DMA. The viscoelastic measurements were performed using a TRITEC8000B DMA (Triton Technology, Lincolnshire, UK), equipped with the compressive mode. The measurements were carried out at 37°C temperature. Samples were cut in cylindrical shapes with approximate 6 mm diameter and 5 mm thickness (measured each sample accurately with a micrometer). Scaffolds were always analyzed by immersing in a liquid bath placed in a Teflon® reservoir. Scaffolds were previously immersed in a PBS solution until equilibrium was reached (37°C overnight). The geometry of the samples was then measured and the samples were clamped in the DMA apparatus and immersed in the PBS solution. After equilibration at 37°C, the DMA spectra were obtained during a frequency scan between 0.1 and 10 Hz. The experiments were performed under constant strain amplitude (50 μm). A small preload was applied to each sample in order to ensure that the entire scaffold surface was in contact with the compression plates before the test. The distance between plates was equal for all scaffolds being tested. A minimum of three samples were used for each condition.

4.8. Determination of the thickness of the shell layer in the core-shell SF hydrogel

In Chapter IX, the prepared core-shell SF hydrogel disc was longitudinally cut, and the soft core layer was separated from the stiff shell layer. The thickness of the wall in the shell layer was measured by a micrometer. Three areas in one disc were measured and the values were averaged. For each group disc, at least 4 specimens were tested.

4.9. Rheological analysis

Rheology studies the relationship between deformation and flow in materials. It gives the elasticity, viscosity and plasticity information of materials under changes of strain, frequency, or time. It is also sensitive to any chemical reactions in the flows, such as gelation and polymerization. In Chapter VIII, the storage and loss moduli of the SF hydrogels were evaluated by using oscillatory model in a rheometer (MCR 300; Anton Paar, Graz, Austria), equipped with a cuvette accessory (CC10/Q1). The radii of the measuring bob and cup were 5.000 and 5.420 mm, respectively. The length of the gap was 14.985 mm, with a cone angle of 120°. For each measurement, 1 mL SF solution was mixed with varied amount HRP and H₂O₂, and then 1 mL of the mixture was transferred into the cup. The bob was immersed into the solution, followed by addition of one drop dodecane onto the surface of the solution. For the measurement of the modulus, the time sweep was first performed under constant strain (0.1%) and frequency (0.5 Hz) until the gel formed and reached a stable state, indicating by appearing a plateau in the storage and loss moduli curves. The storage and loss moduli were determined by averaging the values in the plateau. After the plateau of the storage modulus was reached, the frequency sweep (from 0.1-20 Hz) was conducted for 5 minutes with strain fixed at 0.1%. The strain sweep (0.1-100%) was following the frequency sweep and carried out for another 5 minutes under constant frequency at 1 Hz. All the data points were collected twice per minute for time sweep, frequency, and strain sweep. All measurements were conducted at 37°C.

4.10. Hydration degree of the scaffolds

In Chapter III, IV and VI, the hydration degree of the SF scaffolds and the Silk-NanoCaP scaffolds were assessed after immersion into an isotonic saline solution (ISS, 0.154 M sodium chloride aqueous solution, pH 7.4), for time periods ranging from 3 hours up to 12 months. All experiments were conducted at 37°C and dynamic condition (60 rpm) in a water bath (GFL 1086; GFL, Burgwedel, Germany). After each time point, the specimens were removed from the ISS and the weights were determined immediately after adsorption of the excess of surface water using a filter paper. The hydration degree was calculated as following Equation 3 (Eq. 3).

$$\text{Hydration degree} = \left(\frac{m_{w,t} - m_i}{m_i} \right) \times 100\% \quad (3)$$

In Eq. 3, m_i is the initial weight of the specimen before hydration, and $m_{w,t}$ is the wet weight of the specimens at time t after being removed from the ISS. At least five specimens were used for each condition.

In Chapter VII, the hydration degree of the bilayered Silk/Silk-NanoCaP scaffolds was evaluated by immersion the scaffolds in ISS overnight at 37°C in the same water bath mentioned above, but without shaking. The dried weight of the scaffolds was measured before immersion. The wet weight of the scaffolds was recorded after overnight immersion. The hydration degree was calculated using Eq. 3.

4.11. Degradation analysis on the scaffolds

4.11.1. Degradation analysis in isotonic saline solution (ISS)

In Chapter III, IV and VI, after the determination of the hydration degree, the specimens were washed with distilled water and dry in an oven at 60°C for 24 hours. The weight loss was determined using Equation 4 (Eq. 4).

$$\text{Weight loss ratio} = \left(\frac{m_i - m_{d,t}}{m_i} \right) \times 100\% \quad (4)$$

In Eq.4, m_i is the initial weight before degradation, and $m_{d,t}$ is the dry weight of the specimen been degraded for a certain period of time and after drying until constant weight is reached. At least five specimens were used for each condition.

4.11.2. Enzymatic degradation

In human body, the proteolysis is mainly conducted by enzyme. Enzyme can degrade protein in high efficiency. SF degrades very slowly by hydrolysis in physiological condition. In order to evaluate the subtle differences in biostability of different SF based materials, enzymatic degradation is used. In Chapter V and VII, the biostability of SF scaffold (S16), the Silk-NanoCaP (SC16), and the bilayered Silk/Silk-NanoCaP scaffolds were analyzed by enzymatic degradation in protease XIV solution. The scaffolds used for the degradation study were of 6 mm in diameter and 2 mm in height for S16 and SC, and 6 mm in diameter and 5 mm in height for the bilayered scaffolds (Silk layer: 2 mm in height; Silk-NanoCaP layer: 3 mm in height). Each specimen was placed into a vial supplemented with 5 mL protease XIV solution (1 U/mL or 4 U/mL). The initial dry weight of each specimen was measured first. When 1 U/mL protease solution was used, the scaffolds degradation profile was investigated for 0.5, 1, 2, 3, 5 and 7 days. In experiments using a 4 U/mL protease solution, the samples were analyzed after 3, 6, 12, 24 and 48 hours of soaking. All the enzyme solutions were refreshed every 24 hours. At the end of each time point, the samples were removed from the enzyme solution, and then rinsed by distilled water. The remaining mass of the specimen was measured after drying it at 60°C in an oven overnight. The weight loss ratio (%) was calculated as Eq. 4. At least five specimens were used for each group at each time point.

4.12. *In vitro* mineralization

In Chapter IV, VI and VII, the *in vitro* mineralization of the SF scaffolds, the Silk-NanoCaP scaffolds, and the bilayered Silk/Silk-NanoCaP scaffolds was evaluated. The scaffolds were immersed in a simulated body fluid (SBF) solution for different time in an oven at 37°C, following the method proposed by Kokubo *et al.* [54] and adapted by Oliveira *et al.* [38]. In Chapter, the samples were immersed in SBF solution for 7 days. In Chapter VI and VII, the samples were immersed in SBF solution for 1, 3, 7 and 14 days. At each

time point, the specimens were removed from the SBF solution and washed by distilled water. The samples were frozen at -80°C and then lyophilized (CRYODOS-80; Telstar, Barcelona, Spain). Then, the surfaces of the samples were analysed by SEM and EDX.

4.13. Hydration degree of the SF hydrogels

In Chapter VIII, the prepared discs Silk-10, Silk-12 and Silk-16 were used for the hydration degree analysis. Three formulations were used for each group hydrogels: 1/0.26‰/1.1‰, 1/0.52‰/1.1‰ and 1/0.26‰/1.45‰ (SF/HRP/H₂O₂, by wt.). The swelling ratios of the hydrogels were tested in both ultrapure water and PBS solution. Each piece of hydrogel was placed in a tube with 50 mL PBS solution or ultrapure water (0.55 uS/cm) prepared by a ultrapure water system (Genpure UV/UF; TKA GenPure, Niederelbert, Germany), subsequently the samples were placed in a thermostatic water bath (OLS200; Grant Instruments, Cambridgeshire, UK) at 37°C. The wet weight of the sample was measured at 1, 3, 6 and 12 hours. Before weighting, surface liquid in the hydrogels were absorbed by tissue. The ultrapure water was refreshed at the end of the first and the third hour. After 12 hours, the samples were dried in the oven at 70°C overnight. The swelling ratio at each time point was calculated as Equation 5 (Eq. 5).

$$\text{Hydration degree} = \left(\frac{w_t - w_d}{w_d} \right) \times 100\% \quad (5)$$

In Eq. 5, w_t referred to the wet weight of the sample tested in different time point, and w_d is the dry weight of the sample. It was assumed that the dry weight of each specimen was constant during the tested time period.

In Chapter IX, the hydration degree of the shell layer and the inner core layer of the hydrogels were evaluated. The samples were immersed in PBS solution for 1 hour, and then the wet weights were recorded after removing the surface liquid by filter paper. In the following, the samples were dried at 70°C in an oven overnight. The dry weight of each sample was measured. The hydration degree was calculated using Eq. 5.

4.14. Enzymatic degradation of the SF hydrogels

In Chapter VIII, protease XIV from *Streptomyces griseus* was used for the degradation of the SF hydrogels. Each specimen was immersed in 5 ml PBS solution and placed in the oven at 37°C overnight. And then the wet weight of each specimen was recorded before the addition of 5 mL protease XIV solution. The protease XIV solution was prepared in PBS solution and yielded a concentration of 0.005 U/mL. The samples were placed in a thermostatic water bath (OLS200; Grant Instruments, Cambridgeshire, UK) at 37°C. The wet weight of each specimen was measured at 1, 2, 4, 6 and 12 hours. The weight loss ratio was defined using Equation 6 (Eq. 6).

$$\text{Weight loss ratio} = \left(\frac{w_i - w_t}{w_i} \right) \times 100\% \quad (6)$$

In Eq. 6, w_i meant the initial wet weight of the hydrogel, and w_t was the wet weight tested at each time point.

In Chapter IX, the shell layer of the core-shell SF hydrogel was degraded in protease XIV solution. The non-treated SF hydrogel and the core layer of the core-shell SF hydrogels which immersed in methanol for 10 minutes were also tested. Around 50 mg hydrogel (wet weight after removing surface liquid by filter paper) was immersed in 5 mL protease XIV solution and kept in a thermostatic water bath (OLS200; Grant Instruments, Cambridgeshire, UK) at 37°C. The enzyme solutions of 0.2 U/mL and 0.005 U/mL were used for the shell layer and the core layer hydrogels, respectively. The samples were degraded for 1, 2, 4, 6 and 12 hours, and the weight loss ratio was calculated using Eq. 6.

4.15. Ionic strength response examination

In Chapter VIII, the Silk-16 hydrogels with formulation of 1/0.26‰/1.45‰ (SF/HRP/H₂O₂, by wt.) were used for the ionic strength response test. This test included two parts. In the first part, each discs hydrogels was immersed in 5 mL PBS solution (pH 7.4) in a vial and kept in the oven at 37°C overnight, followed by measuring the diameter of the hydrogels

and subsequently placing each hydrogels in 100 mL distilled water (pH 7.0-7.1, conductivity: 2.0 $\mu\text{S}/\text{cm}$) in a plastic bottle. And then the hydrogels were alternately immersed in distilled water and PBS solution every 12 hours. Before every change between distilled water and PBS solution, the diameter of the hydrogel was measured by a micrometer. The samples were placed in the thermostatic water bath at 37°C during the test. During each immersion procedure, the distilled water or PBS solution was refreshed at the third hour.

For the second part, the prepared hydrogels were immersed in 5 mL 0.154 M sodium chloride solution (pH 7.4, adjusted by 1.0 M sodium hydroxide) in a vial and placed in the oven at 37°C overnight, and then the wet weight of each hydrogel was measured. Each hydrogel was then alternately immersed in 100 mL 2 M sodium chloride solution (pH 7.4, adjusted by 1 M sodium hydroxide) and 100 mL 0.154 M sodium chloride solution (pH 7.4) every one hour. The samples were placed in the thermostatic water bath at 37°C during the test. Before every change of the solution, the wet weight of the hydrogel was recorded. And the wet weight variation ratio was calculated using Equation 7 (Eq. 7).

$$\text{Weight variation ratio} = \left(\frac{w_i - w_t}{w_i} \right) \times 100\% \quad (7)$$

In Eq. 7, w_i means initial weight of the hydrogel after overnight immersion in 0.154 M sodium chloride solution, and w_t refers to the wet weight tested at time t during the immersion. The prepared discs hydrogels were also immersed in methanol for 3 hours, or in hydrochloric acid solution (pH 2.0) overnight to undergo β -sheet conversion, and then the opaque hydrogels were used as control for the ionic strength response test, as well as for hydration degree and degradation tests.

4.16. pH response analysis

In Chapter VIII, hydrogels of the same formulation for ionic strength response test were used in pH response evaluation. This test had two parts. In the first part, the hydrogels were immersed in solutions of the same ionic strength but of different pH values. In the

second part, the hydrogels were alternately immersed in basic and acid sodium chloride solutions. Before the test, the specimens were immersed in 0.154 M sodium chloride solution (pH 7.4, adjusted by 1 M sodium hydroxide) in the oven at 37°C overnight. The initial wet weights of the hydrogels were measured. For the first part, each hydrogel disc was immersed in 100 mL sodium chloride solutions of different pH values of: 2.5, 3.0, 4.0, 7.4, 9.0, 10.0 and 10.5. The ionic strength of these solutions was fixed at 0.154 M. The samples were stayed in the thermostatic water bath at 37°C for 2 hours. Subsequently, the wet weights of the hydrogels were recorded after absorbed the surface liquid by tissue. The wet weight variation after immersion in solution of different pH values was calculated using Eq. 7.

For the second part, the overnight immersed hydrogels were also alternately immersed in the above mentioned 100 mL basic (pH 10.5) and acid (pH 3.0) sodium chloride solutions, after removing the surface liquid by tissue and subsequently measuring the initial wet weight. Before each change of the solutions, the wet weights of the samples were noted after removing the surface liquid by tissue. The wet weight variation ratio was calculated using Eq. 7. The prepared discs hydrogels were also immersed in methanol for 3 hours to undergo β -sheet conversion, and then the opaque hydrogels were employed as control for the pH response test.

The basic solutions (pH 7.4, 9.0, 10.0 and 10.5) were prepared by addition of disodium hydrogel phosphate into the sodium chloride solution (0.137 M) and the pH values were adjusted by using 2 M sodium hydroxide solution. And the acid solutions (pH 2.5, 3.0, 4.0) were produced by supplementation of sodium dihydrogen phosphate into the sodium chloride solution (0.137 M) and the pH values were tuned by employing 2 M hydrochloric acid solution. The concentration of the phosphate buffered saline in the acid or basic solutions was fixed at 1 mM. The contribution of the addition of sodium hydroxide or hydrochloric acid solution to the ionic strength was taken into account, and sodium chloride was added to modulate the final ionic strength to 0.154 M, if necessary.

4.17. Drug delivery in the core-shell SF hydrogels

In Chapter IX, the Albumin-FITC release profiles in the non-treated and core-shell hydrogels were evaluated by immersion of each specimen in PBS solution. For the non-

treated specimens and specimens treated by methanol for 3 minutes, 4 mL PBS solution was used for each disc. Due to the low amount of albumin incorporation in the specimens with methanol treatment for 5 and 10 minutes, 2 mL PBS solution was used for each disc in these two groups. The samples were kept in a water bath at 37°C. The release of Albumin-FITC was tested after immersion for 2, 4, 6, 24, 48, 72, 120 and 168 hours. At the end of each time point, the supernatant from each specimen was removed and equal volume of fresh PBS solution was added. For the quantification of the released Albumin-FITC, the fluorescence intensity of 100 μ L supernatant of the removed PBS solution was read by a microplate reader (Synergy HT; Bio-Tek, VT, USA), with the excitation wavelength at 485/20 nm and the emission wavelength at 528/20 nm. The samples without Albumin-FITC incorporation were used as controls. Five specimens were used for each group. For the determination of the total Albumin-FITC in the hydrogels, the discs were immersed in 4 mL PBS solution and the supernatants were analyzed periodically. A series of Albumin-FITC solutions were prepared in PBS solution (from 0 to 15 μ g/mL), and the fluorescence intensity of these solutions were recorded for standard curve preparation. Standard curve was obtained with R^2 of 0.997. After 24 hours release, the Albumin-FITC incorporated non-treated and core-shell hydrogels were longitudinally cut, and observed in a fluorescence microscope with apotome 2 (Axio Imager Z1m; Zeiss, Jena, Germany).

5. *In Vitro* Biological Evaluation

5.1. Cell sources

5.1.1. L929 cell line

In Chapter IV, the mouse lung fibroblasts L929 cell line (European Collection of Cell Cultures, Salisbury, UK) were used to evaluate the cytotoxicity of the leachables from Silk-NanoCaP scaffolds. The cells were cultured as monolayer in a Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Biochrom, Merck, NJ, USA), 1% of antibiotic-antimycotic mixture (Life Technologies, Carlsbad, CA, USA) containing 10,000 U/mL penicillin G sodium, 10 mg/mL streptomycin sulphate and 25 μ g/mL amphotericin B as fungizone® antimycotic in 0.85% saline. The L929 cells were incubated in an atmosphere containing

5% CO₂ at 37°C (MCO-18AIC (UV); Sanyo, Osaka, Japan), and the medium changed every 2 days.

5.1.2. Human adipose tissue derived stem cell (hASCs)

In Chapter V, the cytocompatibility of S16 and SC16 were evaluated by culturing of hASCs. The hASCs were isolated from the adipose tissue which was obtained from the liposuction procedure [55]. The use of the hASCs was approved by the Ethics Committee of University of Minho. The isolated hASCs were expanded and then stored in liquid nitrogen for long-term use. In this study, the hASCs in passage two (P2) were defrost from the liquid nitrogen and expanded in alpha-minimum essential medium (α -MEM) (Gibco®; Life Technologies, Carlsbad, CA, USA). The α -MEM was supplemented with 10% FBS (Life Technologies, Carlsbad, CA, USA), and 1% antibiotic-antimycotic liquid prepared with 10,000 units/mL penicillin G sodium, 10,000 μ g/mL streptomycin sulfate, and 25 μ g/mL amphotericin B as Fungizone(R) in 0.85% saline (Life Technologies, Carlsbad, CA, USA). The cells were cultured in an aseptic condition, at 37°C in an incubator with 5% CO₂ atmosphere (MCO-18AIC (UV); Sanyo, Osaka, Japan). The medium was refreshed every two day until the cells reached around 90% confluence. In the following, the cells were detached from the culture flask by using TrypLE Express (1X) with phenol red (Life Technologies, Carlsbad, CA, USA). The cell number was counted in a cell counter. Afterwards, the cell suspension (Passage 3, P3) was centrifuged at 1200 rpm for 5 minutes (5810R; Eppendorf, Hamburg, Germany). Then, the supernatants were discarded; the cells were re-suspended and subsequently passaged into new flasks. The cells were expanded until P4 before seeding in the scaffolds.

5.1.3. Rabbit bone marrow mesenchymal stromal cells (RBMSCs)

In Chapter VII, the RBMSCs were cultured in the bilayered Silk/Silk-NanoCaP scaffolds for cytotoxicity and differentiation analysis. The RBMSCs were isolated from male New Zealand White rabbits (Senneville, Quebec, Canada). The maintenance and usage of animals were approved by the Ethics Committee of University of Minho. The 9 weeks old rabbits were sacrificed by injection of overdose pentobarbital sodium. All the procedures

were performed under aseptic condition. The femurs were first separated from the hind legs, followed by removing the epiphysis heads and subsequently flushing out the bone marrow plug by using α -MEM (Gibco®; Life Technologies, Carlsbad, CA, USA) [56]. The α -MEM was supplemented with 10% FBS (Life Technologies, Carlsbad, CA, USA), and 1% Antibiotic-Antimycotic liquid prepared with 10,000 units/mL penicillin G sodium, 10,000 μ g/mL streptomycin sulfate, and 25 μ g/mL amphotericin B as Fungizone(R) in 0.85% saline (Life Technologies, Carlsbad, CA, USA). The isolated RBMSCs (Passage 0, P0) from one femur were cultured in one T150 cm² cell culture flask and expanded in 40 mL α -MEM at 37°C in an incubator with 5% CO₂ atmosphere (MCO-18AIC (UV); Sanyo, Osaka, Japan). The medium were changed for the first time after 4 days, and then changed every two day until the cells reached around 90% confluence. And then the cells were detached from the flask by using TrypLE Express (1X) with phenol red (Life Technologies, Carlsbad, CA, USA) and the cell number were counted in a cell counter. In the following, the cell suspension (Passage 1, P1) was centrifuged at 1200 rpm for 5 minutes (5810R; Eppendorf, Hamburg, Germany). Afterwards, the supernatants were removed, and the cells were re-suspended with new culture medium and subsequently passaged into new flasks. The cells were expanded until passage 2 before seeding into the scaffolds.

5.1.4. ATDC-5 cell line

In Chapter VIII, a mouse chondrocyte teratocarcinoma-derived cell line ATDC-5 (European Collection of Cell Cultures, Salisbury, UK) was used for study the cell encapsulation potential and cytotoxicity of the SF hydrogels. ATDC-5 cells were expanded in basal α -MEM (Gibco®; Life Technologies, Carlsbad, CA, USA), supplemented with 10% FBS (Life Technologies, Carlsbad, CA, USA), and 1% Antibiotic-Antimycotic liquid prepared with 10,000 U/mL penicillin G sodium, 10,000 μ g/mL streptomycin sulfate, and 25 μ g/mL amphotericin B as Fungizone(R) in 0.85% saline (Life Technologies, Carlsbad, CA, USA). The cells were incubated in a CO₂ incubator (MCO-18AIC (UV); Sanyo, Osaka, Japan) under an atmosphere of 5% CO₂ at 37°C, with medium change every two days. As the cells reached around 90% confluence, they were detached from the culture flask by using TrypLE Express (1X) (Life Technologies,

Carlsbad, CA, USA) with phenol red, the cell suspension was centrifuged and then re-suspended with new culture medium and subsequently passaged into new flasks.

5.2. Cell seeding techniques

5.2.1. Seeding the L929 cells in the cell culture plate

In Chapter IV, the confluent L929 cells were detached from the culture flasks using trypsin (0.25% trypsin–EDTA solution; Life Technologies, Carlsbad, CA, USA) and a diluted cell suspension was prepared. The cells were seeded in 96-well tissue culture polystyrene (TCPS) plate at a cell density of 20,000 cells/well and with 200 μ L medium /well. The cells were incubated for 24 hours at 37°C in a CO₂ incubator with 5% CO₂ atmosphere.

5.2.2. Seeding the hASCs in the scaffolds

In Chapter V, S16 and SC16 (diameter: 6 mm; height: 2 mm) were sterilized by ethylene oxide before the biological examination. All the procedures were performed under aseptic condition. Before the cell seeding, the scaffolds were degassed by a syringe and hydrated in α -MEM overnight in the CO₂ incubator. In the following day, the hydrated scaffolds were transferred to a 24-well suspension cell culture plate (Cell star; Greiner Bio-One, Kremsmuenster, Austria). The hASCs of P3 were detached and a new cell suspension (P4) was prepared (cell density: 5 million/mL). Each scaffold was seeded with 200,000 cells on its surface, and then the constructs were kept in the CO₂ incubator at 37°C. Three hours later, the constructs were moved to a new 24-well suspension culture plate and 2 mL of α -MEM were added for each construct. The culture medium was changed every two or three days.

5.2.3. Seeding the RBMSCs in the bilayered scaffolds

In Chapter VII, the RBMSCs were seeded into the Bilayered scaffolds and the control scaffolds (S16 and SC16). The bilayered scaffolds for cell seeding were 6 mm in diameter and 5 mm in height (Silk-NanoCaP layer: 3 mm in height; Silk layer: 2 mm in

height). The S16 and SC16 were 6 mm in diameter and 2 mm in height). The scaffolds were sterilized by ethylene oxide. Before the cell seeding, the scaffolds were degassed and hydrated in α -MEM overnight in the CO₂ incubator. Afterwards, the scaffolds were removed from the medium and placed into a 24-well suspension cell culture plate (Cell star; Greiner Bio-One, Kremsmuenster, Austria). RBMSCs of passage 2 were detached from the flasks and a new cell suspension with cell density of 5 million/mL were prepared (P3). The cells were seeded onto the surface of the scaffolds, and then the scaffolds with cells were kept in the CO₂ incubator. For the cell viability assay, 100,000 cells were seeded onto each bilayered scaffold. For the cell proliferation and osteogenic differentiation assay, 200,000 cells were seeded onto each bilayered scaffold, and 10,000 cells were seeded onto each S16 or SC16. After 3 hours, the constructs were transferred to each well of a 24-well suspension culture plate. To each well was added 2 mL of α -MEM. After seeding overnight, the constructs were cultured in basal medium (α -MEM) and osteogenic medium, respectively. The culture medium was refreshed every two or three days.

5.2.4. Encapsulation of ATDC-5 cells in SF hydrogels

In Chapter VIII, the ATCD-5 cells were used for cell encapsulation study in the SF hydrogels. SF solution of 16 wt.% were sterilized by UV radiation for 15 minutes in a sterile vertical laminar airflow cabinet (BH-EN 2000 S/D; Faster, Cornaredo, Italy) and used for the later cell encapsulation. The cell encapsulation procedure was performed in the sterile cabinet. All the solutions and materials used for cell encapsulation were sterile. At first, a water bath was placed inside the cabinet with temperature controlled at 37°C by a heating magnetic stirrer (FB15001; Thermo Fisher Scientific, Waltham, MA, USA). Cells were detached and suspension was prepared. Cell suspension containing 1 million cells was placed in a 1.5 mL centrifuge tube (Eppendorf, Hamburg, Germany) and subsequently centrifuged in a centrifuge (5810R, Eppendorf, Hamburg, Germany), a cell pellet was obtained after remove the supernatant. The SF solution (1 mL) was mixed with the HRP and H₂O₂ solutions in the 1.5 mL centrifuge tube and the mixture was warmed in the water bath for 6 minutes. Two formulations were used: 1/0.26‰/1.1‰ and 1/0.26‰/1.45‰ (SF/HRP/H₂O₂, by wt.). The warmed mixture (1 mL) was mixed with the cell pellet to obtain a homogeneous cell suspension (seeding density: 1 million/mL), and

every 50 μ L of the cell suspension was transferred into one piece of the polystyrene cover slips with 13 mm diameter (Sarstedt, Newton, NC, USA) in a 24-well suspension cell culture plate. The plate was then placed into the CO₂ incubator for around 10-15 minutes to allow the gelation. After the gel was formed, 1 mL basal α -MEM medium was supplemented into each well. The incorporated cells were cultured in the CO₂ incubator and the medium was changed every two days.

5.3. Cytotoxicity examination

5.3.1. MTS assay

The 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay (MTS) is a common method to study the cytotoxicity [57]. In Chapter IV, the cytotoxicity of the Silk-NanoCaP and SF scaffolds was screened by culturing the L929 cells with the extractions from the scaffolds. Briefly, extract fluids were obtained by immersing 1g of scaffolds (sterilized by autoclave) in a 50 mL tube containing 20 mL complete DMEM culture medium. The tubes were incubated in a water bath at 37°C with 60 rpm for 24 hours. A latex rubber extract was used as positive control for cell death. Afterwards, the extract fluids were filtrated by using a 0.45 μ m filter. The culture medium in each well (cultured with L929 cells for 24 hours) was removed and replaced by an identical volume (200 μ L) of the extraction fluids. Cell culture medium was used as negative control. At the end of 1, 3 and 7 days, the extracts were removed and replaced by 300 μ L of mixed solution containing serum-free culture medium (without phenol red) and MTS using the CellTiter 96® AQueous One Solution Cell Proliferation Assay Kit (Promega, Fitchburg, WI, USA). After incubation for 3 hours at 37°C in an atmosphere with 5% CO₂, the optical density (OD) was measured at 490 nm using a plate reader (Molecular Devices, SunnyVale, CA, USA). Cell viability was calculated by subtracting the mean OD value of the blank (MTS solution) from the ones of the scaffolds and controls, followed by normalization with the mean OD value obtained for the negative control (cell culture medium). The MTS assay was performed in triplicate (n=18).

In Chapter VII, the RBMSCs viability on the bilayered scaffolds was tested by MTS, after culturing for 1, 3 and 7 days. The MTS working solution was prepared as mentioned above. At the end of each time point, the constructs were removed from the culture

medium, washed by PBS solution, and then placed into 1 mL working solution in a 48-well cell culture plate and kept in the incubator for 3 hours at 37°C. Afterwards, the supernatant from each well was transferred into a 96-well cell culture plate (100 µL/well) and read in a microplate reader (Synergy HT; Bio-Tek, VT, USA) at 490 nm. The scaffolds without cells were used as control. Three independent experiments were performed for the cell viability assay, and at least three samples were analyzed for each time point in one experiment.

In Chapter VIII, the viability of the ATDC-5 cells encapsulated in the SF hydrogels was studied by MTS assay, after culturing for 1, 4, 7 and 10 days. At each time point, the culture medium was removed, and the hydrogels with cells were washed by PBS solution once. The MTS working solution (500 µL) was added into each well, followed by incubated in the CO₂ incubator for 3 hours before read in a microplate reader (Synergy HT; Bio-Tek, VT, USA) at 490 nm. Hydrogels without cells were used as control. Three independent experiments were performed, with three samples analyzed for each time point in each experiment.

5.3.2. Alamar Blue assay

In Chapter V, the viability of the hASCs seeded in the scaffolds was evaluated after cell seeding for 1, 3, 7, 10 and 14 days, by using the Alamar blue reagent (AlamarBlue®, AbD Serotec, Kidlington, Oxford, UK) [58]. The Alamar blue working solution containing 10% Alamar blue stock solution and 90% α-MEM was prepared and protected from light. At the end of each time point, the constructs were transferred into a new 48-well cell culture plate which was supplemented with 500 µL Alamar blue working solution in each well. The plate was kept in dark and incubated for three hours in the CO₂ incubator. Afterwards, 100 µL supernatant from each construct was transferred into each well of a new 96-well cell culture plate. The constructs were washed by PBS solution for three times and then returned to the corresponding well in the original culture plate. The culture medium was changed accordingly. The reacted AlamarBlue® was read in a microplate reader (Synergy HT; Bio-Tek, VT, USA) at 570 and 600 nm, respectively. And then, the reduction percentage of AlamarBlue® was calculated following the protocol from the manufacturer. Scaffolds without cell seeding were used as controls. Four specimens

were used for each group at each time point. Three independent experiments were performed.

5.3.3. Live/Dead staining assay

In Chapter VII, 100,000 cells were seeded onto the bilayered scaffolds for viability assay. The Live/Dead assay was performed by Calcein AM and propidium iodide (Molecular Probes®; Life Technologies, Carlsbad, CA, USA) staining after cell culturing for 3 days. At first, each construct was washed by PBS solution, and then transferred into 1 mL PBS solution supplemented with 1 µg Calcein AM and 2 µg propidium iodide and subsequently incubated in the incubator for 10 minutes. The samples were observed in a transmitted and reflected light microscope with apotome 2 (Axio Imager Z1m; Zeiss, Jena, Germany), after rinsing by PBS solution twice. By using the accompanying software Zen, a Z-stack function was used to combine images at different depth into one final image. The cells stained in green indicated live and the cells stained in red indicated dead.

In Chapter VIII, The viability of the encapsulated cells was evaluated by Live/Dead after culturing for 1, 3, 7 and 10 days. Calcein AM and propidium iodide staining was used as mentioned above. For this assay, the hydrogels with cells were washed by PBS solution, and then immersed in 1 mL PBS solution supplemented with 1 µg Calcein AM and 2 µg propidium iodide for 10 minutes. The samples were observed in a microscope after washing by PBS solution.

5.4. DNA quantification

In Chapter V, the proliferation of hASCs seeded into the scaffolds was analyzed by the total DNA content, after culturing for 1, 3, 7, 10 and 14 days [57]. At the end of each time point, the constructs were removed from the medium, followed by rinsing with PBS solution. Afterwards, each construct was transferred into one vial containing 1 mL ultrapure water. The vials were stored at -80°C freezer at least for 6 hours before the DNA content determination. For the DNA quantification, the constructs were defrosted firstly, and then underwent ultrasonication treatment for 20 minutes to release the DNA

from the scaffolds. The double-stranded DNA (dsDNA) was quantified by using a Quant-IT PicoGreen dsDNA Assay Kit 2000 assays (Life Technologies, Carlsbad, CA, USA) according to the instruction from the manufacturer. Briefly, 30 μ L supernatant from each vial was mixed with 70 μ L PicoGreen working solution and 100 μ L Tris-EDTA buffer. The fluorescence intensity of the samples was recorded in the microplate reader (Synergy HT, Bio-Tek, VT, USA), with the excitation wavelength at 485/20 nm and the emission wavelength at 528/20 nm. Standard curve was prepared by using standard dsDNA solutions with different concentrations, in order to quantify of the DNA content in the samples.

In Chapter VII, the proliferation of the seeded RBMSCs on the bilayered scaffolds and the controls (S16 and SC16) was screened by DNA quantification, after culturing for 7 and 14 days. At the end of each time point, each construct was removed from the medium and rinsed by PBS solution. After rinse, the silk layer and the Silk-NanoCaP layer were separated by a blade, and each part was placed into 1 mL ultrapure water in a 1.5 mL centrifuge tube. The analysis procedure and the equipment used for DNA quantification was the same as mentioned above. The DNA contents of the bilayered scaffolds were obtained by combining the DNA contents of the corresponding silk layer and Silk-NanoCaP layer. The proliferation studies were repeated twice, with at least three specimens for each time point in one study.

5.5. *In vitro* osteogenesis differentiation of RBMSCs

5.5.1. Osteogenic differentiation culture of RBMSCs in scaffolds

In Chapter VII, the RBMSCs seeded in the bilayered scaffolds and the controls (S16 and SC16) were undergone osteogenic differentiation for 7 and 14 days [57]. After seeding the cells in the scaffolds overnight, the constructs were cultured in basal medium (α -MEM) and osteogenic medium, respectively. The osteogenic medium was based on the α -MEM, and supplemented with 10 mmol/L beta-glycerophosphate, 50 μ g/mL ascorbic acid (Wako Pure Chemicals, Tokyo, Japan), and 10^{-8} mol/L dexamethasone. The medium were changed every two or three days. At the end of each time point, each construct was removed from the medium and rinsed by PBS solution.

5.5.2. Quantification of alkaline phosphatase (ALP)

In Chapter VII, the same lysates for DNA assay were also used for ALP activity quantification. For this assay, 20 μ L supernatant was mixed with 60 μ L 0.2% (wt./vol.) *p*-nitrophenyl phosphate disodium solution (*p*NPP) and incubated at 37°C for 1 hour [57]. The *p*NPP was dissolved in 1 mol/L diethanolamine buffer solution (pH 9.8, adjusted by hydrochloric acid). During the incubation, the *p*NPP was hydrolyzed by the ALP and the yellow *p*-nitrophenol (*p*NP) was formed. The reaction was stopped by the addition of 80 μ L of 2 mol/L sodium hydroxide solution into each well. The absorbance of each well at 405 nm was read in the microplate reader (Synergy HT, Bio-Tek, VT, USA). The standard solutions were prepared with the 10 mmol/L *p*NP solution. And the absorbance of these standard solutions was read in order to prepare the standard curve. The ALP activity from each sample was reflected by the amount of the formed *p*NP. The ALP activity of the samples was normalized by their corresponding DNA contents. The ALP activities of the bilayered scaffolds were obtained by combining the ALP activities of the corresponding silk layer and Silk-NanoCaP layer. The differentiation studies were repeated twice, with at least three specimens for each time point in one study.

5.6. Cell attachment and migration evaluation

In Chapter V, the attachment and migration of the hASCs in S16 and SC16 were observed by SEM, after culturing for 1, 3, 7, 10 and 14 days. At the end of each time point, the constructs were removed from the medium and rinsed by PBS solution, followed by fixing in 10% formalin solution for at least overnight. In order to dehydrate the specimens, the fixed constructs were immersed in a serial of aqueous ethanol solutions with gradient increased concentration in ethanol (from 30% to 100%). The samples were dried in a flow chamber. And then the surface of the constructs was observed by SEM (Nova NanoSEM 200; FEI, Hillsboro, OR, USA). Prior to the analysis, the specimens were coated with Au/Pd SC502-314B in a high vacuum evaporator coater (E6700; Quorum Technologies, East Grinstead, UK).

In Chapter VII, the cells' attachment on the bilayered scaffolds in both basal and osteogenic conditions was observed by SEM, after culturing for 7 days. The procedures

for preparation the specimen and the equipments used were the same as for hASCs attachment observation in S16 and SC16.

In Chapter VIII, the hydrogels encapsulated with cells were frozen and then lyophilized, after culturing for 6 and 10 days, respectively. The morphology of the hydrogels was observed by SEM after surface coating, using the same equipments as for the hASCs attachment observation in S16 and SC16.

5.7. Biomechanical analysis

In Chapter V, the compressive modulus of the S16 and SC16 after culturing with hASCs for two weeks was examined. At the 14th days, the constructs were removed from the culture medium and subsequently rinsed by PBS solution. The specimens were tested in a universal testing machine (Instron 4505; Instron, Norwood, MA, USA), after removing the surface liquid by filter paper. The samples were screened under a compressive rate of 2 mm/minute until reaching 60% strain. The slope of the initial linear domain in the compressive curve was used to determine the elastic modulus of each specimen. Scaffolds kept in culture medium for two weeks but without cell seeding were used as controls. At least six specimens were analyzed for each group.

5.8. Histological analysis

5.8.1. Haematoxylin and Eosin (H&E) staining

In Chapter V, the scaffolds cultured with the hASCs for 3, 7 and 14 days were analyzed by H&E staining. At the end of each time point, the constructs were removed from the culture medium and washed by PBS solution. Afterwards, the constructs were fixed in 10% formalin overnight then immersed in paraffin after dehydration. Slides of 4 μ m in thickness were prepared by a microtome (HM355S Microtome; Thermo Fisher Scientific, Waltham, MA, USA), and then the slides were subjected to H&E staining which was performed in a automatic staining (Robot-Stainer HMS740; Thermo Fisher Scientific, Waltham, MA, USA). In the end, the slides were mounted.

5.8.2. Toluidine blue staining

In Chapter V, the scaffolds cultured with the hASCs were also undergone Toluidine blue staining. Slides were prepared using the same procedure as for the H&E staining. After removing the paraffin, the sections were subsequently rinsed with ultrapure water. Afterwards, the sections were stained with 0.05% Toluidine blue solution. After mounting, the sections were observed under microscope.

6. *In Vivo* Studies

6.1. Subcutaneous implantation

In Chapter VII, the bilayered scaffolds were subcutaneously implanted in rabbit [56]. The bilayered scaffolds of 6 mm in diameter and 8 mm in height (Silk layer: 3 mm; Silk-NanoCaP: 5 mm) were used for the subcutaneous implantation in New Zealand White rabbits (Charles River, Senneville, Quebec, Canada). All the rabbits for the *in vivo* studies were male and of 9-11 weeks old, with average weight 2.4 Kg at the implantation time. The maintenance and usage of animals were approved by the Ethics Committee of University of Minho. The scaffolds were sterilized by ETO and all the procedures were performed in an aseptic condition. For the implantation, six bilayered scaffolds were implanted into three rabbits (2 pieces/rabbit). Each rabbit was anesthetized by intravenous injection of 1.375 mL mixture of Imalgene (Ketamina, 75 mg/Kg) and Domitor (Medetomidina 1 mg/Kg). The hair of the rabbit was cut at the implantation area, followed by washing with 70% ethanol and iodine. In each rabbit, two skin incisions were made below the ears in the back (one in the left and the other in the right), each around 2 cm in length. The scaffolds were subcutaneously implanted into each pocket. And the skin was sutured by using bioresorbable silk suture. After 4 weeks, the rabbits were euthanized by injection of overdose anesthesia and the implanted scaffolds were retrieved.

In Chapter VIII, the SF hydrogels were implanted subcutaneously in mice. The maintenance and use of animals were in accordance to the Ethics Committee of University of Minho. Two formulations of the Silk-16 hydrogels were used for the *in vivo* implantation: 1/0.26‰/1.1‰ and 1/0.26‰/1.45‰ (SF/HRP/H₂O₂, by wt.). The hydrogel discs (Diameter: 8 mm; Height: 3 mm) were prepared in a sterile condition using the sterilized silk solution. In this study, 4 Mice Hsd:ICR (CD-1) of 5 weeks old and average

weight of 32 g (Charles River, Senneville, Quebec, Canada) were used. Each mouse was anesthetized by intraperitoneal injection of 100 μ L of a mixture of Imalgene (Ketamina, 75 mg/Kg) and Domitor (Medetomidina 1 mg/Kg). If necessary, 50 μ L Antisedan (Atipamezol, 1 mg/Kg) was used to reverse the anesthesia. The hair in the implantation area of the mouse was removed by shaving, followed by disinfection via scrubbing with tincture of iodine. In each mouse, 4 skin incisions were made in the back near the midline below the ear, two in the right side and another two in the left side. In the following, 4 pieces of hydrogel discs were implanted subcutaneously into respective pocket and the skin was sutured. For each formulation, 8 pieces of hydrogel discs were implanted. After 2 weeks post-surgery, the mice were euthanized by injection of overdose pentobarbital sodium, and the implants were retrieved.

6.2. Implantation in bone defects

In Chapter VI, the S16 and SC16 (4 mm in diameter and 3 mm in height) were implanted in the femur bone defects of rat [59]. The scaffolds were sterilized by ethylene oxide. Young male Wistar rats (n=6 per group) with an a body weight of 125 to 150 g were purchased from Charles River (Senneville, Quebec, Canada), housed in light- and temperature-controlled rooms, and fed a standard diet. Bone defects were drilled bilaterally in each distal femur, proximal to the epiphyseal plate, of every rat. The defects were made using a low speed drill (2.3 mm in diameter) with copious saline irrigation. The defects were made until it reached the bone marrow domain. The scaffolds were then pressed fit into the defects. The maintenance and use of animals were in accordance to the Ethics Committee of University of Minho. Animals were sacrificed after 3 weeks and the femurs were removed.

6.3. Implantation in the osteochondal defects (OCD)

In Chapter VII, the bilayered scaffolds of 5 mm in diameter and 5 mm in height (Silk layer: 2 mm; Silk-NanoCaP: 3 mm) were implanted in the OCD in the knee of the New Zealand White rabbits (Charles River, Senneville, Quebec, Canada) [60]. The rabbits for this study were the same condition as the ones for subcutaneous implantation. The maintenance and usage of animals were approved by the Ethics Committee of University

of Minhø. The scaffolds were sterilized by ETO and all the procedures were performed in an aseptic condition. The implantation was performed in critical size OCD (4.5 mm in diameter and 5 mm in depth), 9 bilayered scaffolds were implanted into 3 rabbits (3 pieces/rabbit). The anesthesia of the rabbits was administered intravenously with a mixture of Imalgene (Ketamina, 75 mg/Kg) and Domitor (Medetomidina 1 mg/Kg), with 1.375 mL/animal. The hair of the rabbit was cut at the implantation area, followed by washing with 70% ethanol and iodine. The rabbits were anesthetized and the hair in the knee joints of the hind legs was cut. The skin was washed with 70% ethanol and iodine. And then the knee joints were exposed through a medial parapatellar longitudinal incision. Two OCD were created in each femur using a Brace manual drill, one located between the lateral and the medial condyle, the other was in the opposite site of the patellar. The bilayered scaffolds were implanted into the defects by press fit. The skin was sutured. In each rabbit, one of the defects was empty and used as control. Four weeks post-operation, the rabbits were euthanized with an overdose of pentobarbital sodium, and the knees were excised.

6.4. Explants characterization

6.4.1. Histological examination

In Chapter VI, The femurs of the rats (n=5/group) were fixed in neutral formalin, decalcified in a 1:1 mixture of 45% formic acid and 20% sodium citrate, dehydrated and embedded in paraffin. Five-micrometer-thick serial sections perpendicular to the long axis of the implant were cut with a Spencer 820 microtome (Spencer 820, American Optical Company, NY, USA). Sections were then stained with Masson's Trichrome stain to selectively stain muscle, collagen fibers, fibrin, and erythrocytes respectively. A green color is attributed to collagen in the newly formed bone.

In Chapter VII, the explants from the rabbit subcutaneous implantation were fixed in 10% formalin, and then dehydrated through graded ethanol, and finally embedded in paraffin. Sections were prepared by cutting the specimen into sections of 5 μ m thick using a microtome (Spencer 820, American Optical Company, NY, USA). The obtained sections were stained with H&E.

In Chapter VII, three explants from the rabbit OCD were fixed by 10% formalin and then immersed in paraffin after dehydration. Slides were prepared, and H&E and Masson's Trichrome staining were performed.

6.4.2. Histomorphometry.

In Chapter VI, the bone histomorphometry was evaluated via IMAGE J (National Institutes of Health, Bethesda, MD). The images of the Masson's Trichrome slides (area for each slide: 0.45 mm*0.35 mm) of each explants were first converted to gray-value images. And then proper threshold values were selected for each image in order to best match the new bone area in original images. The new bone area in each slide image was calculated by the software. Slides from 4 explants were used for each group, and at least 10 slides were evaluated per explant.

6.4.3. Micro-CT analysis of the explants

In Chapter VII, three explants from the OCD were used for micro-CT observation in wet state, under 100 keV and 98 μ A. The explants were loaded by a parafilm during the scanning to avoid the evaporation of liquid. The integration time was fixed at 1.3 second and the pixel resolution was 19.13 μ m. The specimens were first scanned and the data sets were processed following the procedure for scaffold scanning as mentioned above (Section 4.1.2 in this Chapter). The 3D micro-CT images of the explants were obtained by using the CTvox software (Skyscan). In order to calculate the porosity and CaP content in the interested regions, the data set of each specimen was re-arranged by standard software (Dataviewer, Skyscan). The porosity and CaP contents of the defect controls and the defects implanted with scaffolds were analyzed in standardized software (CT Analyser, version 1.5, Skyscan), and the thresholds used were the same as mentioned above (Section 4.1.2 in this Chapter). In each specimen, a cylinder model region (Height: 4 mm; Diameter: 4 mm) was used for the evaluation of porosity and CaP distribution. For the quantification calculation of the porosity or the CaP content, the top 2 mm region in the cylinder model region was considered as cartilage domain in defect controls or as silk layer in defects implanted with scaffolds, and the down 2 mm region

was considered as subchondral bone domain in defect controls or as Silk-NanoCaP layer in defects implanted with scaffolds.

6.4.4. SEM observation

In Chapter VII, the explants from the rabbit subcutaneous implantation were lyophilized and then coated with Au/Pd and observed by SEM, followed the protocol as mentioned above for cell attachment observation in Section 5.6 in this Chapter.

6.4.5. ATR evaluation

In Chapter VIII, the SF hydrogels after two weeks subcutaneous implantation were evaluated by ATR. The SF hydrogels for this test were without fixation in formalin. Before the analysis, the surface of the hydrogels was cleaned by removing the wrapping tissues and washing by PBS solution. This examination were followed the same procedure as mentioned above for ATR test on SF hydrogel (Section 4.3. in this Chapter).

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Section 3.

Chapter III

Macro/Microporous Silk Fibroin Scaffolds with Potential for
Articular Cartilage and Meniscus Tissue Engineering
Applications

Chapter III

Macro/Microporous Silk Fibroin Scaffolds with Potential for Articular Cartilage and Meniscus Tissue Engineering Applications

Abstract

This study describes the developmental physicochemical properties of silk fibroin scaffolds derived from high concentration aqueous silk fibroin solutions. The silk fibroin scaffolds were prepared with different initial concentrations (8%, 10%, 12% and 16%, in wt.%) and obtained by combining the salt-leaching and freeze-drying methodologies. The results indicated that the antiparallel β -pleated sheet (silk-II) conformation was present in the silk fibroin scaffolds. All the scaffolds possessed macro/micro porous structure. Homogeneous porosity distribution was achieved in all the groups of samples. As the silk fibroin concentration increased from 8% to 16%, the mean porosity decreased from $90.8 \pm 0.9\%$ to $79.8 \pm 0.3\%$, and the mean interconnectivity decreased from $97.4 \pm 0.5\%$ to $92.3 \pm 1.3\%$. The mechanical properties of the scaffolds exhibited a concentration dependence. The dry state compressive modulus increased from 0.81 ± 0.29 MPa to 15.14 ± 1.70 MPa, and the wet state dynamic storage modulus increased around 20-30 folds at each testing frequencies when the silk fibroin concentration increased from 8% to 16%. The hydration degree decreased by means of increasing silk fibroin concentration. The scaffolds present favorable stability as their structure integrity, morphology and mechanical properties were maintained after *in vitro* degradation for 30 days. Based on these results, the scaffolds developed in this study are herein proposed to be used in meniscus and cartilage tissue engineering scaffolding.

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1. Introduction

The development of novel 3-dimensional degradable porous scaffolds is of great interest for tissue engineering and regenerative medicine [1]. There are several critical requirements in the design and preparation of the scaffolds [2-3]. With those requirements in mind, different biomaterials have been explored as matrices to be used in tissue engineering scaffolding, such as synthetic and natural occurring polymers, and bioactive calcium phosphate ceramics [4-10]. Among those, silk fibroin derived from silkworm *Bombyx mori* has proved to be a promising candidate as a scaffolding material [11,12]. *In vivo*, its foreign body response is dependent on the implantation site and chosen model, and in most cases, the response is low and subsides with time [11]. Additionally, it is a versatile material for tissue engineering scaffolding as its degradability and mechanical properties can be tailored by chemical cross-linking or by the introduction of β -sheet conformation [13]. Moreover, it can be processed easily into various structures, such as fiber meshes, membranes, hydrogels, 3-dimensional porous scaffolds, and microspheres [14-21]. For the above reasons, silk-based scaffolds have been successfully applied in tissue engineering of skin, bone, cartilage, tendon and ligament [11,12]. These structures produced favorable outcomes in the previous biomedical explorations [22-26].

In order to produce porous silk fibroin scaffolds, a diversity of methods have been used, such as: salt leaching, gas foaming, freeze-drying and rapid prototyping [14,19,26-28]. Kim *et al.* [14] proposed a new strategy to prepare porous silk fibroin scaffolds by means of using aqueous derived silk fibroin solutions and salt leaching method. The whole preparation procedure was in aqueous environment, and the scaffolds produced presented new features regarding the biodegradation and mechanical properties [14,17]. Makaya *et al.* [28] developed a modified method to prepare salt leached silk fibroin scaffolds via a size-reduced porogen (250-500 μm) for cartilage regeneration. Wang *et al.* [29] further studied the synergistic effects of salt leached silk fibroin and hydrodynamic environment in cartilage tissue regeneration. However, to the authors' knowledge, salt leached porous scaffolds prepared with more than 10% aqueous silk fibroin solution were not reported yet [14,17]. Although there were a few reports using high concentration silk fibroin solution [15,19,23,30,31], no processing routes to form different structures by comprising combination of salt-leaching and freeze-drying methodologies.

The previous studies indicated that the compressive modulus values of the salt leached silk fibroin/cell constructs were still very low although they were higher as compared to the silk scaffold controls, as reported by Marolt *et al.* [32], and Kim *et al.* [33]. To prepare silk or silk-based scaffolds with initial improved mechanical properties for specific tissue engineering applications can be of high interest, as reported by Collins *et al.* [34] and Rajkhowa *et al.* [35]. In the present work, highly concentrated aqueous silk fibroin solutions were used to prepare silk-based scaffolds aiming at improving the obtained physicochemical properties. The mechanical properties and three-dimensional architecture were tailored to make them suitable for cartilage and meniscus tissue engineering. The aqueous derived silk fibroin scaffolds were prepared via salt leaching method, with different initial concentrations (8, 10, 12 and 16%, in wt.%) followed by freeze-drying. The structural conformation of silk fibroin was confirmed by Fourier transform infra-red spectroscopy (FTIR) and X-ray diffraction (XRD). The morphology and microstructure of the scaffolds were assessed by scanning electron microscopy (SEM) and micro-computed tomography (micro-CT). The static and dynamic mechanical properties were characterized by both compressive tests and dynamic mechanical analysis (DMA). The hydration degree and degradation ratio were registered for different time periods, ranging from 3 hours to 30 days. Finally, the morphology and mechanical properties of the scaffolds were also analyzed by SEM and DMA, respectively.

2. Materials and Methods

2.1. Materials

Cocoons of *Bombyx mori* were supplied by the Portuguese Association of Parents and Friends of Mentally Disabled Citizens (APPACDM, Castelo Branco, Portugal). In this study, commercial grade granular sodium chloride (Portugal) was used. Silicon tubing was purchased from Deltalab (Barcelona, Spain). The remaining materials and reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless indicated otherwise.

2.2. Preparation of concentrated silk fibroin aqueous solution

Bombyx mori silk fibroin was prepared as reported elsewhere with minor modifications [16]. In brief, cocoons were boiled for 1 hour in an aqueous sodium carbonate solution (0.02 M), and then rinsed thoroughly with distilled water in order to extract the glue-like protein sericine and wax. The purified silk fibroin was dissolved in 9.3 M lithium bromide solution at 70°C for 1 hour yielding a 16% (wt./vol.) solution. The solution was dialyzed in distilled water using a benzoylated dialysis tubing (molecular weight cut-off: 2 kDa), for the period of 48 hours. Afterwards, the silk fibroin aqueous solution was dialyzed against a 20 wt.% poly(ethylene glycol) solution (20,000 g/mol) for around 6 hours [31]. Finally, the dialysis tubing was carefully washed in distilled water, and silk fibroin solution was collected to a flask. The final concentration of the concentrated silk fibroin was about 20 wt.%, as determined by measuring the dry weight of the silk fibroin solutions. The prepared silk fibroin solution was stored at 4°C until further use.

2.3. Preparation of salt leached silk fibroin scaffolds

Granular sodium chloride was prepared by sieving the sodium chloride in an analytical sieve shaker (Retsch, Haan, Germany) in the range of 500-1000 µm. The prepared concentrated silk fibroin solution was diluted into 8, 10, 12 and 16 wt.%, respectively. The scaffolds were prepared by transferring 1 mL of silk fibroin solution (8-16%) into a silicon tubing (inner diameter: 9 mm), followed by addition of 2 g of granular sodium chloride (500-1000 µm) [14]. In the case of the preparation of scaffolds from silk fibroin solutions of 12% and 16%, the sodium chloride particles were slowly added into the silicon tubing and this was gently tapped to facilitate the precipitation of the salt particles. Afterwards, the silicon tubing was placed in a Petri dish and dried at room temperature for 48 hours. In order to extract the sodium chloride, the tubing was immersed in distilled water for 3 days. Finally, the scaffolds were obtained by using a stainless steel punch (inner diameter: 6 mm) in order to remove the outer skin that is generated, followed by freezing at -80°C during 1 day and freeze-drying (CRYODOS-80; Telstar, Barcelona, Spain). The prepared silk fibroin scaffolds are here designated as silk-8, silk-10, silk-12 and silk-16, according to their initial concentrations (in wt.%), respectively (Figure S1). The air dried scaffolds were also prepared as control (Figure S2).

2.4. Physicochemical characterization

2.4.1. XRD

X-ray diffractometer (Philips PW 1710; Philips, Amsterdam, Netherlands) employing Cu-K α radiation ($\lambda=0.154056$ nm) was used to analyze the crystallinity of the silk scaffolds on powder. Data was collected from 0 to 60° 2 θ values, with a step width of 0.02° and a counting time of 2 second/step. The test was repeated three times for each condition.

2.4.2. FTIR

The infrared spectra of the silk fibroin powders were recorded on a FTIR spectroscopy (Perkin-Elmer 1600 series equipment; Perkin-Elmer, MA, USA). Prior to the analysis, the silk fibroin powders were mixed with potassium bromide in a ratio of 1:100 (by wt.) followed by uniaxially pressing into a disk. All spectra were obtained between 4000 to 400 cm⁻¹ at a 4 cm⁻¹ resolution with 32 scans. Each condition was examined for at least three times.

2.4.3. SEM

The cross-sectional morphology of the prepared scaffolds was observed under the scanning electron microscope (Leica Cambridge S-360; Leica Manufacturer, Cambridge, UK). Prior to the analysis, specimens were coated with gold using a Fisons Instruments Coater (Polaron SC 502; Fisons plc, Ipswich, UK). The cross-sectional morphology of scaffolds after 30 days of degradation was also observed under the SEM (Nova NanoSEM 200; FEI, Hillsboro, OR, USA). The specimens were coated with Au/Pd SC502-314B using a high vacuum evaporator coater (E6700; Quorum Technologies, East Grinstead, UK). Three samples were tested for each condition.

2.4.4. Micro-CT

The architecture of the silk scaffolds was evaluated using a high-resolution μ -CT Skyscan 1072 scanner (Skyscan, Kontich, Belgium) possessing a resolution of pixel size of ~ 8 μ m

and integration time of 1.3 second. The X-ray source was set at 40 keV and 248 μ A. Approximately 300 projections were acquired over a rotation range of 180° with a rotation step of 0.45°. Data sets were reconstructed using standardized cone-beam reconstruction software (NRecon v1.4.3, SkyScan). The output format for each sample was 300 serial 1024 x 1024 bitmap images. Representative data set of the slices was segmented into binary images with a dynamic threshold of 40-255 (grey values). Then, the binary images were used for morphometric analysis (CT Analyser, v1.5, SkyScan), and to build the 3D models (ANT 3D creator, v2.4, SkyScan). Three samples were tested for each condition.

2.4.5. Compression tests

Compressive tests (dry state) were performed by using a Universal Testing Machine (Instron 4505; Instron, Norwood, MA, USA) with a 1kN load cell at room temperature. The size of the tested specimens was measured with a micrometer. The length of the tested specimens for silk-8, silk-10, silk-12 and silk-16 were 5.593 ± 0.242 mm, 5.593 ± 0.330 mm, 5.935 ± 0.257 mm and 5.503 ± 0.187 mm, respectively. The diameter of the tested specimens for silk-8, silk-10, silk-12 and silk-16 were 5.355 ± 0.182 mm, 5.534 ± 0.154 mm, 5.435 ± 0.093 mm and 5.203 ± 0.062 mm, respectively. The cross-head speed was set at 2 mm/minute and until 60% reduction in specimen height. The elastic modulus (E) was defined by the slope of the initial linear section of the stress-strain curve. A minimum number of 7 specimens were tested. Then, E was averaged from the measurements.

2.4.6. DMA

The viscoelastic measurements were performed using a TRITEC8000B DMA (Triton Technology, Lincolnshire, UK), equipped with the compressive mode. The measurements were carried out at 37°C temperature. Samples were cut in cylindrical shapes with approximate 6 mm diameter and 5 mm thickness (measured each sample accurately with a micrometer). Scaffolds were always analyzed immersed in a liquid bath placed in a Teflon® reservoir. Scaffolds were previously immersed in a phosphate buffered saline (PBS) solution until equilibrium was reached (37°C overnight). The

geometry of the samples was then measured and the samples were clamped in the DMA apparatus and immersed in the PBS solution. After equilibration at 37°C, the DMA spectra were obtained during a frequency scan between 0.1 and 10 Hz. The experiments were performed under constant strain amplitude (50 µm). A small preload was applied to each sample to ensure that the entire scaffold surface was in contact with the compression plates before testing and the distance between plates was equal for all scaffolds being tested. A minimum of three samples were used for each condition.

2.4.7. Hydration degree and weight loss-related tests

The hydration degree and degradation behaviour of the silk fibroin scaffolds were assessed after immersion into an isotonic saline solution (ISS, 0.154 M sodium chloride aqueous solution, pH 7.4), for time periods ranging from 3 hours until 30 days [36]. All experiments were conducted at 37°C and dynamic condition (60 rpm) in a water bath (GFL 1086). After each time point, the specimens were removed from the ISS and the weights were determined immediately after adsorption of the excess of surface water using a filter paper. The hydration degree was calculated as following expression:

$$\text{Hydration degree} = \left(\frac{m_{w,t} - m_i}{m_i} \right) \times 100\% \quad (1)$$

Where m_i is the initial weight of the specimen before hydration, and $m_{w,t}$ is the wet weight of the specimens at time t after being removed from the ISS.

After the determination of the hydration degree, the specimens were washed with distilled water and dry in an oven at 60°C for 24 hours. The weight loss was determined using the following expression:

$$\text{Weight loss ratio} = \left(\frac{m_i - m_{d,t}}{m_i} \right) \times 100\% \quad (2)$$

Where m_i is the initial weight of the specimen before hydration, and $m_{d,t}$ is the dry weight of the specimen been degraded for a certain period of time, after drying at 60°C until constant weight is reached. Six specimens were used for each condition.

The surface morphology and dynamic mechanical properties of the specimens were analyzed as aforementioned, after 30 days of soaking. Three specimens were tested for each condition.

2.5. Statistical analysis

The mean pore size, mean pore size distribution, mean trabecular thickness, mean trabecular thickness distribution, mean porosity, mean interconnectivity, mechanical results, hydration degree and degradation ratio were presented as means $\pm$ standard deviation. At first, a one-way analysis of variance (ANOVA) was used to evaluate the data. And then a comparison between two means was analyzed using Tukey's test with statistical significance set at $p < 0.05$. At least three specimens were used in each condition.

3. Results and Discussion

3.1. Chemical structure

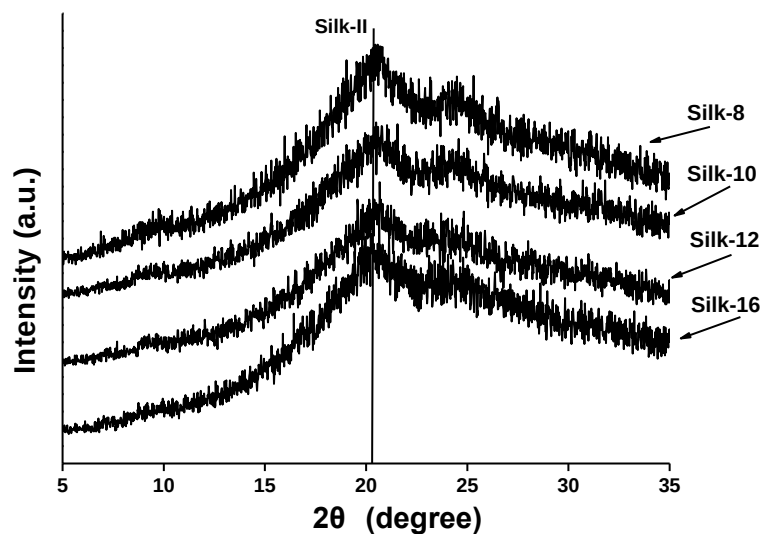


Figure 1. X-ray diffraction patterns of the silk fibroin scaffolds obtained by combining salt-leaching and freeze-drying methodologies.

Several conformations (random coil, silk-I, silk-II and 3_{10} -helix) of silk fibroin have been identified previously by means of X-ray diffraction, Infra-red spectroscopy, and ^{13}C nuclear magnetic resonance (NMR) [37-42]. Random coil is an amorphous structure presented in aqueous silk fibroin solution of low concentration, in lyophilized silk fibroin, and also in silk fibroin films casted under controlled conditions [31,43,44]. Silk-I is a metastable form which can be produced by drying the silk gland contents or by controlling the water annealing of silk fibroin films at room temperature [42-44]. Silk-II is an antiparallel β -pleated sheet structure which exists in natural silk fibroin fibers or can be produced from aqueous silk fibroin solutions treated with physical shear or organic solvents [31,38]. The 3_{10} -helix structure can be produced by casting silk fibroin solution in a fluoro-based solvent system [41,42].

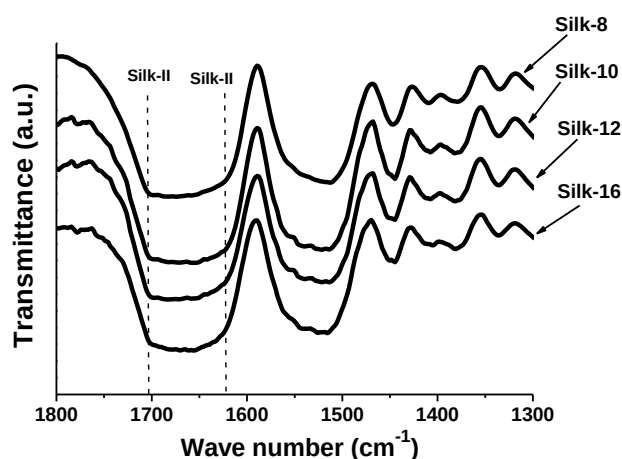


Figure 2. Fourier transform infra-red spectra of the silk fibroin scaffolds obtained by combining salt-leaching and freeze-drying methodologies.

Jin *et al.* [31] listed the fingerprint reflection of XRD for silk-I and silk-II (in angstroms): 9.8 (II), 7.4 (I), 5.6 (I), 4.8 (II), 4.4 (I), 4.3 (II), 4.1 (I), 3.6 (I), 3.2 (I), 2.8 (I). Kim *et al.* [14] defined the crystal structure of silk fibroin in the aqueous derived salt leached scaffold as silk-II evidenced by XRD peaks at $2\theta = 8.5^\circ$ (10.37 Å), 20.8° (4.35 Å) and 24.6° (3.62 Å). Previous studies [43,44] described the preparation of water-insoluble silk fibroin, mainly of silk-I structure. These studies reported that XRD peaks (2θ) at 24.2° (3.7 Å), and at around 22.2° and 25° were assigned to silk-I structure. Moreover, these studies showed that both silk-I and silk-II structures coexisted in the methanol annealing silk fibroin film. Tamada [45] reported that $2\theta = 24-25^\circ$ was attributed to silk-I structure and both the silk-I and silk-II conformations presented in the same scaffold. These observations were

supported by another interesting study [37], which reported the production of silk fibroin with variable amounts of silk-I and silk-II.

In this study, XRD analysis was performed to determine the crystalline structure in the scaffolds (Figure 1). From Figure 1, it is possible to observe that there were no significant differences between the four groups in respect to the peak positions. The peaks at 20.5° - 20.8° can be assigned to silk-II based on the previous studies in the literature [14,31,37,43,44]. All these peaks are broad and of low intensity, which is an indication that the prepared scaffolds possess low crystallinity and uncertain amount of random coil.

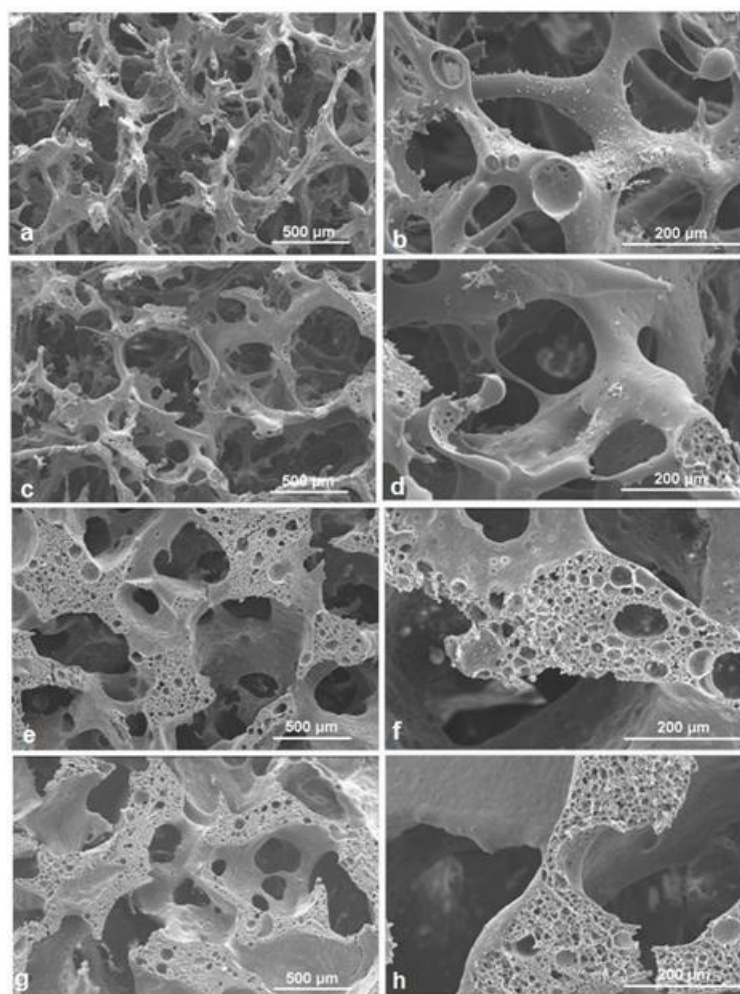


Figure 3. Scanning electron micrographs of the cross-sectional morphology of the silk fibroin scaffolds obtained by combining salt-leaching and freeze-drying methodologies. (a, b) Silk-8; (c, d) silk-10; (e, f) silk-12; (g, h) silk-16.

FTIR is also a reliable technique to further confirm the crystal conformation in silk fibroin [37,43-45]. Figure 2 shows the FTIR spectra of silk fibroin scaffolds obtained by combining salt-leaching and freeze-drying methodologies. The peaks located at 1701 -

1704 cm^{-1} , 1622-1627 cm^{-1} can be attributed to silk-II structure [43,44,46]. The corresponding peak positions of the main groups are mostly the same for all scaffolds. It should be addressed that the way the FTIR was performed can also affect the final spectra as reported by Demura *et al.* [47].

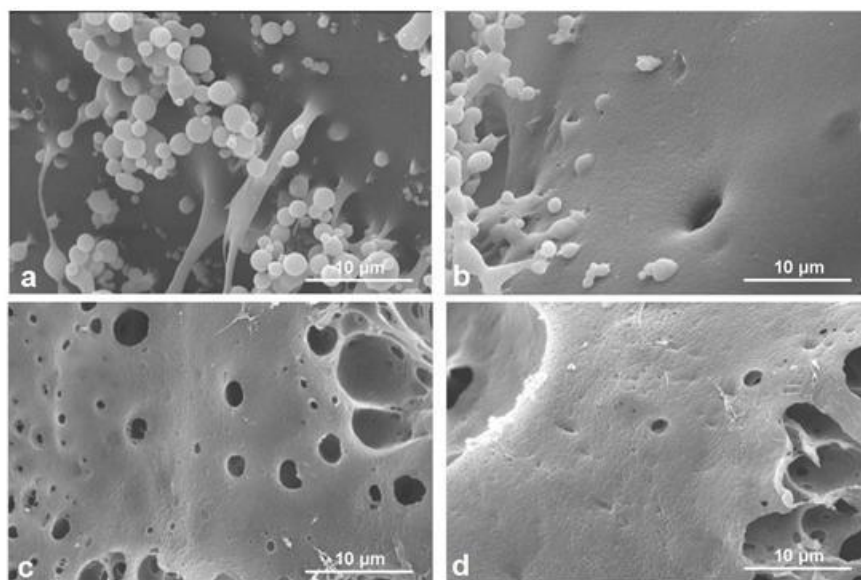


Figure 4. Scanning electron micrographs of the surface of the silk fibroin scaffolds obtained by combining salt-leaching and freeze-drying methodologies. (a) silk-8; (b) silk-10; (c) silk-12; (d) silk-16.

By correlating the XRD and FTIR results, it is possible to state that the prepared silk fibroin scaffolds possess silk-II structure. This observation is consistent with those reported in previous studies using the salt leaching methodology [14,28]. In this study, it was not possible to determine the content of the structure conformation in the different scaffolds. Further quantitative ^{13}C NMR analysis [28,37] and studies on conformational changes in a real-time manner need to be addressed.

3.2. Morphology and microstructure

Salt leaching method is an versatile route that has been attracting a great deal of attention in tissue engineering scaffolding [14,19,28]. In this study, the pores morphology of the prepared silk fibroin scaffolds was investigated using SEM. From the obtained images, mainly two types of pore size were observed among the cross-section of the scaffolds (Figure 3). The morphology of the developed scaffolds varied among the different initial concentrations used. The silk-8 and silk-10 presented branched-like

morphology (Figure 3a, c), while silk-12 and silk-16 seemed to possess thicker trabecular structures compared to silk-8 and silk-10, based on SEM visual observation (Figure 3e, g). From Figure 3, pores of several hundred micrometers were observed (named L-pore, Figure 3a, c, e and g). There were also pores with size less than 100 μm (named S-pore) distributed inside the trabeculae of the L-pore (Figure 3b, d, f and h).

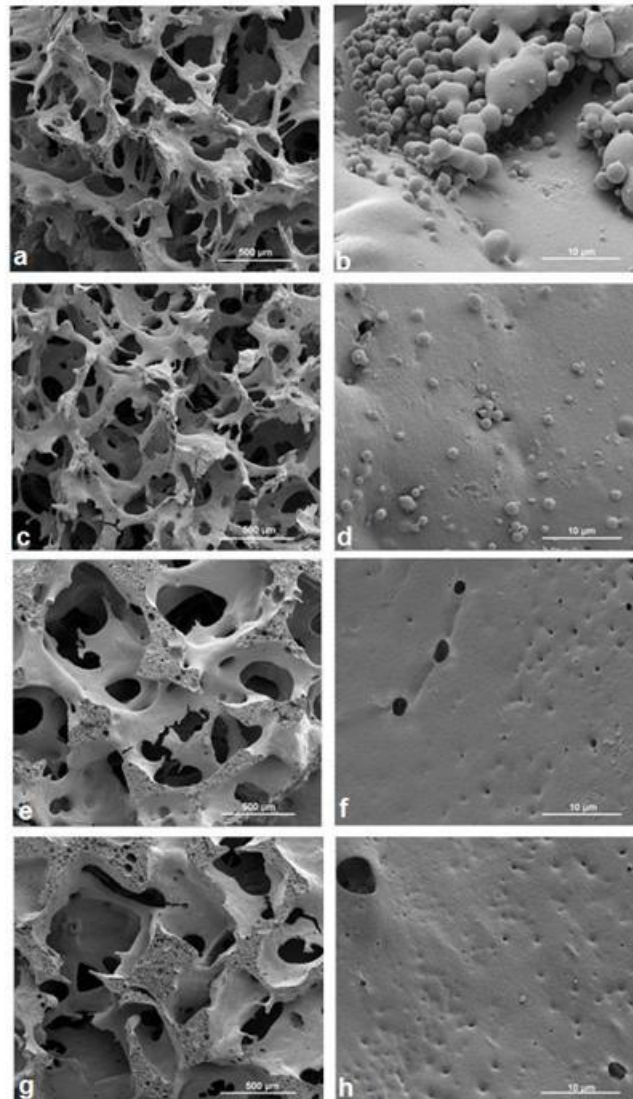


Figure 5. Scanning electron micrographs of the cross-sectional morphology of the silk fibroin scaffolds obtained by combining salt-leaching and freeze-drying methodologies after 30 days degradation. (a, b) Silk-8; (c, d) silk-10; (e, f) silk-12; (g, h) silk-16.

Figure 4 shows the SEM images of the surface of silk fibroin scaffolds obtained by combining salt-leaching and freeze-drying methodologies. From Figure 4, it can be seen that the surface of the different scaffolds are distinct. An interesting finding was the presence of silk fibroin microspheres on the surface of silk-8 and silk-10, which possess size ranging from several hundred nanometers to several micrometers (Figure 4a, b).

Additionally, it was observed pores with size less than 10 μm on the surface of silk-12 and silk-16 (Figure 4c, d).

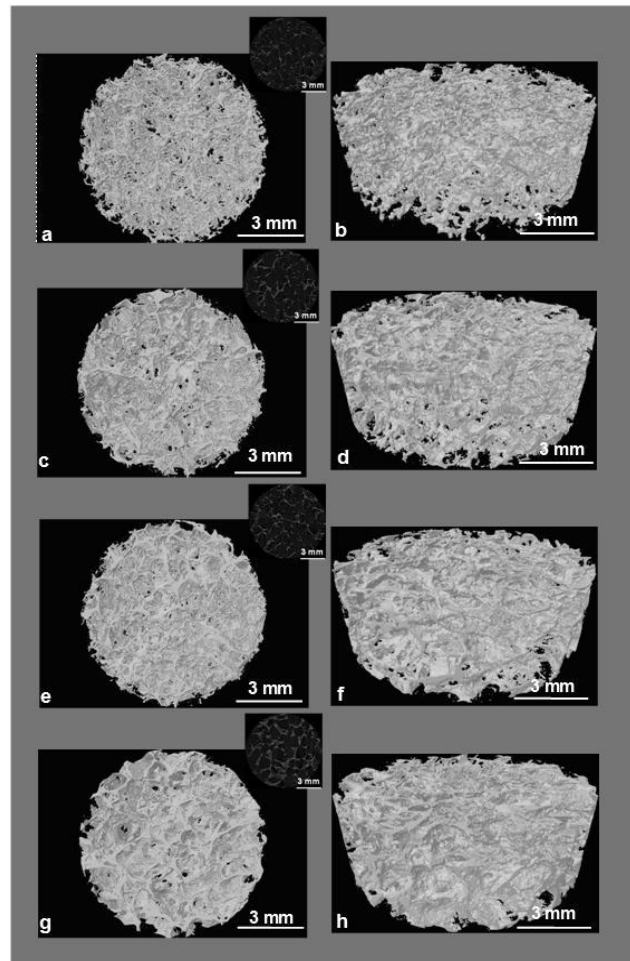


Figure 6. Micro-computed tomography 3-D images of the silk fibroin scaffolds obtained by combining salt-leaching and freeze-drying methodologies. (a, b) Silk-8; (c, d) silk-10; (e, f) silk-12; (g, h) silk-16. The inserted images are the 2-D images of the scaffolds.

In previous studies [14,28], uniform pore size distribution was achieved since the salt particles used were comprised within a narrow size range. The pore size of the scaffolds produced in the present study is not as homogeneous as the one found in the literature, since NaCl particles of a wide size range were used in this study. The L-pore is formed by extraction of the salt particles, and since the salt particles partially dissolve during the precipitation, the L-pore is not of the same size of the NaCl particles [14,28]. The size of the L-pore in this work is adequate for bone tissue engineering, as proposed elsewhere [2,48]. The finding of the S-pore in the trabeculae of the L-pore is consistent with the observations reported by Makaya *et al.* [28], though presenting different morphology.

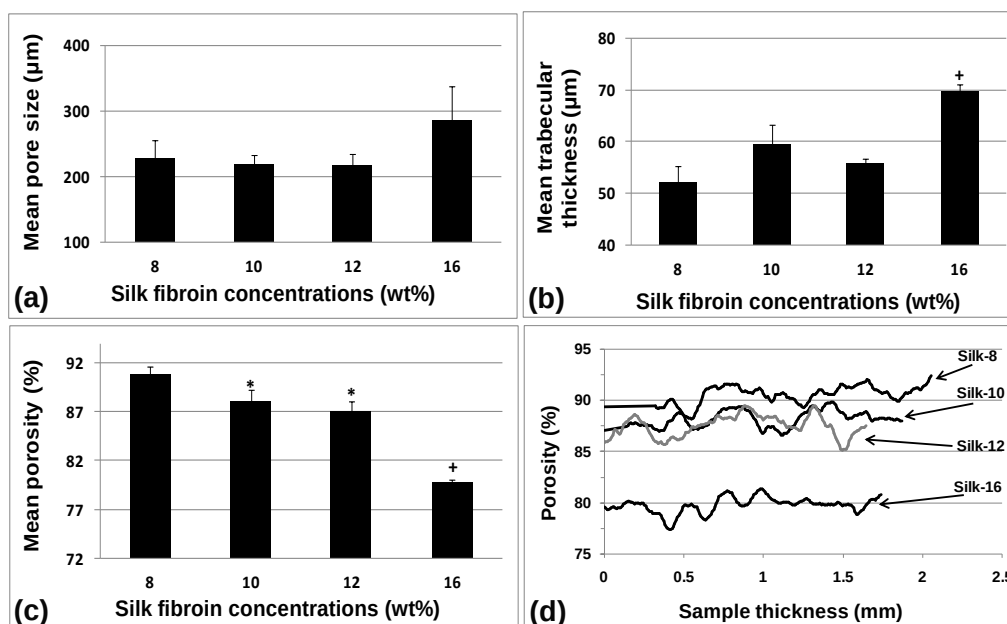


Figure 7. (a) Mean pore size, (b) mean trabecular thickness, (c) mean porosity and (d) representative porosity distribution of the silk fibroin scaffolds obtained by combining salt-leaching and freeze-drying methodologies. * indicates statistical significance when compared with silk-8 ($p < 0.05$), + indicates statistical significance when compared with silk-8, silk-10 and silk-12 ($p < 0.05$).

As can be seen in Figure S2, there are also microporous structures in the trabeculae of all the air dried scaffolds that were produced by salt leaching methodology. In this case the porosity is explained as a result of some re-crystallization of the dissolved salt in the system inside of the silk structure. When comparing the S-pore within the scaffolds produced by the combination of salt leaching and freeze-drying methodologies, the latter one seems to possess high porosity in the trabeculae. Thus, it is clear that the microporosity presented by the scaffolds produced combining salt leaching and freeze-drying may result from the combined effect of the re-crystallization of the dissolved salt particles in the system and the lyophilization process. This unique macro/micro porous structure is of great interest for tissue engineering. The size of the macro-pores (L-pores) is adequate for the transmission of nutrients and metabolic products, for cell in-growth, as well as for the growth of new vessels [2,48]. The micro-pores (S-pores) might help to tailor the degradation of the scaffolds, increase the cell seeding efficiency and enhance the cells' adhesion in the future application.

Regarding the formation of the silk fibroin microspheres, our observations were in agreement with previous findings [14,31]. During the precipitation of the silk fibroin,

residue silk fibroin in aqueous solution tends to form micelles, which subsequently will self-assemble into microspheres with the increase of ion concentration. In the case of highly concentrated silk fibroin solutions, such as silk-12 and silk-16, the gelation of the silk fibroin was dominant without the formation of the self-assembled microspheres at the surface.

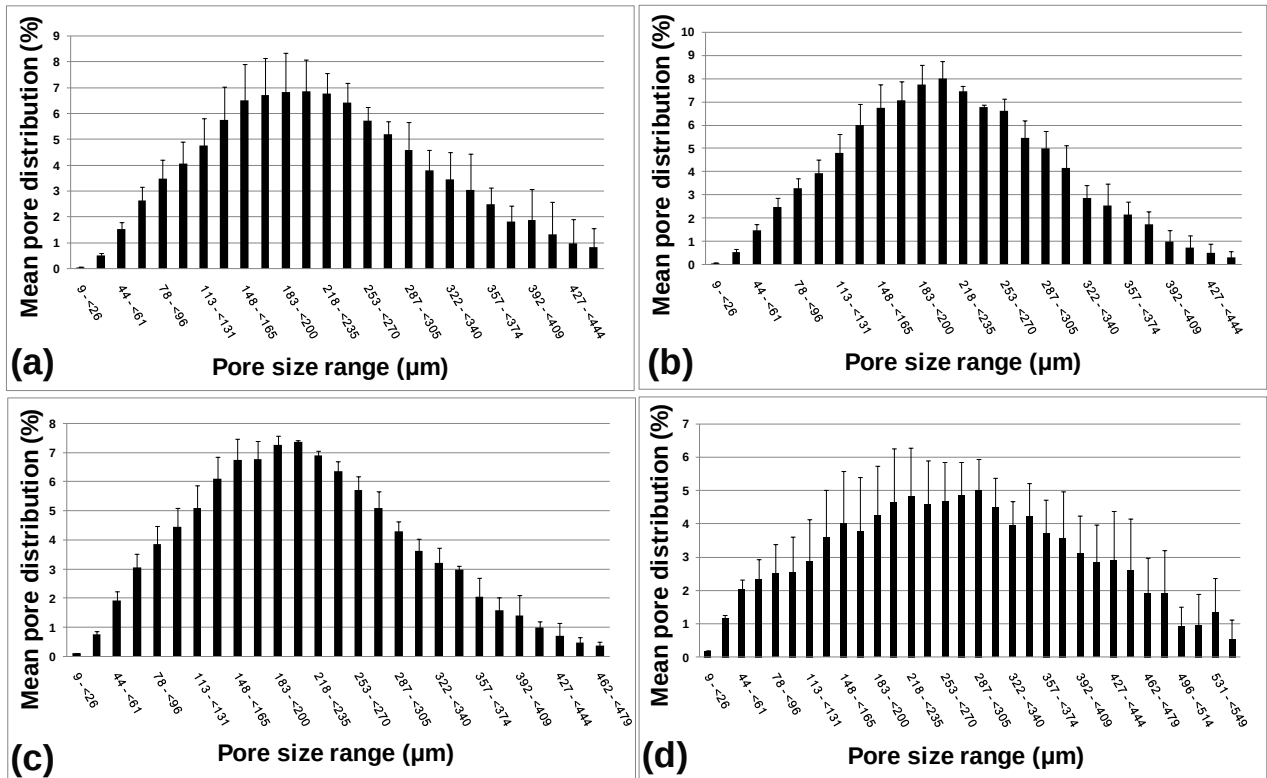


Figure 8. Mean pore distribution of silk fibroin scaffolds obtained by combining salt-leaching and freeze-drying methodologies, as determined by micro-computed tomography. (a) Silk-8; (b) silk-10; (c) silk-12; (d) silk-16.

The microstructure and architecture of the scaffolds are crucial parameters for tissue engineering applications since they can affect the final outcome of the tissue regeneration. Comparing the conventional methods in determination of the pore size and porosity of the scaffold, such as liquid displacement, mercury and flow porosimetry, gas pycnometry gas adsorption, and SEM (combine with computer software), micro-CT emerges as a promising alternative [49,50]. It is not only non-destructive, fast, and accurate, but also provides a comprehensive overview of the microstructure of the scaffolds. In this study, micro-CT was employed to investigate the architecture of the scaffolds (Figure 6). From the 3-D and 2-D images (Figure 6, inserted images), it was observed that the scaffolds were highly porous and presented interconnected pores, and the thickness of the pore walls for the larger pores (L-pore) seemed to increase when

increasing the silk fibroin concentration. These results were consistent with the SEM observations.

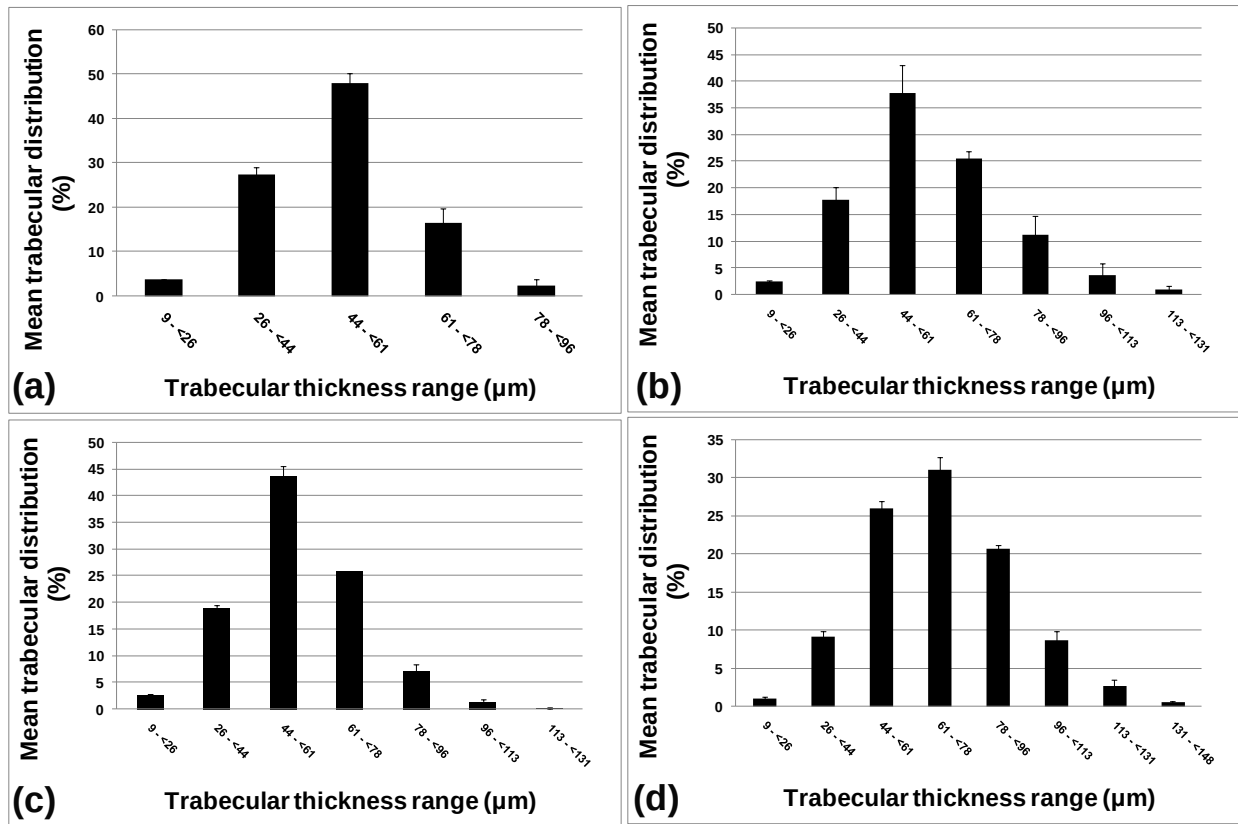


Figure 9. Mean trabecular distribution of silk fibroin scaffolds obtained by combining salt-leaching and freeze-drying methodologies, as determined by micro-computed tomography. (a) Silk-8; (b) silk-10; (c) silk-12; (d) silk-16.

Micro-CT morphometric analysis of the silk fibroin scaffolds obtained by combining salt-leaching and freeze-drying methodologies can be seen in Figures 7-10. The mean pore size of the scaffolds was comprised between 200 and 300 μm (Figure 7a). No statistical significant differences for pore size were found among the scaffolds, though silk-16 presented the highest mean pore size. Silk-16 also presented a wider pore distribution as compared to the other scaffolds (Figure 8). A higher mean trabecular thickness (Figure 7b) and a wider trabecular distribution (Figure 9) in silk-16 were also observed. As it can be seen in Figure 7c, the porosity decreased from $90.8 \pm 0.9\%$ to $79.8 \pm 0.3\%$, when increasing silk fibroin concentration from 8% up to 16%. The porosity is homogeneously distributed (Figure 7d) in the core of all the developed scaffolds. In this study, the interconnectivity of the prepared scaffolds was also evaluated (Figure 10). The interconnectivity values of the prepared scaffolds were comprised between $92.3 \pm 1.3\%$

and $97.4 \pm 0.5\%$. As the silk fibroin concentration increased, the mean interconnectivity tended to decrease. Even though the lowest interconnectivity was observed in silk-16, it was still as high as $92.3\% \pm 1.3\%$.

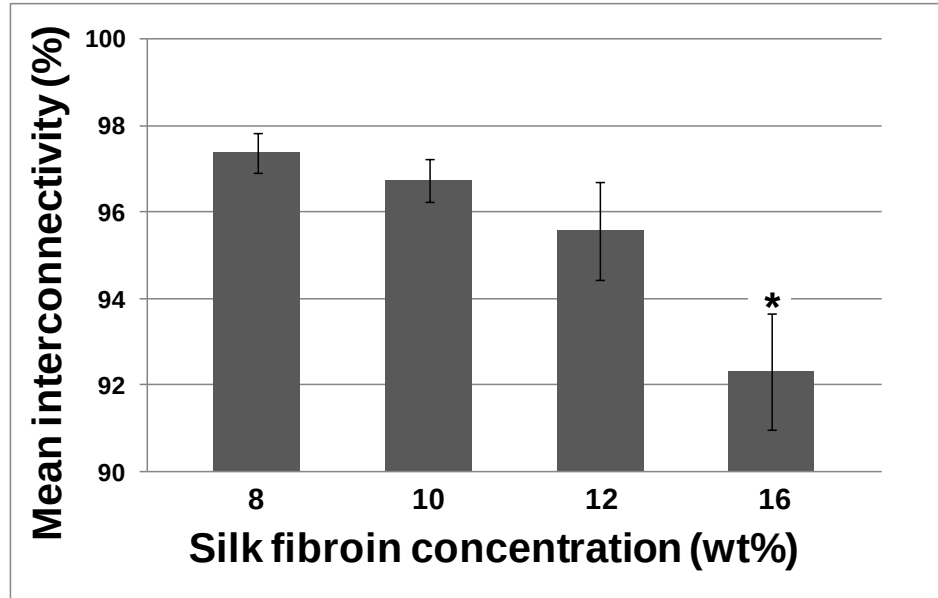


Figure 10. Mean interconnectivity of the silk fibroin scaffolds obtained by combining salt-leaching and freeze-drying methodologies, as determined by micro-computed tomography. * indicates statistical significance when compared with silk-8, silk-10 and silk-12 ($p < 0.05$).

The microstructure results were related to the initial silk fibroin concentrations. During the precipitation, the amount of silk fibroin precipitated increased by means of increasing the concentration of silk solution. The higher the concentration of silk fibroin solutions used, the lower the porosity and higher trabecular thickness can be achieved. Since the salt particles used in each case were in the same range of size, the mean pore size of the scaffolds was of no statistical difference. The mean pore size was a statistical data obtained from the measured size of the L-pore and the S-pore. This explained why this value was lower than the size of the L-pore observed under SEM. In this study, both the L-pores and the S-pores contributed to the interconnectivity of the scaffolds. From the SEM images (Figure 3), the L-pores were nearly completely interconnected, while the S-pores inside the trabeculae of the L-pores were not as interconnected as the L-pores. Silk-16 presented the highest trabecular thickness (Figure 7b) which could result in the highest amount of S-pores (Figure 3b, d, f and h). This explains the lowest interconnectivity of the silk-16. Moreover, the homogenous porosity distribution inside the

scaffolds indicated that the wide size range of salt particles did not affect the homogeneity of the scaffolds.

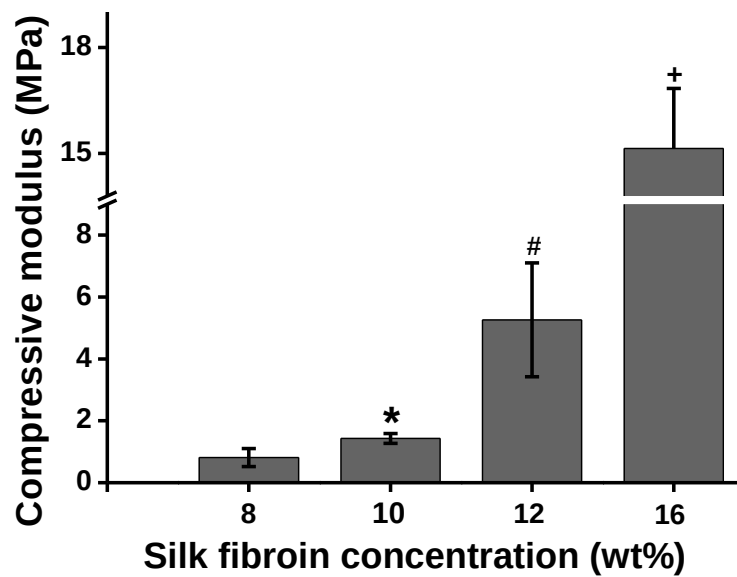


Figure 11. Compressive modulus of the silk fibroin scaffolds obtained by combining salt-leaching and freeze-drying methodologies. * indicates statistical significance when compared with silk-8 ($p < 0.05$), # indicates statistical significance when compared with silk-8 and silk-10 ($p < 0.05$), + indicates statistical significance when compared with silk-8, silk-10 and silk-12 ($p < 0.05$).

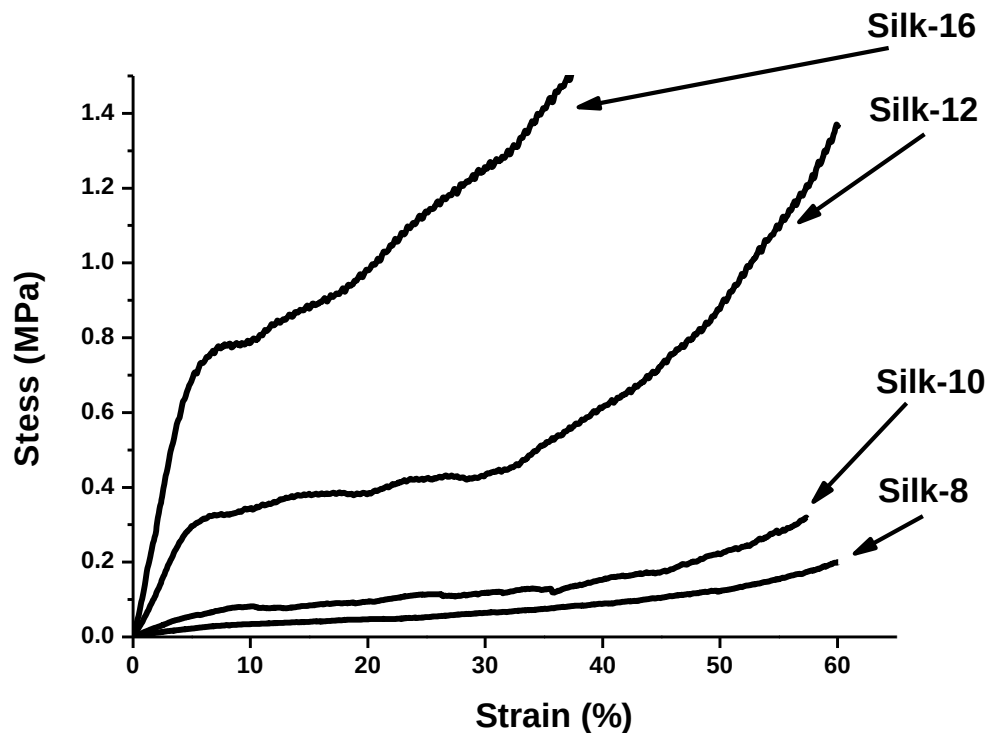


Figure 12. Stress-strain plot of the silk fibroin scaffolds obtained by combining salt-leaching and freeze-drying methodologies.

It has been reported that pore size larger than 300 μm is suitable for the formation of new bone and capillaries [48]. In Figure 8, it was found that silk-8, silk10 and silk-12 possessed about 15% pores with size more than 300 μm , while silk-16 presented an even higher ratio. It was also suggested that highly interconnected pore network with high porosity would benefit the cells growth, the transport of nutrients or metabolic waste, the deposit of cellular matrix, and the ingrowth of the new formed tissue [2, 28]. In this study, by developing silk fibroin scaffolds combining together a high interconnectivity (all above 90%), a high porosity (all above 79%) and a macro/micro-porous architecture, we firmly expect to obtain promising scaffold candidates for tissue engineering applications.

3.3. Mechanical properties

Figure 11 shows the mechanical properties of silk fibroin scaffolds obtained by combining salt-leaching and freeze-drying methodologies evaluated under compression testing. The static compressive modulus of the dried silk fibroin scaffolds increased dramatically as the increase of the silk fibroin concentration. The modulus increased from 0.81 ± 0.29 MPa to 15.14 ± 1.70 MPa as the silk fibroin concentration increased from 8% up to 16%. The representative stress-strain plot (Figure 12) shows that the compressive strength of the scaffolds remarkably improved from 0.05 MPa to 0.79 MPa when increasing the silk fibroin concentration from 8% to 16%. Regardless the different characterization conditions, the compressive modulus of silk-8 and silk-10 were lower as compared to that of scaffolds with the same concentrations reported in the previous studies [14]. This can be explained by the homogeneous pore size distribution, as reported by Kim *et al.* [14]. The compressive modulus of silk-16 was higher as compared to other previously reported data for pure silk fibroin scaffolds prepared by salt leaching or gas forming method [14,19,28]. Notably it was higher than that of the scaffolds prepared with 17% silk fibroin in hexafluoroisopropanol [19].

Since the scaffolds are expected to be used in a hydrated environment, it is of relevance to predict their biomechanical behavior namely by testing the mechanical properties in a more realistic condition, using DMA analysis. Figure 13 shows the mechanical properties of silk fibroin scaffolds obtained by combining salt-leaching and freeze-drying methodologies determined by DMA analysis. From the obtained data, we can observe that the storage modulus of all the groups increased by the increase of the frequency

from 0.1 to 10 Hz, but the increase profiles were different (Figure 13a). The modulus values of silk-8 and silk-10 increase at a lower rate compared to what is observed for silk-12 and silk-16. For the tested frequencies, the moduli were from 12.8 ± 4.2 to 33.7 ± 7.5 kPa, 37.6 ± 1.7 to 77.9 ± 4.4 kPa, 158.0 ± 16.8 to 264.1 ± 26.8 kPa, and 399.2 ± 19.6 to 630.3 ± 49.8 kPa for silk-8, silk-10, silk-12 and silk-16, respectively. These results proved that the stiffness of the scaffolds improved with the increase of silk fibroin concentrations.

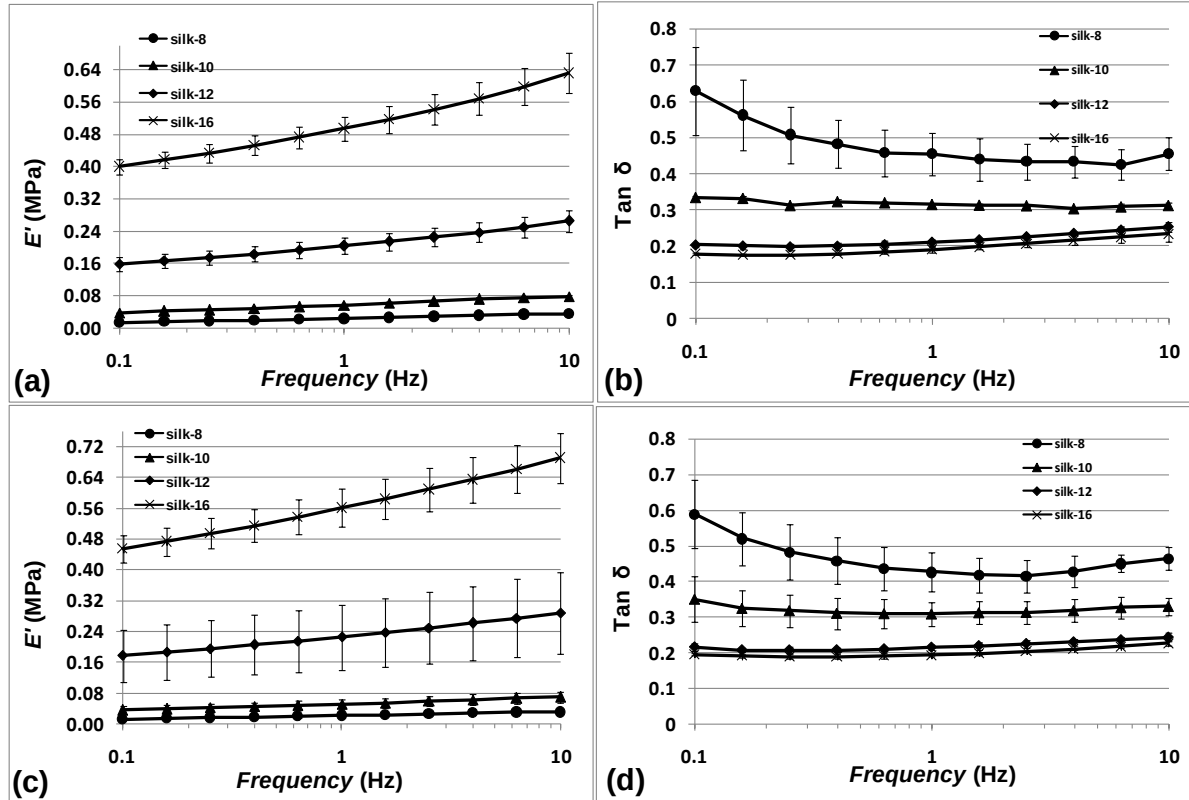


Figure 13. Dynamic mechanical analysis of the silk fibroin scaffolds obtained by combining salt-leaching and freeze-drying methodologies. (a) Storage modulus (E') and (b) loss factor ($\tan \delta$) of the silk fibroin scaffolds before degradation. (c) Storage modulus (E') and (d) loss factor ($\tan \delta$) of the silk fibroin scaffolds after 30 days of degradation.

Additionally, at each testing frequency, the modulus of the scaffolds exhibited concentration dependence and its trend was the same as the one observed in the static and dry status compressive test (Figure 11). The distinct mechanical properties of the developed scaffolds can be explained by the differences in the porosity and microstructure for each group. On the other hand, previous study shows that the value of the equilibrium compressive modulus of silk fibroin scaffolds (prepared from 17% silk fibroin in hexafluoroisopropanol) is less than 10 kPa which is lower than the values

obtained for human meniscus(23.6-47.8 kPa) and articular cartilage (0.4-0.8 MPa) [32,51-53]. Although the analysis in this study was not performed in equilibrium conditions, the obtained values of compressive modulus of silk-12 and silk-16 are comparable with those found in the literature [14,19]. Based on the higher compressive modulus values of silk-12 and silk-16 compared to the values found in the literature [14,19], the equilibrium modulus of silk-12 and silk-16 is expected to be higher than those of silk fibroin scaffolds prepared in previous studies, which make them suitable to be used in meniscus (silk-10 and silk12) and cartilage (silk-16) tissue engineering. At the present, studies are ongoing to evaluate the aggregate and equilibrium modulus of the silk fibroin scaffolds, as well as to test the biological performance.

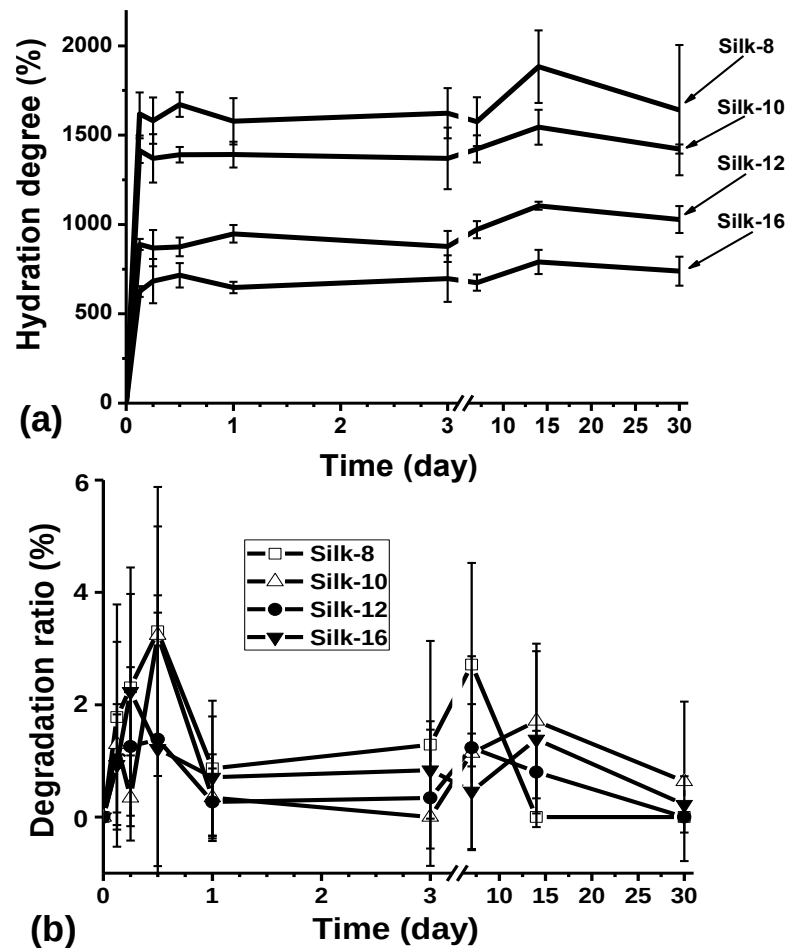


Figure 14. (a) Hydration degree and (b) degradation profile of the silk fibroin scaffolds obtained by combining salt-leaching and freeze-drying methodologies during immersion up to 30 days.

Loss factor is the ratio of the amount of energy dissipated by viscous mechanisms relative to energy stored in the elastic component. Comparing the loss factor data of the

four groups of scaffolds, it is found that the viscosity values decreased as the silk fibroin concentration increased in the tested frequency (Figure 13b). Concerning the damping property of each group, it is shown that there are not many differences in silk-10, silk-12 and silk-16 at all the tested frequencies, evidencing that these three groups of scaffolds present stable elasticity and viscosity. This property endows the prepared scaffolds with potential to be applied for engineering elastic tissues, such as articular cartilage and meniscus. Although with higher standard deviations, the loss factor of silk-8 seems to decrease with increasing frequency, indicating the weaker stiffness of this group comparing with the other groups.

There were distinct mechanical performances between the scaffolds tested in dry and in wet status. These differences can be associated to the 7 smaller internal hydrophilic blocks and 2 large hydrophilic blocks at the chain ends among the silk fibroin heavy chain [14]. In wet status, the hydrophilic groups in silk fibroin are hydrated and consequently the stiffness of the scaffolds decreases.

The mechanical properties of the scaffolds were also investigated by DMA analysis, after 30 days of soaking (Figure 13c, d). It was observed that all the scaffolds maintained their original mechanical strength, after 30 days of soaking. There were no statistical differences in respect to mechanical properties before and after soaking. The ability of the scaffolds to maintain their mechanical performance during tissue regeneration is very important.

By correlating the previous analysis on the conformation and microstructure of the scaffolds, it is found that the mechanical properties of these scaffolds greatly depended on their conformation and porosity. The obtained crystal conformation is responsible for the presented water-stability, while the decrease in porosity resulted in improved mechanical properties, both in wet and in dry states.

3.4. Hydration degree and degradation related properties

The ability to uptake fluids from the surrounding medium plays an important role in tissue engineering. As it can be found in Figure 14a, the hydration degree of all the scaffolds reached equilibrium only after 3 hours of immersion in aqueous solutions and can be maintained until 30 days. This result reveals that the scaffolds possess a good hydration

capability and are able to maintain their structural integrity. The hydration degree of the scaffolds decreased with the increasing silk fibroin concentration (Figure 14a). The hydration degree differences can be attributed to the differences in the porosity of the scaffolds. It was observed that for the scaffolds with higher porosity, the hydration degree increased. This trend is in agreement with observations previously reported elsewhere [14].

All the scaffolds maintained their original weights after soaking in aqueous solutions for 30 days (Figure 14b). From XRD and FTIR data, it was possible to observe that the silk fibroin crystal conformation in the scaffolds is responsible for the stability of the scaffolds during the *in vitro* degradation test. Furthermore, the morphology of the scaffolds after immersion in ISS for 30 days was assessed by SEM (Figure 5). It was possible to observe that there were no differences in the scaffolds' morphology before and after 30 days degradation, which is an evidence of their stability.

The stable hydration degree, the negligible weight loss and the maintenance of the original morphology of the produced scaffolds during the degradation study are clearly related with the silk fibroin crystal conformation. The differences in the hydration degrees were related with their varied porosities. These results can provide valuable reference for the future application of these structures in cartilage and meniscus tissue engineering scaffolding.

4. Conclusions

In this study, an initial physicochemical characterization is presented of silk fibroin scaffolds derived from high concentration aqueous silk fibroin solution and prepared combining salt leaching and freeze-drying methodologies. The results indicated that the developed scaffolds presented silk-II conformation, as confirmed by FTIR and XRD. The morphological study revealed that the scaffolds possessed both macro- and micro-porous structures, and the morphology varied depending on the initial concentration. The micro-CT analysis further demonstrated the prepared scaffolds possessed high porosity and interconnectivity, which seemed to decrease with increasing silk fibroin concentration. An opposite trend was exhibited in terms of the trabecular thickness of the scaffolds. The compressive test and DMA analysis showed that the mechanical

properties of the silk fibroin scaffolds increased dramatically with the increasing of silk fibroin concentration. The viscosity properties of silk-10, silk-12 and silk-16 were stable for the testing frequencies. The hydration degree data demonstrated that the scaffolds presented a high swelling capability that increased with increasing porosity. It should be highlighted that the prepared scaffolds were able to keep their original structure and morphology, as well as their original mechanical properties, after 30 days of immersion. Therefore, the developed silk fibroin scaffolds are good candidates to be used in tissue engineering scaffolding, namely for cartilage and meniscus regeneration.

This study also opens a new window to prepare load-bearing multifunctional silk fibroin based scaffolds for other specific tissue engineering applications. Based on the promising physicochemical performance of the developed scaffolds, further *in vitro* (with cell lines, primary cells) and *in vivo* studies are envisioned in order to fully evaluate the biological performance of the developed silk scaffolds.

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Supplementary Data

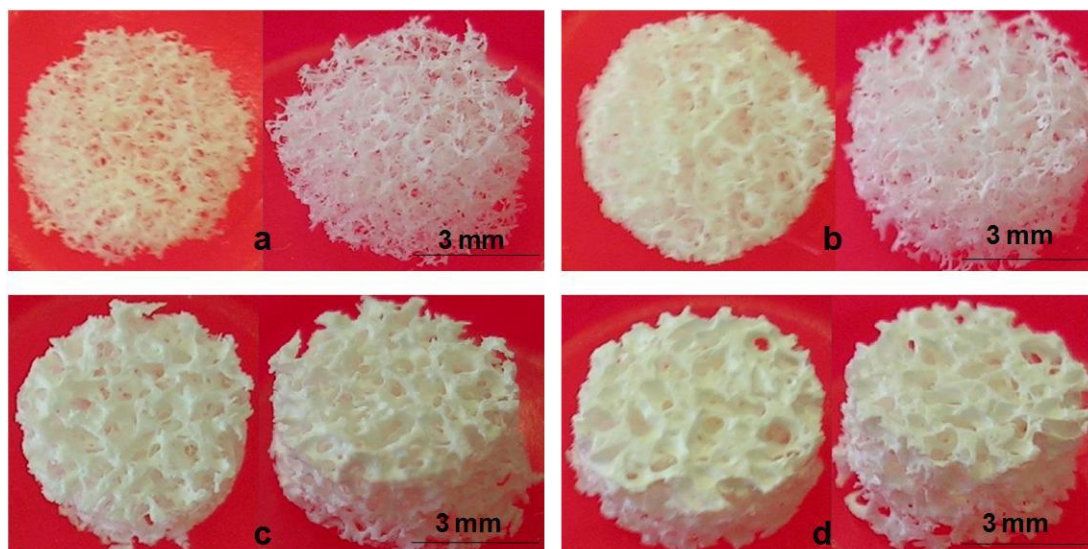


Figure S1. Macroscopic images of the silk fibroin scaffolds obtained by combining salt-leaching and freeze-drying methodologies: (a) Silk-8, (b) silk-10, (c) silk-12 and (d) silk-16.

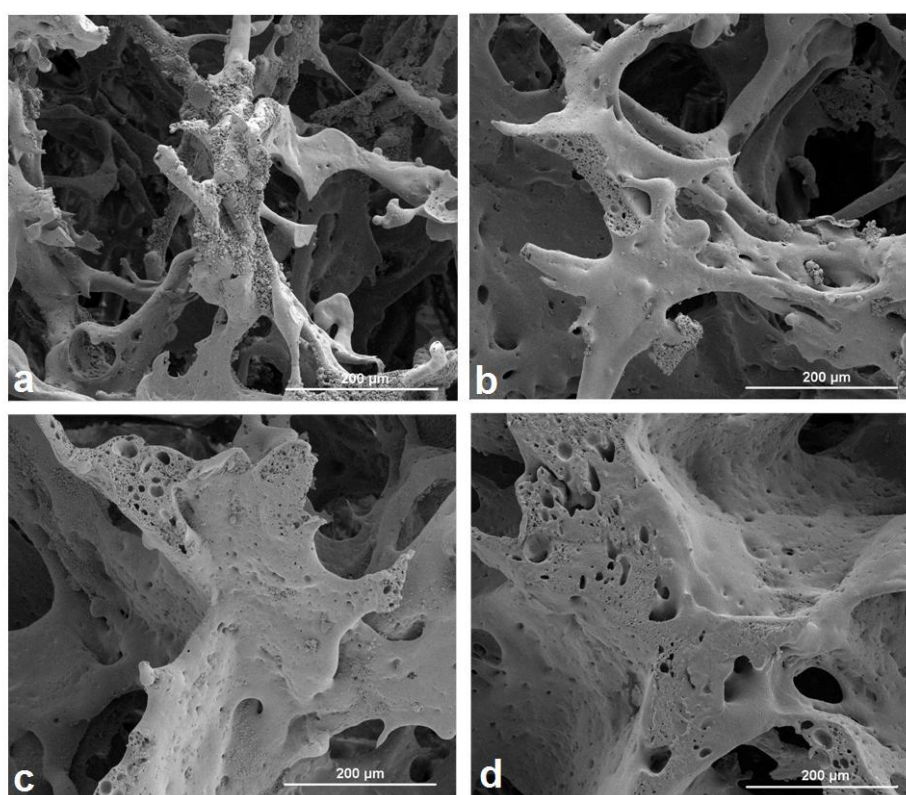


Figure S2. Scanning electron micrographs of the cross-sectional morphology of the air dried salt leached silk fibroin scaffolds. (a) Silk-8, (b) silk-10, (c) silk-12, (d) silk-16.

Chapter IV

Bioactive Macro/Microporous Silk Fibroin/Nano-Sized
Calcium Phosphate Scaffolds with Potential for Bone
Tissue Engineering Applications

Chapter IV

Bioactive Macro/Microporous Silk Fibroin/Nano-Sized Calcium Phosphate Scaffolds with Potential for Bone Tissue Engineering Applications

Abstract

This study aimed to develop novel silk/nano-sized calcium phosphate (Silk-NanoCaP) scaffolds with highly dispersed CaP nanoparticles in the silk fibroin (SF) matrix for bone tissue engineering. Nano-CaP was incorporated in a concentrated aqueous SF solution (16 wt.%) by using an *in-situ* synthesis method. The Silk-NanoCaP scaffolds were then prepared through a combination of salt-leaching/lyophilization approaches. The CaP particles presented good affinity to SF and their size was inferior to 200 nm when theoretical CaP/silk ratios were between 4 and 16 wt.%, as determined by scanning electron microscopy (SEM). The CaP particles displayed a uniform distribution in the scaffolds at both microscopic and macroscopic scales as observed by Backscattered SEM and micro-computed tomography, respectively. The prepared scaffolds presented self mineralization capability and no cytotoxicity confirmed by *in vitro* bioactivity tests and cell viability assays, respectively. These results indicated that the produced Silk-NanoCaP scaffolds could be suitable candidates for bone tissue engineering applications.

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1. Introduction

Bone tissue engineering requires the development of three-dimensional porous scaffolds with osteogenic properties [1]. Calcium phosphate-based (CaP) inorganic component have shown their osteogenesis potential in bone regeneration [2,3]. But the fragile nature and slow degradation profile limit the application of these ceramic scaffolds. Combining natural or synthetic polymer with calcium phosphate is a promising strategy for bone tissue engineering scaffolding. A great deal of degradable polymers has been explored for this purpose [4]. Among those, silk fibroin (SF) derived from silkworm *Bombyx mori* has been considered as a versatile biomaterial for tissue engineering applications [5-13].

Regarding the preparation of silk/CaP composite scaffolds, there are some challenging issues should be solved. For instance, it is crucial to achieve good affinity between SF and CaP particles, as well as to maintain the porous structure and mechanical properties of the scaffold. On the other hand, the aggregation of CaP particles in the scaffold must be prevented. Furthermore, it is also important to achieve homogeneous distribution of the CaP particles inside the scaffold, both at macroscopic and microscopic scales. Many attempts have been made to improve the interface compatibility of the two phases in the scaffolds. Oliveira *et al.* [14] synthesized hydroxyapatite (HA) in SF by the addition of phosphate ions into the calcium chloride/ethanol/water solution with dissolved SF. This strategy allowed the formation of nano-sized HA inside the SF matrix. Kim *et al.* [15] prepared aqueous derived salt-leached SF scaffolds with the addition of polyaspartic acid, followed by the consecutive immersion the scaffolds in calcium chloride and sodium phosphate monobasic solutions in order to form calcium phosphate crystal on the surface of the scaffolds. The introduction of polyaspartic acid compromised the mechanical properties of the scaffolds. Collins *et al.* [16] prepared the first load-bearing silk/CaP scaffold via an integrated procedure. The generated scaffold presented mechanical properties comparable to cancellous bone, and a pore size range between 50 and 100 μm . In another interesting work, Zhang *et al.* [17] tried to improve distribution homogeneity of the CaP particle in the scaffold by using the silk/CaP hybrid particle instead of the pure CaP particle. Actually, it was shown that the CaP/silk composite scaffold enhanced the osteogenic differentiation of human bone mesenchymal stromal cells and promoted the cancellous bone formation in calvarial defect in SCID mice. However, the compressive strength of the scaffolds was less than 80 kPa. The above-

mentioned studies somehow solved one or more challenges in the preparation of silk/CaP scaffolds, but had not yet reached the ideal level.

In previous study, we have developed porous SF scaffolds with superior mechanical properties by using highly concentrated aqueous SF solution, via salt-leaching/freeze-drying methods [18]. In this study, we aim to solve the above-mentioned challenges by introduce of nano-sized CaP in SF via an *in-situ* synthesis method, followed by preparation the porous scaffolds through salt-leaching/freeze-drying approaches. The structural conformations of SF and CaP were investigated by Fourier transform infra-red spectroscopy (FTIR) and/or X-ray diffraction (XRD) analysis. The morphology and microstructure of the Silk-NanoCaP scaffolds were evaluated by scanning electron microscopy (SEM) and micro-computed tomography (micro-CT). The CaP content and Ca/P ratio in the scaffold were determined by thermal gravimetric analysis (TGA) and energy dispersive X-ray detector (EDX), respectively. The size and the microscopic distribution of the CaP nano-particles were also investigated by Backscattered SEM. The macroscopic distribution of the CaP nano-particles in the scaffold was analyzed by micro-CT. The mechanical properties in dry state and wet state were characterized by compressive tests and dynamic mechanical analysis (DMA) at pH 7.4 and 37°C, respectively. Additionally, the hydration degree was registered from 3 hours up to 30 days and the weight loss was recorded from 1 up to 30 days. Finally, the cytotoxicity of the Silk-NanoCaP scaffolds along with the silk control scaffolds were evaluated by carrying out a cellular viability test by a -(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophynyl)-2H-tetr-azolium) assay, MTS) using mouse lung fibroblasts (L929 cell line) cells, which were previously in contact with the scaffolds' extract fluids.

2. Materials and Methods

2.1. Materials

Cocoons of *Bombyx mori* were offered by the Portuguese Association of Parents and Friends of Mentally Disabled Citizens (APPACDM, Portugal). In this study sodium chloride particles (Portugal) of commercial grade were used. Silicone tubing (9 mm inner

diameter) was obtained from Deltalab (Barcelona, Spain). The other materials and reagents were supplied by Sigma-Aldrich (MO, USA) unless mentioned otherwise.

2.2. Preparation of high concentration SF aqueous solution

Bombyx mori SF was extracted from cocoons as reported previously with slight modifications [12]. Briefly, the sericin was eliminated by boiling the cocoons for 1 hour in 0.02 M sodium carbonate solution, and then rinse thoroughly with distilled water. The purified SF was dissolved in 9.3 M lithium bromide solution at 70°C for 1 hour. Then, the SF solution was dialyzed against distilled water by using a benzoylated dialysis tubing (MWCO: 2000) for the period of 2 days. Afterwards, the SF solution was concentrated by dialysis in a 20 wt.% poly(ethylene glycol) solution (20,000 g/mol) for 6 hours [19]. Finally, the tubing was carefully rinsed in distilled water, and the concentrated solution was collected. The concentration of the final SF solution was calculated by dividing the dry weight by the initial weight of the SF solution. The concentrated SF solution was kept at 4°C before use.

2.3. Preparation of salt-leached Silk-NanoCaP scaffolds

Silk-NanoCaP composite was prepared via an *in-situ* synthesis method. At first, the concentrated SF aqueous solution was diluted to 16 wt.%. Different amounts of a calcium chloride solution (6 mol/L) were mixed with the SF solution followed by the addition of different amounts of an ammonia dibasic phosphate solution (3.6 mol/L). The theoretical calcium to phosphate atomic ratio was maintained at 1.67 in each group. The pH value of the system was adjusted to 8.5 by the addition of ammonia (30%). The suspension was stirred for 30 minutes and subsequently aged for 24 hours at room temperature. The theoretical content of the CaP formed in the SF solution was determined based on the hypothesis that the calcium and phosphate species would react completely to form stoichiometric HA, $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$. Silk-NanoCaP composites possessing a theoretical CaP content (theoretical CaP mass divided by the total mass of SF) of 4, 8, 16 and 25 wt.% were prepared. Fraction of sodium chloride particles having a size in the range of 500-1000 μm were obtained by using an analytical sieve shaker (Retsch, Haan, Germany). The Silk-NanoCaP scaffolds were prepared by addition of 2.0 g of sodium

chloride granule (500-1000 μm) to 1 mL Silk-NanoCaP suspension, in a silicone tubing of 9 mm inner diameter; followed by drying the material inside the silicone tubing at room temperature, for 2 days. Sodium chloride and the by-products were removed by immersion in distilled water for 2 days. The skin of the Silk-NanoCaP scaffolds was removed by a stainless steel punch of 6 mm inner diameter. Finally, the scaffolds were frozen at -80°C followed by lyophilization in a freeze-drier (CRYODOS-80; Telstar, Barcelona, Spain). The prepared Silk-NanoCaP scaffolds were designated as silk/CaP-4, silk/CaP-8, silk/CaP-16 and silk/CaP-25, according to their initially incorporated amount of CaP, respectively (Figure 1). The SF scaffolds (control) without CaP were also prepared from a 16 wt.% aqueous solution following our previously reported method [18].

2.4. Characterization of the physicochemical properties

2.4.1. XRD

The crystallinity of the Silk-NanoCaP scaffolds on powder was investigated using a X-ray diffractometer (Philips PW 1710; Philips, Amsterdam, Netherlands) with Cu-K α radiation ($\lambda=0.154056$ nm). Data was collected from 0 to 60° 2θ values, and the step width and counting time were set at 0.02° and 2 seconds per step, respectively. The analysis was repeated twice for each formulation.

2.4.2. FTIR

The Silk-NanoCaP scaffolds were first reduced to powder by using a mortar. The powders were mixed with potassium bromide (1:100, by wt.) and then uniaxially pressed to obtain a transparent disk. The infrared spectra were obtained by FTIR (Perkin-Elmer 1600 series equipment; Perkin-Elmer, MA, USA), between 4000 to 500 cm^{-1} . Spectra were recorded at a resolution of 4 cm^{-1} with 32 scans. Each formulation was screened three times.

2.4.3. SEM

The cross-sectional morphology of the control scaffold and Silk-NanoCaP scaffolds were observed under SEM (Nova NanoSEM 200; FEI, Hillsboro, OR, USA). Prior to the analysis, the specimens were coated with Au/Pd SC502-314B in a high vacuum evaporator coater (E6700; Quorum Technologies, East Grinstead, UK). The size and the microscopic distribution of the CaP particle in the Silk-NanoCaP scaffolds were determined. For the purpose of this study, Silk-NanoCaP scaffolds were milled into powder followed by observation of the CaP particles in the composite powder via Backscattered SEM (Nova NanoSEM 200; FEI, Hillsboro, OR, USA) without any coating. The calcium and phosphate content in the powder was investigated by EDX during the SEM observation.

2.4.4. CaP content and Ca/P atomic ratio in the Silk-NanoCaP scaffolds

The CaP content in the Silk-NanoCaP scaffolds was determined by TGA (TGA Q500, TA Instruments, USA). Each specimen was placed in a platinum pan and equilibrated at 50°C for 2 minutes, followed by increasing the temperature to 700 °C at a rate of 20°C/minute in air atmosphere. The CaP content in the scaffolds (CaP mass divided by the mass of SF) and the CaP incorporation efficiency were determined using equation (Eq.) 1 and 2, respectively.

$$CaP\ content = \left(\frac{m_r}{m_i - m_r} \right) \times 100\% \quad (1)$$

$$CaP\ incorporation\ efficiency = \frac{CaP\ content}{Theoretical\ CaP\ content} \times 100\% \quad (2)$$

In Eq.1, m_r is the weight of the residual, and the m_i is the initial dried weight of the material. The theoretical contents for silk/CaP-4, silk/CaP-8, silk/CaP-16 and silk/CaP-25 are 4, 8, 16 and 25 wt.%, respectively. Three specimens were evaluated for each formulation.

For the determination of the Ca/P atomic ratio in the scaffold, the Silk-NanoCaP scaffolds were burned at 700°C for 40 minutes in a furnace (Fornoceramica, Leiria, Portugal) to remove the SF. The obtained residual CaP was adhered in a cooper support for the analysis of the Ca/P atomic ratio by EDX (NanoSEM-FEI Nova 200). In each condition, 5 independent areas (200 × 200 µm) of the residual CaP were selected.

2.4.5. Micro-CT

The microstructure of the Silk-NanoCaP scaffolds was qualitatively and quantitatively investigated by employing a high-resolution micro-CT (1072 scanner; Skyscan, Kontich, Belgium) possessing a resolution of pixel size of ~6.7 µm and time of integration of 1.3 second. The X-ray source was fixed at 61 keV and 163 µA. Around 300 projections were obtained over a 180° rotation with a step width of 0.45°. Data sets were rebuilt employing standardized software (NRecon v1.4.3, SkyScan) in a cone-beam model. The format of the output for each specimen was 300 serial bitmap images with 1024 x 1024 pixels. Representative serial images in each data set was transferred into binary images by using a grey values (dynamic threshold) of 40-255. Finally, the binary images were used for microstructural analysis (CT Analyser, version 1.5, SkyScan), and to establish the 3D models (ANT 3D creator, version 2.4, SkyScan). At least three specimens were used for each condition. The macroscopic distribution of CaP in the Silk-NanoCaP scaffolds was also assessed by micro-CT. The test followed the same procedure as mentioned above, but the dynamic threshold was set between 120 and 255 (grey values).

2.4.6. Mechanical properties

2.4.6.1. Compressive tests (dry state)

The tests were conducted in an unconfined mode by using a Universal Testing Machine (Instron 4505; Instron, Norwood, MA, USA) with a load cell of 1kN and at ambient temperature. The height and the diameter of the tested specimens were measured by a micrometer. The length and diameter of the tested specimens were approximately 6.50 mm and 5.30 mm, respectively. The cross-head speed was fixed at 2 mm/minute and until 60% deformation in specimen height. The elastic modulus (*E*) was obtained from the

slope of the initial linear domain of the stress-strain curve. The compressive strength was determined when the scaffold crushed. At least 7 specimens for each formulation were tested.

2.4.6.2. DMA (wet state)

The viscoelastic mechanical properties of the scaffolds were measured by TRITEC8000B DMA (Triton Technology, Lincolnshire, UK) in a compressive mode. Scaffolds (cylindrical shape with 6 mm diameter and 4 mm thickness) were kept in a phosphate buffered saline (PBS) solution at 37°C overnight. Afterwards, the specimens were fixed in the DMA apparatus and kept immersing in the PBS solution during the measurement. After reaching the equilibrium at 37°C, the DMA spectra were recorded during a frequency sweep, ranging from 0.1 up to 25 Hz. Constant strain amplitude of 50 µm was used in all the experiment. Before the test, a small amount of preload was applied to each specimen to allow the complete contact of the scaffold surface with the compression plates. The distance between the plates was the same for all the tested scaffolds. A minimum of 4 samples was used for each formulation.

2.4.7. Hydration degree and weight loss ratio

The hydration degree (time period: 3 hours until 30 days) and degradation behaviour (time period: 1 to 30 days) of the Silk-NanoCaP scaffolds were assessed after immersion into a 0.154 M sodium chloride isotonic saline solution (ISS, pH 7.4) [18]. These experiments were conducted at 37°C and dynamic condition (60 rpm) in a water bath (GFL 1086). In the end of each time point, the specimen was removed from the ISS and the wet weight was measured immediately after removing the excess surface water by using a filter paper. The hydration degree was calculated using Eq.3.

$$\text{Hydration degree} = \left(\frac{m_{w,t} - m_i}{m_i} \right) \times 100\% \quad (3)$$

In Eq.3, m_i is the initial weight of the specimen before hydration, and $m_{w,t}$ is the wet weight of the specimens at time t after being removed from the ISS. After the determination of the hydration degree, the specimens were rinsed with distilled water several times and dried in an oven at 60°C for 24 hours. The weight loss ratio was determined using Eq.4.

$$\text{Weight loss ratio} = \left(\frac{m_i - m_{d,t}}{m_i} \right) \times 100\% \quad (4)$$

In Eq.4, m_i is the initial weight before degradation, and $m_{d,t}$ is the dry weight of the specimen been degraded for a certain period of time and after drying at 60°C for 24 hours. Five specimens were measured for each formulation.

2.4.8. *In vitro* mineralization

The Silk-NanoCaP scaffolds were immersed in a simulated body fluid (SBF) [20] solution for 7 days in an oven at 37°C, following the method proposed by Kokubo *et al.* and adapted by Oliveira *et al.* [20,21]. At each timepoint, the specimens were removed from the SBF solution and washed by distilled water. The samples were frozen at -80°C and lyophilized (CRYODOS-80; Telstar, Barcelona, Spain). Then, the surfaces of the samples were analysed by SEM and EDX (NanoSEM-FEI Nova 200). Prior to the SEM and EDX analysis, the samples were coated with carbon in a high vacuum evaporator coater (E6700; Quorum Technologies, East Grinstead, UK). For the EDX analysis, the data were collected by scanning three independent areas (5 × 5 µm) in each specimens for 90 seconds. Three specimens were analyzed in each assay for each group of scaffolds.

2.5. *In vitro* cytotoxicity screening

A-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (MTS) assay was performed to evaluate cytotoxicity of the silk/CaP-16 and silk control scaffold in comparison to latex (positive control for cell death), in accordance

with ISO/EN 10993 (1992) Part 5 guidelines. Mouse lung fibroblasts (L929 cell line) were cultured as monolayer in a Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Biochrom, Merck, NJ, USA), 1% of antibiotic-antimycotic mixture (Life Technologies, Carlsbad, CA, USA) containing 10,000 U/mL penicillin G sodium, 10 mg/mL streptomycin sulphate and 25 µg/mL amphotericin B as fungizone[®] antimycotic in 0.85% saline. The L929 cells were incubated in an atmosphere containing 5% CO₂ at 37°C, and the medium changed every 2 days.

The extract fluids were prepared as previously reported by Oliveira *et al.* [21]. Briefly, extract fluids were obtained by immersing 1g of scaffolds (sterilized by autoclave) in 50 mL tubes containing 20 mL complete DMEM culture medium. The tubes were incubated in a water bath at 37°C and 60 rpm for 24 hours. A latex rubber extract was used as positive control. Afterwards, the extract fluids were filtrated by using a 0.45 µm filter. Confluent L929 cells were detached from the culture flasks using trypsin (0.25% trypsin – EDTA solution) and a diluted cell suspension was prepared. The cells were seeded in a 96-well tissue culture polystyrenes (TCPS) plate (six replicates per sample) at a cell density of 20,000 cells/well and incubated for 24 hours at 37°C in an atmosphere with 5% CO₂. The culture medium in each well was removed and replaced by an identical volume (200 µL) of the extraction fluids. Cell culture medium was used as negative control. After 1, 3 and 7 days, the extracts were removed and replaced by 300 µL of mixed solution containing serum-free culture medium (without phenol red) and MTS using the CellTiter 96[®] AQueous One Solution Cell Proliferation Assay Kit (Promega, Fitchburg, WI, USA). After incubation for 3 hours at 37°C in an atmosphere with 5% CO₂, the optical density (OD) was measured at 490 nm using a plate reader (Molecular Devices, SunnyVale, CA, USA). Cell viability was calculated by subtracting the mean OD value of the blank (MTS solution) from the ones of the scaffolds and controls, followed by normalization with the mean OD value obtained for the negative control (cell culture medium). The MTS assay was performed in triplicate (n=18).

2.6. Statistical analysis

All data were presented as average and standard deviation. A one-way analysis of variance (ANOVA) was used to assess the data obtained from TGA analysis, micro-CT

analysis, compressive tests, and cytotoxicity test. The comparison between two average values was evaluated using Tukey's test with $p < 0.05$ as statistical significance.

3. Results

3.1. Chemical structure

Figure 2a shows the XRD patterns of the silk and Silk-NanoCaP porous scaffolds. The characteristic peaks of silk-II structure located at 9.0° and 20.5° were detected for all the scaffolds [12,19]. The broad peak width and low intensity of these two peaks indicate that the SF was of low crystallinity comprising uncertain amount of random coil. It was observed that as the amount of incorporated CaP increased, the intensity of the peaks located at 25.9° , 32.1° and 39.7° slightly increased. These peaks indicate that the CaP incorporated in the scaffold is a HA presenting low crystallinity [23]. The FTIR spectra (Figure 2b) corroborated the XRD analysis that revealed the conformation of silk-II in the SF in all the scaffolds. It can be observed peaks located at 1704 cm^{-1} and 1622 cm^{-1} attributed to silk-II [24,25]. The absorption area attributed to the ν_3 vibration of the PO_4^{3-} bond (between the absorption range of 970 cm^{-1} and 1100 cm^{-1}) increased with the increasing CaP content [17,21,22]. The ν_4 vibration of the PO_4^{3-} bond located at 607 cm^{-1} and 560 cm^{-1} [17,21,22] was distinct in the spectrum of Silk/CaP-25, while the spectra of silk/CaP-4, silk/CaP-8 and silk/CaP-16 presented a lower intensity for this absorption. The XRD and FTIR results demonstrated that the CaP particles were successfully generated within the scaffolds.

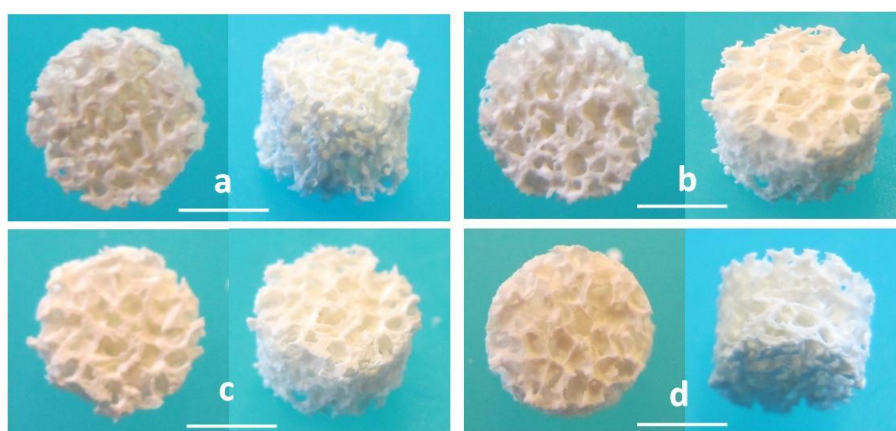


Figure 1. Macroscopic images of the Silk-NanoCaP scaffolds. (a) Silk/CaP-4, (b) silk/CaP-8, (c) silk/CaP-16 and (d) silk/CaP-25. Scale bar: 3 mm.

3.2. Morphology and microstructure

Figure 3 shows SEM images of the different Silk-NanoCaP scaffolds. All the scaffolds presented a macro/micro porous structure. The size of the macro-pores is around 500 μm and highly interconnected (Figure 3a, d, g and j). The trabeculae of the macro-pores were composed of partially interconnected micro-pores with a size ranging from 10 μm to 100 μm (Figure 3b, e, h and k). An interesting finding is the formation of cauliflower-like apatite clusters on the surface of silk/CaP-25, with size around 700 nm (Figure 3l, inserted image). The clusters are composed of flake-like and worm like apatite crystal.

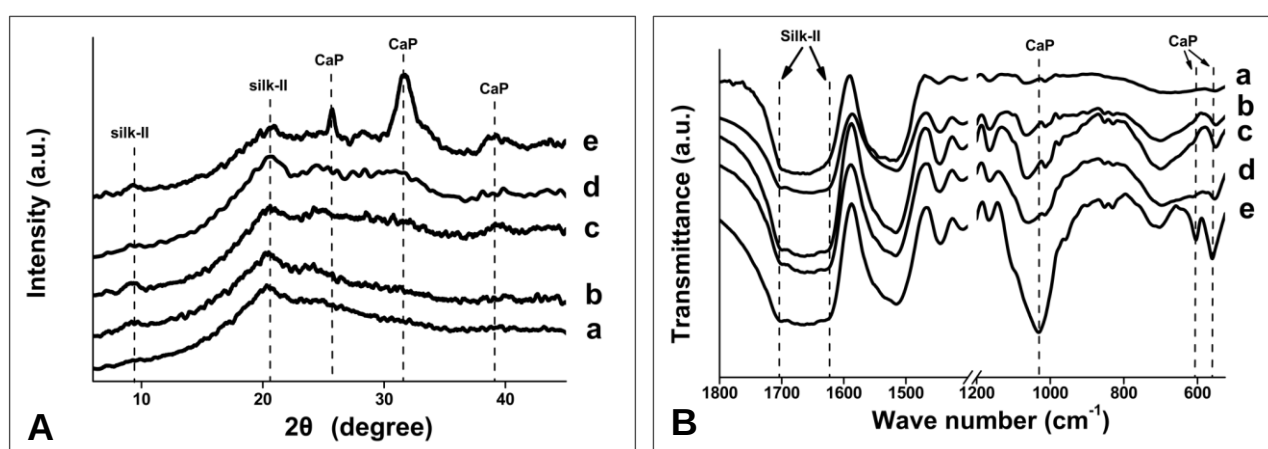


Figure 2. XRD patterns (A) and FTIR spectra (B) of the silk and Silk-NanoCaP scaffolds. (a) Control, (b) silk/CaP-4; (c) silk/CaP-8; (d) silk/CaP-16; (e) silk/CaP-25.

The microstructure of the control and the Silk-NanoCaP scaffolds was qualitatively and quantitatively studied by micro-CT (Figure 4-5). A highly porous structure was observed in all the Silk-NanoCaP scaffolds from the two-dimensional and three-dimensional micro-CT images (Figure 4). From the three-dimensional micro-CT images, it is also possible to observe that the macro-pores are interconnected. Silk/CaP-4 possessed the highest porosity in the trabeculae of the macro-pores as compared to the other groups (Figure 4a-d). The two-dimensional micro-CT images also confirmed this observation (Figure 4e-h). Figure 5a shows that silk/CaP-4 presented the highest mean porosity ($77.61 \pm 0.72\%$), while silk/CaP-16 exhibited the lowest one ($63.56 \pm 2.43\%$) among the Silk-NanoCaP scaffolds. The representative porosity distribution profile (Figure 5b) shows that a homogeneous porosity was observed for all the scaffolds. Figure 5c showed that all the scaffolds presented interconnectivities higher than 70%. Silk/CaP-4 and silk/CaP-8 presented higher interconnectivity as compared to silk/CaP-16 and silk/CaP-25. The

control possessed higher porosity and interconnectivity as compared to all the Silk-NanoCaP scaffolds.

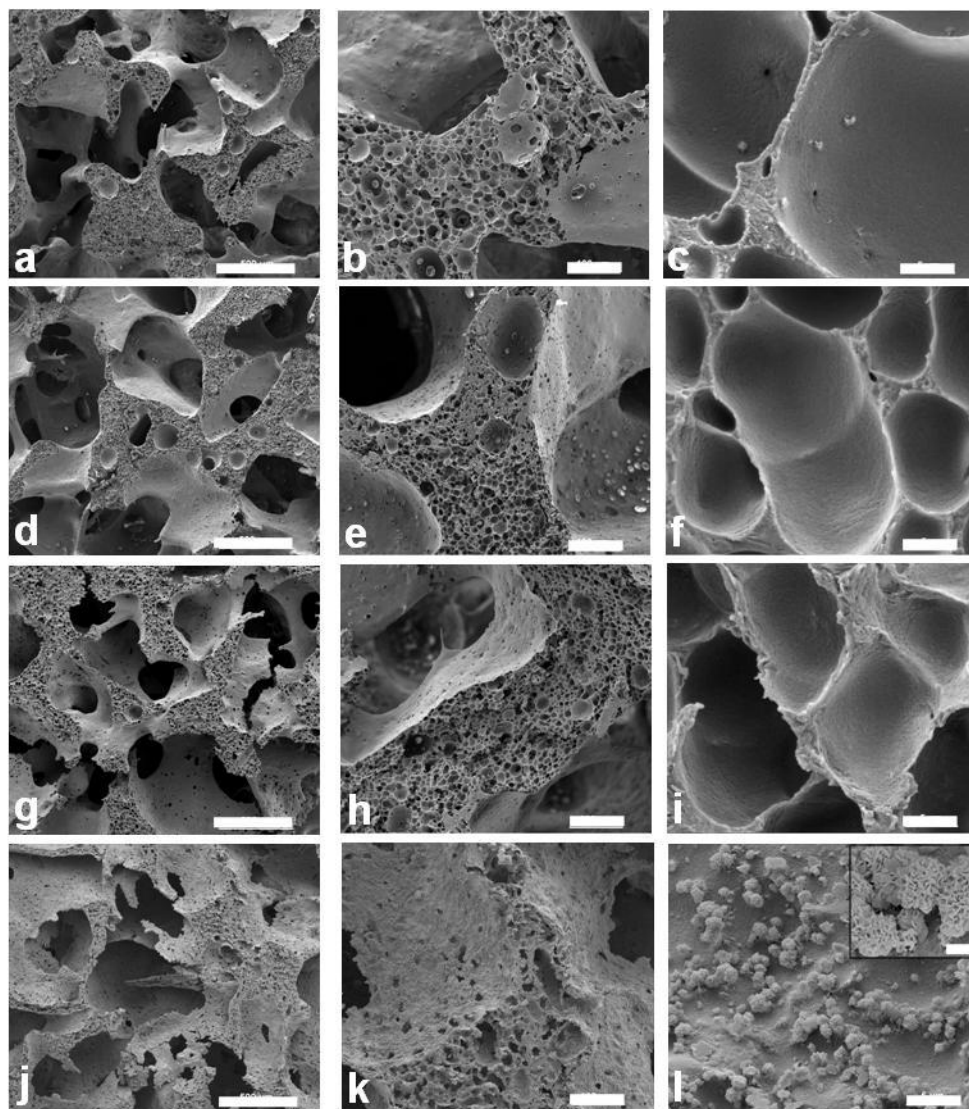


Figure 3. Morphology of the Silk-NanoCaP scaffolds determined by SEM. (a, d, g and j) Overview of silk/CaP-4, silk/CaP-8, silk/CaP-16 and silk/CaP-25, respectively (Scale bar: 500 μm); (b, e, h and k) trabecular structure of silk/CaP-4, silk/CaP-8, silk/CaP-16 and silk/CaP-25, respectively (Scale bar: 100 μm); (c, f, i and l) surface of the micro-pores of silk/CaP-4, silk/CaP-8, silk/CaP-16 and silk/CaP-25, respectively (Scale bar: 5 μm). The inserted image in (l) is the amplified image of (l) (Scale bar: 500 nm).

3.3. Characterization of the CaP in the scaffold

Table 1 shows the CaP content and the incorporation efficiency for the different Silk-NanoCaP scaffolds. After the extraction of sodium chloride particles, the CaP content was measured by TGA. This result demonstrated that most of the CaP formed was

retained in the silk/CaP-8 and silk/CaP-16 as compared to silk/CaP-4 and silk/CaP-25. The Ca/P atomic ratio determined by EDX analysis is also shown in Table 1. The Ca/P atomic ratio was approximately 1.67 for all the Silk-NanoCaP scaffolds, indicating the CaP synthesized in SF is similar to HA in respect to its chemical composition.

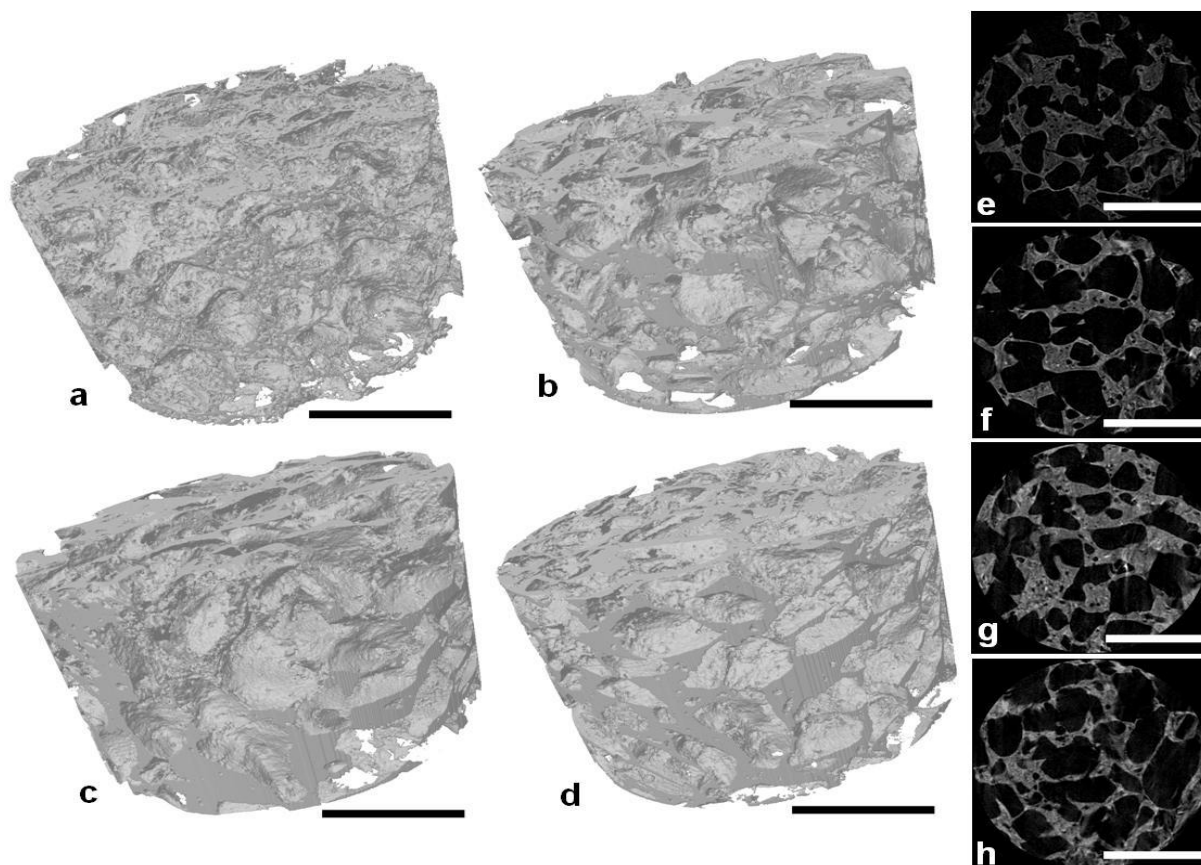


Figure 4. Three dimensional and two dimensional images of the Silk-NanoCaP scaffolds determined by micro-CT. (a, b, c and d) Three dimensional images of silk/CaP-4, silk/CaP-8, silk/CaP-16 and silk/CaP-25, respectively. (e, f, g and h) Two dimensional images of silk/CaP-4, silk/CaP-8, silk/CaP-16 and silk/CaP-25, respectively. Scale bar: 3 mm.

The backscattered SEM was used to identify the CaP particles and observe their microscopic distribution in the Silk-NanoCaP composite powder based on the contrast differences of each element. EDX was employed to confirm the presence of the CaP particles together with backscattered SEM. The amount of CaP particles (white regions) in the composite powder increased when increasing the amount of initially incorporated CaP in the SF (Figure 6a-h). The size of the CaP particles in silk/CaP-4, silk/CaP-8 and silk/CaP-16 was inferior to 200 nm. Despite, it was possible to observe particles with a

size close to 1 μm in silk/CaP-25. The distribution of the CaP particles in silk/CaP-8, silk/CaP-16 and silk/CaP-25 was uniform at a microscopic scale (Figure 6c-h). The EDX spectra (Figure 6i-l) also showed that the CaP particles observed in the Silk-NanoCaP scaffolds were generated without any by-product present, *i.e.*, no chloride (from ammonia chloride and sodium chloride) and sodium ions (from sodium chloride) were detected. The CaP particles were well integrated into the SF matrix (Figure 6m, n).

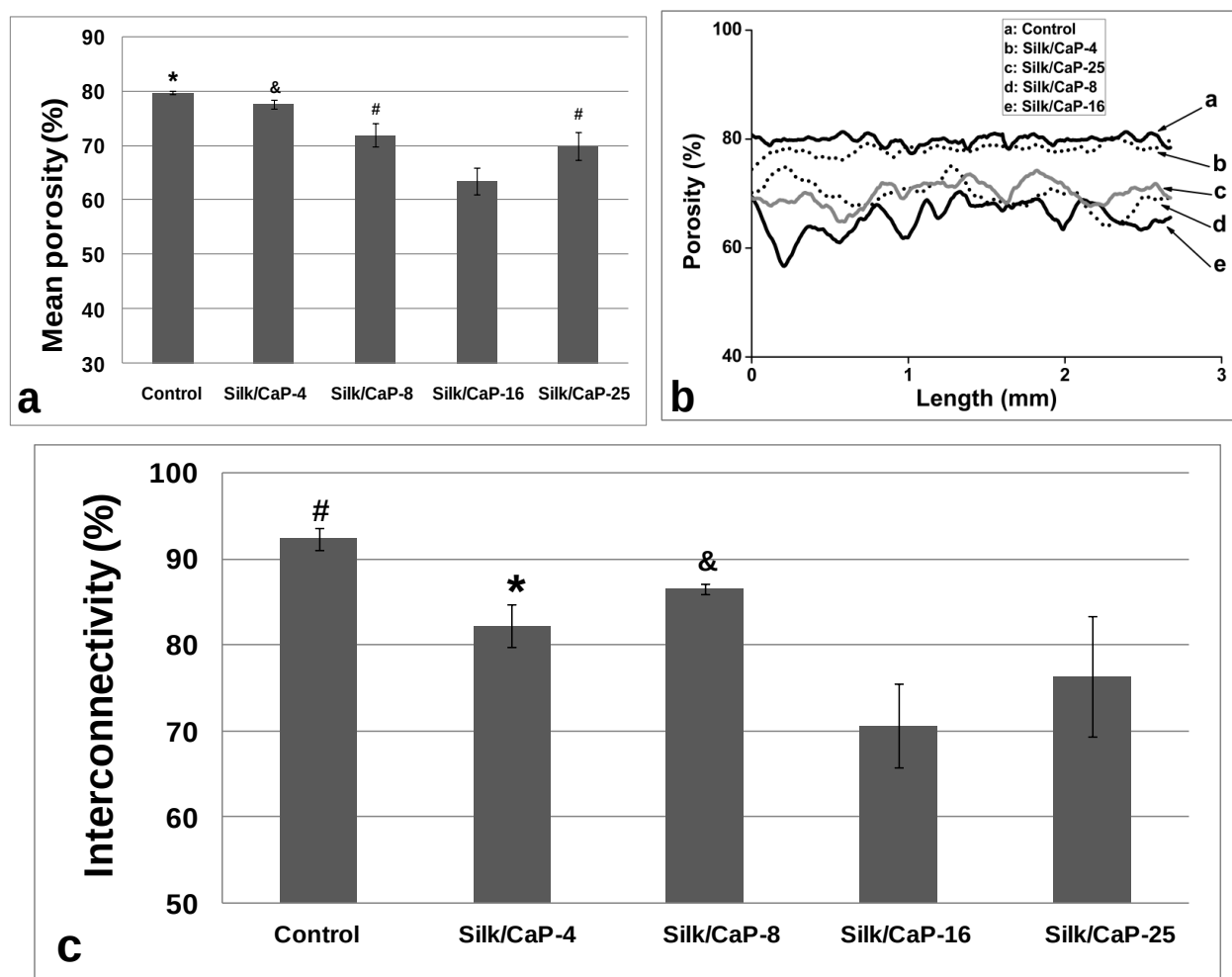


Figure 5. (a) Mean porosity, (b) representative porosity distribution along the length ,and (c) interconnectivity of the Silk-NanoCaP porous scaffolds determined by micro-CT. (a) * indicates significant differences compared with silk/CaP-4, silk/CaP-8, silk/CaP-16 and silk/CaP-25, **&** indicates significant differences compared with silk/CaP-8, silk/CaP-16 and silk/CaP-25, **#** indicates significant differences compared with silk/CaP-16. (c) **#** significant differences compared with silk/CaP-4, silk/CaP-8, silk/CaP-16 and silk/CaP-25; ***** significant differences compared with silk/CaP-8 and silk/CaP-16; **&** significant differences compared with silk/CaP-16.

Micro-CT analysis was performed in order to determine the distribution of CaP particles in the different Silk-NanoCaP scaffolds. The sole CaP distribution profile (Figure 7A) shows that the CaP content (vol. %) increased from silk/CaP-4 to silk/CaP-16, and the distribution of CaP in the Silk-NanoCaP scaffolds was homogeneous in each group. The combined SF and CaP distribution profile (Figure 7B) revealed that CaP content increased from silk/CaP-4 to silk/CaP-16 and the CaP presented homogeneous distribution at a macroscopic scale.

Table 1. The CaP content, CaP incorporation efficiency and Ca/P atomic ratio in the Silk-NanoCaP scaffolds determined by TGA and EDX analyses

Groups	Theoretical CaP content (wt.%) ⁺	Final CaP content (wt.%) [‡]	CaP incorporation efficiency (%) [§]	Ca/P atomic ratio [¶]
Silk/CaP-4	4	2.57±0.04	64.17±0.88	1.66±0.06
Silk/CaP-8	8	6.92±0.35 [#]	86.44±4.44	1.68±0.06
Silk/CaP-16	16	13.92±0.76 ⁺⁺	87.01±4.72	1.67±0.05
Silk/CaP-25	25	18.16±1.00 ^{**}	72.63±4.01	1.65±0.03

⁺The theoretical CaP content was calculated based on the hypothesis that the added Ca and P ions would reacted completely and that the formed CaP would be $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ (hydroxyapatite), which is the most stable phase under the processing conditions. The values were obtained by dividing the mass of the theoretical formed CaP by the mass of silk fibroin in each condition.

[‡]The final CaP content is determined by dividing the mass of residual CaP obtained from TGA assay by the mass of silk fibroin (determined by total mass of the scaffold minus the mass of residual CaP).

[§]The CaP incorporation efficiency was calculated by dividing the final CaP content by the theoretical CaP content.

[¶]The Ca/P atomic ratio was determined by analysis of the CaP residual by EDX after burning the scaffolds in a furnace. In each condition, 5 independent areas (200 × 200 μm) of the CaP residual were selected for this assay.

[#]Indicates significant difference compared with silk/CaP-4.

⁺⁺Indicates significant differences compared with silk/CaP-4 and silk/CaP-8.

^{**}Indicates significant differences compared with silk/CaP-4, silk/CaP-8 and silk/CaP-16.

3.4. Mechanical properties

Figure 8 shows the compressive mechanical properties of the control and different Silk-NanoCaP scaffolds determined in dry conditions. The values of the compressive modulus (Figure 8a) were 15.14±1.70 MPa, 16.76±4.58 MPa, 13.75±2.99 MPa, 19.02±5.77 MPa and 4.87±0.95 MPa for the control, silk/CaP-4, silk/CaP-8 and silk/CaP-16 and silk/CaP-

25, respectively. And the values of the compressive strength (Figure 8b) were 0.69 ± 0.12 MPa, 0.59 ± 0.14 MPa, 0.63 ± 0.11 MPa, 0.62 ± 0.11 MPa and 0.25 ± 0.03 MPa for the control, silk/CaP-4, silk/CaP-8 and silk/CaP-16 and silk/CaP-25, respectively. Regarding the compressive modulus and strength, there were no significant differences among the control, silk/CaP-4, silk/CaP-8 and silk/CaP-16.

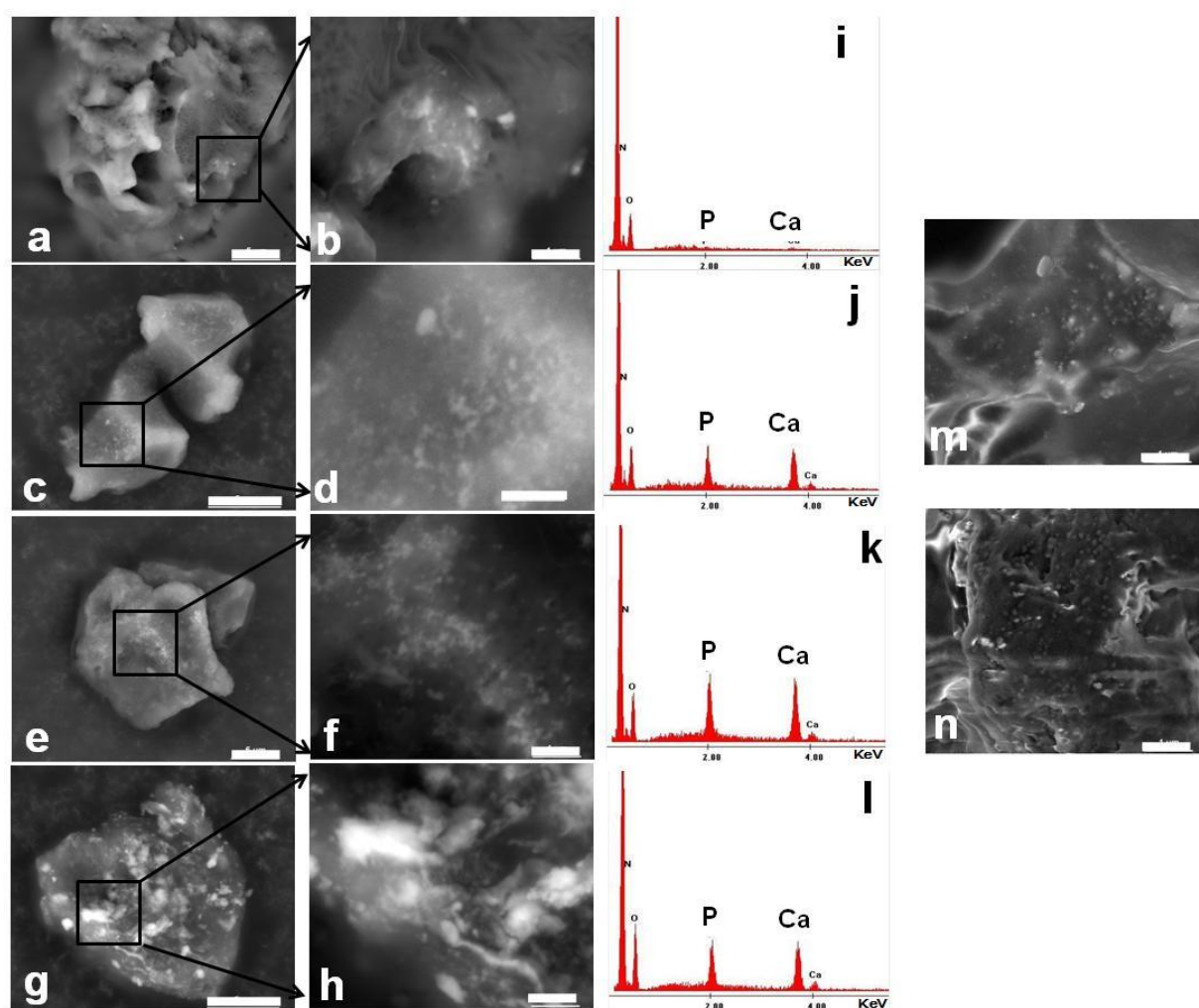


Figure 6. Distribution and particle size of the CaP particle in the Silk-NanoCaP scaffolds determined by SEM and EDX analyses. (a-h) were observed in a Backscattered SEM model, while (m, n) were observed in a secondary electron SEM model. (a, c, e and g) SEM images for silk/CaP-4, silk/CaP-8, silk/CaP-16 and silk/CaP-25, respectively (Scale bar: 5 μ m). (b, d, f and h) Amplified SEM images of (a, c, e and g), respectively (Scale bar: 1 μ m). (m, n) Secondary electron SEM images of (d, f), respectively (Scale bar: 1 μ m). (i, j, k and l) EDX spectra of (b, d, f and h), respectively.

The dynamic mechanical properties (wet conditions) of the control and different Silk-NanoCaP scaffolds were also assessed by DMA (Figure 9). The storage modulus (E') of

silk/CaP-4, silk/CaP-8 and silk/CaP-16 increased with the increasing of tested frequency (from 0.1 Hz to 25 Hz), while the E' for silk/CaP-25 presented no significant differences in all the tested frequencies. When the CaP content increased from 4% up to 16%, the storage modulus also increased for all the tested frequencies. Silk/CaP-16 presented the highest value in respect to storage modulus, which varied from 0.53 ± 0.15 MPa to 0.87 ± 0.12 MPa when the frequency increased from 0.1 Hz to 25 Hz. The loss factor ($\tan \delta$) of silk/CaP-4, silk/CaP-8 and silk/CaP-16 were comprised between 0.16 and 0.2 when the tested frequency was inferior to 10 Hz.

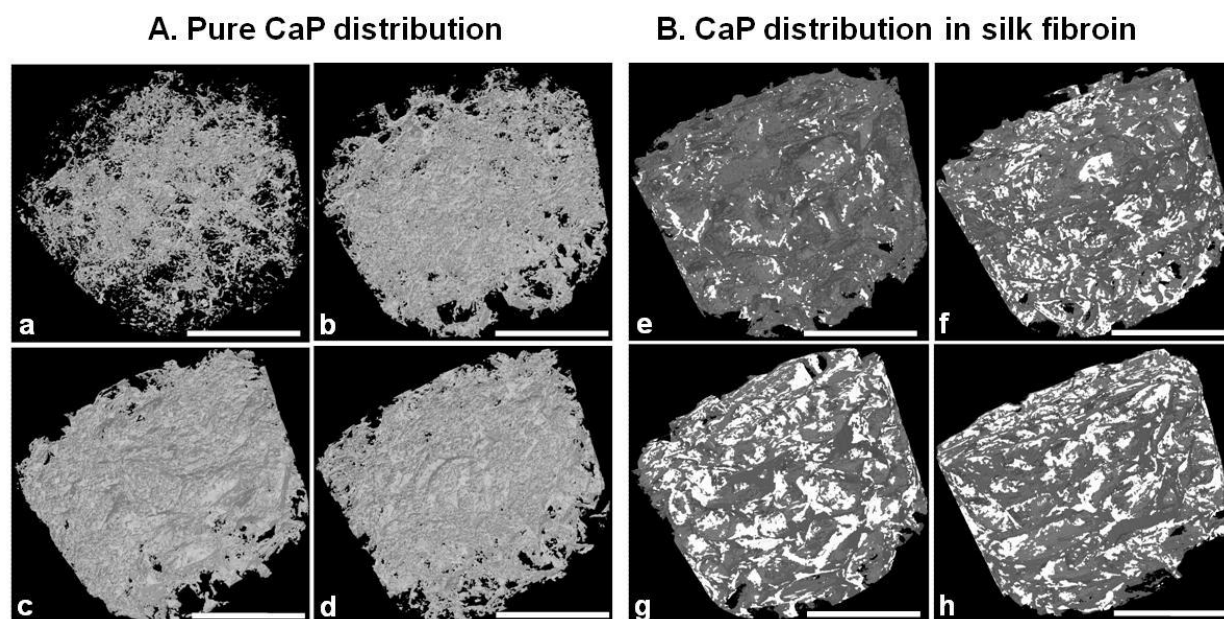


Figure 7. Three dimensional images of (A) pure CaP distribution and (B) CaP distribution in silk fibroin in the Silk-NanoCaP porous scaffolds, determined by micro-CT. (B) Silk fibroin: the gray domain; CaP: the white domain. (a, e) Silk/CaP-4; (b, f) silk/CaP-8; (c, g) silk/CaP-16; (d, h) silk/CaP-25 (Scale bar: 3 mm).

3.5. Hydration degree and weight loss ratio

Figure 10 shows the hydration degree and weight loss ratio for the control and several Silk-NanoCaP scaffolds. The hydration degree of each group scaffolds were maintained after immersion in sodium chloride solution after 6 hours (Figure 10a). As increasing CaP content from 4 wt.% up to 16 wt.%, the hydration degree of the Silk-NanoCaP scaffolds decreased after 6 hours immersion. The hydration degree behavior of silk/CaP-25 was similar to silk/CaP-16 and the control. Regarding the weight loss profile, silk/CaP-4

presented the lowest weight loss ratio as compared to the other Silk-NanoCaP scaffolds at day 1 and day 3 (Figure 10b). After 7 days of soaking, the weight loss value of all the Silk-NanoCaP scaffolds presented no significant differences, while the control group presented lower weight loss profile as compared to all the Silk-NanoCaP scaffolds after immersion for 7 days.

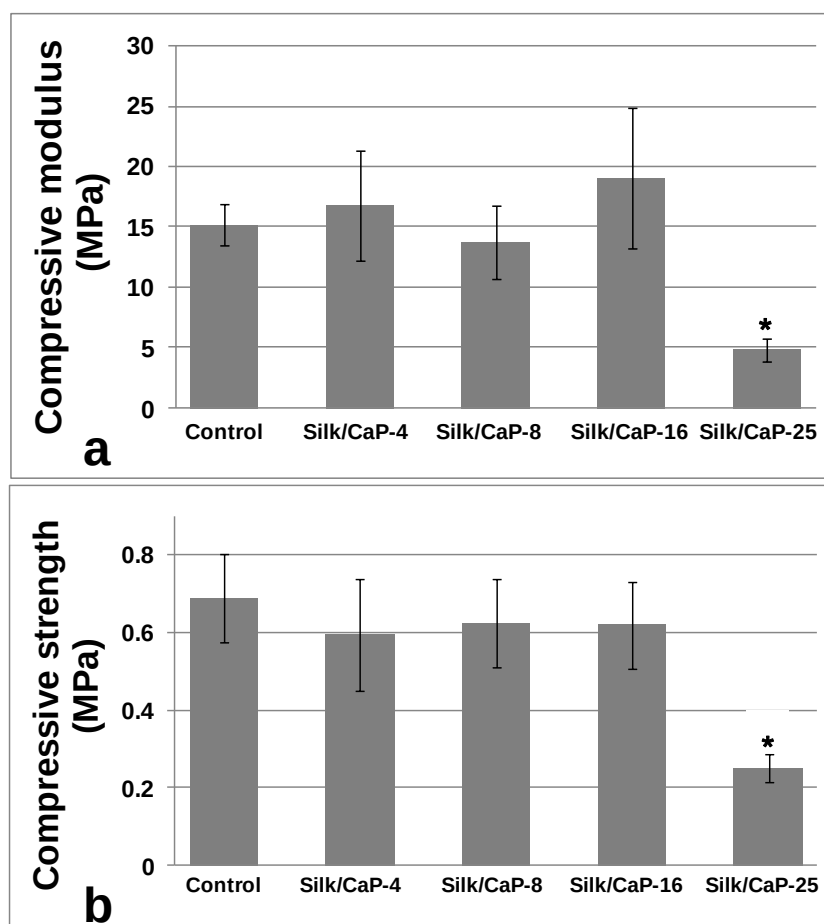


Figure 8. (a) Compressive modulus and (b) compressive strength of the silk and Silk-NanoCaP scaffolds. * indicates significant differences compared with silk/CaP-4, silk/CaP-8 and silk/CaP-16.

3.6. *In vitro* mineralization

After immersion of the Silk-NanoCaP scaffolds in SBF solution for 7 days, mineralized nuclei have grown on the surface of all the Silk-NanoCaP scaffolds, as shown in Figure 11 a-d. The EDX spectra confirmed that no other species than calcium and phosphorus have mineralized in the surface of the scaffolds (Figure 11e-h). The intensity of calcium and phosphorus signals increased from silk/CaP-4 to silk/CaP-25 (Figure 11e-h). Since EDX can detect elemental content under the surface up to 2 μm , the nano-CaP from the scaffolds might also contribute to the detected calcium and phosphorus signals. Further

quantification analysis of the CaP minerals formed on the surface of the scaffolds should be then performed. The apatite minerals exhibited worm-like or flake-like morphology, with size less than 500 nm (Figure 11a-d). It was also found that the minerals grown into cauliflower-like clusters, which were dominant in silk/CaP-25. This typical morphology has been described in previous studies [26] and results from the ability of a surface to induce *per se* the nucleation and growth of an apatite layer.

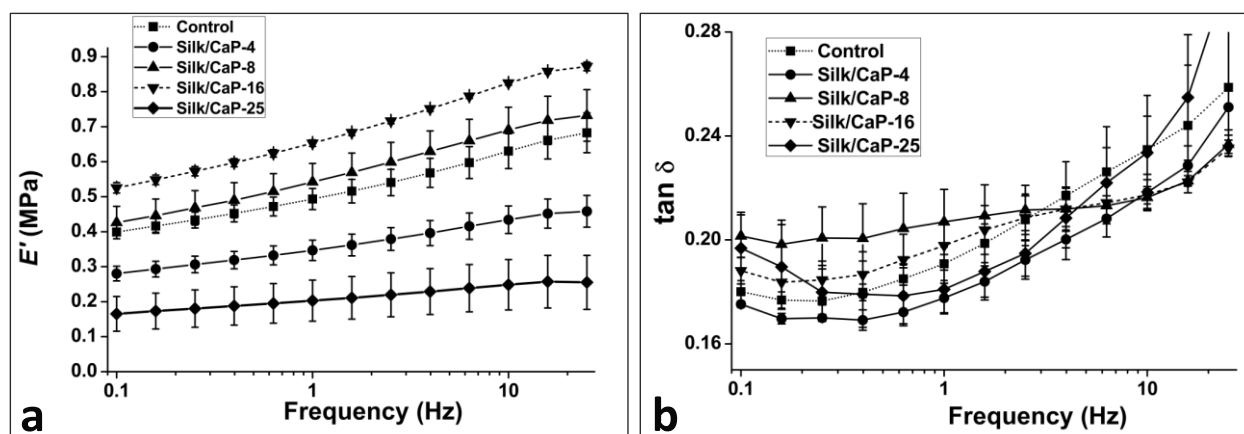


Figure 9. (a) Storage modulus and (b) loss factor of the silk and Silk-NanoCaP scaffolds determined by DMA at 37°C in PBS solution.

3.7. Cytotoxicity assessment

Latex leachables was used as control for cell death (positive control) in this study. This material has been described as cytotoxic and it has long been used [21] as control of cell death, in standard cytotoxicity assays. Figure 12 shows the cytotoxicity results of the silk control and silk/CaP-16. Cell viability of the silk control increased from day 1 to day 3 ($p < 0.05$), without significant difference between day 3 and day 7. Cell viability of silk/CaP-16 seemed to increase from day 1 to day 3, but without significant statistical difference. At day 7, silk/CaP-16 presented higher cell viability as compared to values obtained at day 1 and day 3, and as well as to those of the silk control at all the tested time points. Furthermore, both silk control and silk/CaP-16 showed higher cell viability as compared to the negative control at day 3 and day 7. The positive control (latex) presented negative value in cell viability at all the tested time points ($p < 0.05$). The negative value (%) of the positive control was due the OD value of the latex was lower than the one of the blank (MTS), thus it would generate negative value following the calculation procedure (2.5. in Materials and Methods)

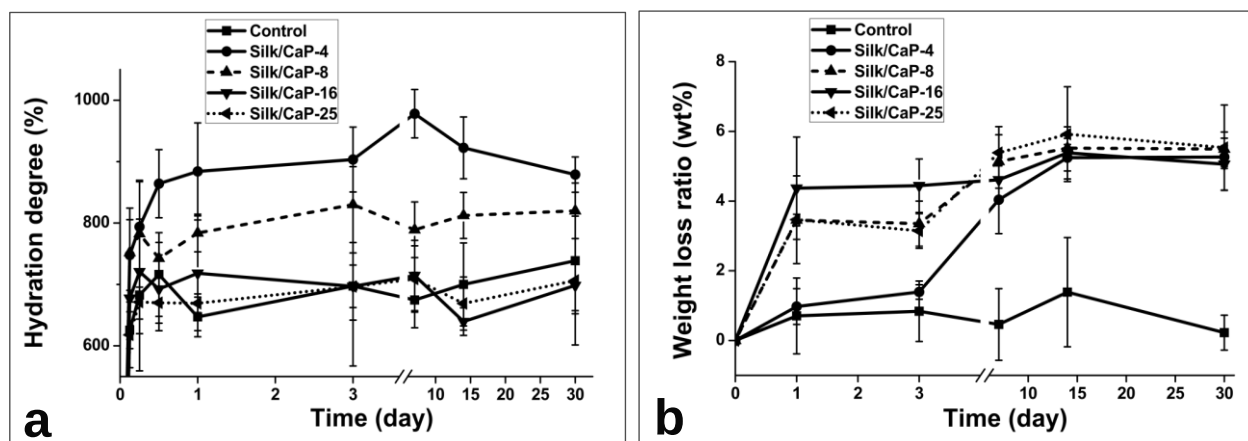


Figure 10. (a) Hydration degree and (b) weight loss ratio of the silk and Silk-NanoCaP scaffolds determined by immersion the scaffolds in sodium chloride solution in a water bath at 37°C (60 rpm) for different time period.

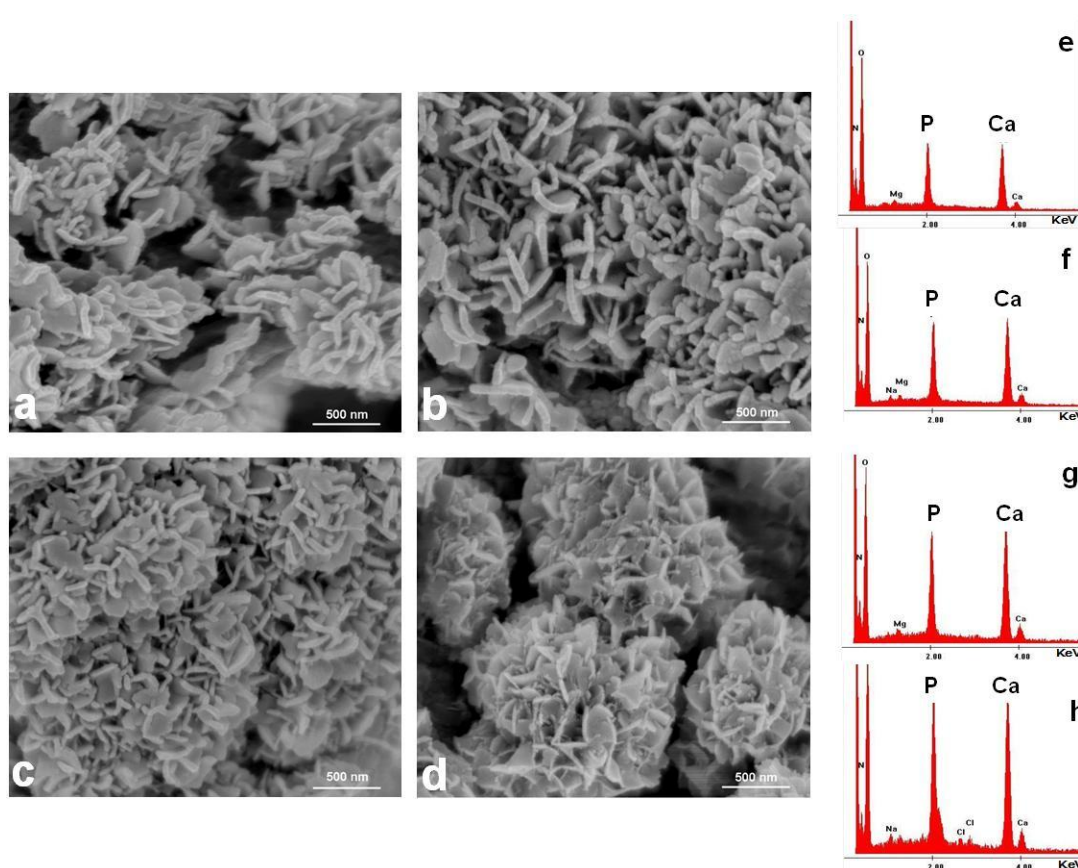


Figure 11. Mineralization of the Silk-NanoCaP porous scaffolds determined by SEM and EDX, after immersion in a simulated body fluid (SBF) solution for 7 days. (a, b, c and d) are the SEM images of the mineral on the surface of silk/CaP-4, silk/CaP-8, silk/CaP-16 and silk/CaP-25, respectively. (e, f, g and h) are EDX spectra corresponding to (a, b, c and d), respectively.

4. Discussion

In previous studies, porous silk/CaP scaffolds have shown promising potential in bone tissue engineering scaffolding [14-17,27]. However, a silk/CaP scaffold possessing suitable mechanical properties, proper microstructure and homogeneous distribution of the CaP particles to better match bone tissue engineering scaffolding demand has not been developed yet. Previously, macro/microporous silk scaffolds with good mechanical properties and high interconnectivity were developed by means of using high concentration aqueous SF solution (up to 16 wt.%) and a combination of salt-leaching/lyophilization approaches [18]. In the present study, we propose an *in-situ* synthesis method for the formation of nano-sized CaP particles within the matrix of macro/micro porous SF scaffolds.

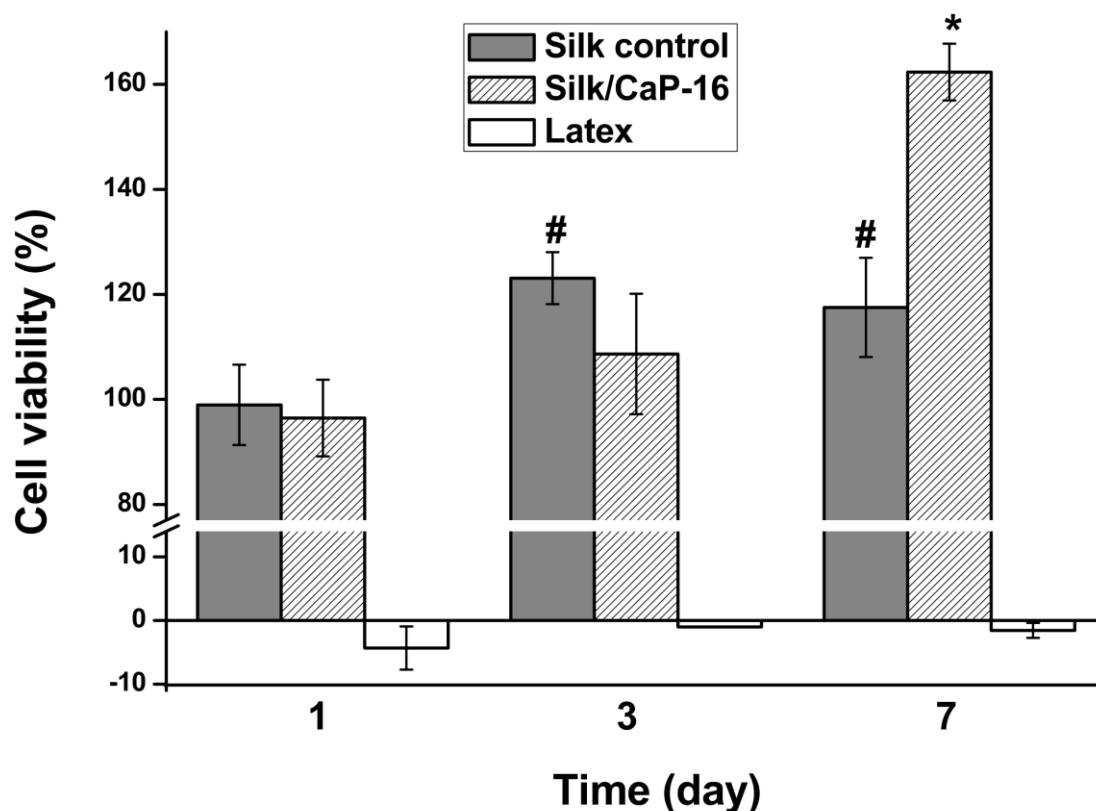


Figure 12. Cytotoxicity assessment of the leachables from control and silk/CaP-16 using L929 cells.

* indicates significant differences as compared to the cell viability of the control at all the tested time points, and as well as the cell viability of silk/CaP-16 at day 1 and day 3. # indicates significant differences as compared with the cell viability at day 1. Extract fluid of latex used as positive control.

Figures 2 showed that the CaP particles were successfully incorporated in the SF scaffolds. The XRD analysis indicated that the incorporated CaP particles could be assigned to a low crystalline HA, which is of great biomedical relevance as it can mimic closer bone apatite [23]. Dorozhkin [23] reported that mixing the calcium and phosphate ions in aqueous solution would first form amorphous calcium phosphates which were thermodynamically unstable compounds and spontaneously tended to transform into crystalline apatite. In the present work, the CaP particles (amorphous CaP) were aged in the SF solution for 24 hours (HA) prior to their incorporation into the SF scaffold. The XRD results show that the CaP did not change its structure during the salt-leaching in distilled water for 2 days. This result is consistent with the previous study [28], which studied the SF regulated mineralization process of calcium phosphate. The XRD and FTIR results also indicate that the incorporation of CaP did not impair the formation of β -sheet conformation in the SF. The formation of β -sheet is critical for the maintenance of the mechanical properties and structure stability of the Silk-NanoCaP scaffolds.

Kim *et al.* [12] found that the addition of sodium chloride particles into SF solution would induce the formation of β -sheet conformation in the SF. Based on this finding, they were able to generate aqueous derived porous SF scaffolds. But with this method, they only prepared SF scaffolds using aqueous SF solution with no more than 10 wt.% concentration. In our recent study, it was shown that SF scaffold obtained combining salt-leaching and lyophilization methods can be prepared by using aqueous SF solution up to 16 wt.% [18]. In the present study, we demonstrated that the salt-leached porous Silk-NanoCaP scaffolds derived from 16 wt.% SF aqueous solution can also be prepared by a similar approach (Figures 1 and 3). The SEM analysis of the Silk-NanoCaP scaffolds revealed that the morphology of the silk/CaP-4, silk/CaP-8 and silk/CaP-16 was similar to that of pure SF scaffold (16 wt.%) [18]. The formation of the apatite crystals on the surface of silk/CaP-25 can occur during the extraction procedure. During the salt extraction, the CaP particles would partially dissolved in water, increasing the local concentration of calcium and phosphate ions. The local enrichment of calcium and phosphate ions was favorable for the nucleation of apatite on the surface of the scaffolds, similar to the process observed for the studies on the biomineralization of HA scaffolds in a simulated body fluid solution [21]. The size of the macro-pores (around 500 μm) in all the Silk-NanoCaP scaffolds is adequate for bone scaffolding as it has been shown to support new bone formation and vascularization [29]. The size of the micro-pores in the

Silk-NanoCaP scaffolds is suitable for promoting the cell attachment and proliferation [29]. Furthermore, the macro-pores are highly interconnected (Figure 3) which would benefit cell ingrowth and tissue regeneration.

It has been shown that micro-CT is a powerful tool to quantitatively and qualitatively characterize the microstructure of scaffolds [30]. The observation from the three-dimensional and two-dimensional micro-CT images (Figure 4) corroborates the data obtained from the SEM images in respect to scaffolds morphology (Figure 3). Silk/CaP-4 possessed the lowest amount of CaP content as compared to the other groups, thus its porosity (Figure 5a) is closed to that of the control [18]. As the initially incorporated content of the CaP increased from 4 wt.% to 16 wt.%, the total porosity of the scaffolds decreased (Figure 5). This is because the trabeculae of the macro-pores of the scaffolds were impregnated with increased CaP particles. Actually, this observation is supported by the data obtained from micro-CT analysis (Figure 4). As the initially incorporated CaP content increased up to 25 wt.%, silk/CaP-25 presented lower structural integrity compared to other group (Figures 3, 8 and 9), thus induced the a higher porosity as compared to silk/CaP-16 (Figure 5a). Moreover, it was also observed that the distribution of the porosity along the scaffold is homogenous in all the groups (Figure 5b) and the interconnectivity remained at a high level (Figure 5c) indicating that the incorporation of CaP particles did not affect the foreseen architecture of the scaffolds.

The SF retained large amount of the initially incorporated CaP in the scaffolds (Table 1) within the SF matrix. The highly concentrated SF aqueous solution played an important role in preventing the leaching out of the CaP particles from the scaffolds. In our previous study [18], it was found that the thickness of the trabeculae of the macro-pores in the salt-leached SF scaffolds increase with the increase of SF concentration, being higher for scaffolds derived from 16 wt.% SF solutions. We observed that the thicker the trabeculae thickness, the higher the amount of CaP can be retained in the scaffolds (data not shown). This justifies the use of a highly concentrated SF aqueous solution in this study. It should be addressed that the CaP vol.% shown in Figure 7, cannot represent the real CaP particles contents in each group scaffolds. This is due to the fact that the resolution of the micro-CT equipment used in this study is 6 μm . Since the size of most of the CaP particles is less than 1 μm , thus it is impossible to quantitatively determine the CaP content in the Silk-NanoCaP scaffolds by micro-CT. Similar observation was reported in previous studies, Oliveira *et al.* [30] quantified the biomimetic CaP coating by micro-CT

for the first time. It was found that the micro-CT equipment was still capable of detecting the CaP coating, even the thickness of the biomimetic CaP coating was around 8 μm and the resolution of the micro-CT instrument was 11 μm , due to the high diffraction of the ceramic when comparing with the polymer. This observation was consistent with the result in this study (Figure 7). On the other hand, Bhumiratana *et al.* [27] also found that not all the HA micro-particles were detected by micro-CT since the resolution of micro-CT (21 μm) is a little higher than the approximate size of HA (20 μm). That is the reason why we used TGA to quantitatively evaluate the CaP content of the Silk-NanoCaP scaffolds. On the other hand, the formed CaP presented a Ca/P atomic ratio close to 1.67 which is in good agreement of the initial Ca/P atomic ratio (Table 1), and corresponds to the value calculated for stoichiometric HA.

A previous study by Kong *et al.* [28] has shown that the *in-situ* synthesis approach allowed for the formation of nano-sized HA crystal in the diluted SF solution (less than 2 wt.%). But with low concentration SF solution, it is difficult to generate a mechanical stable porous scaffold [14]. In this study, we performed the first time on synthesis nano-sized CaP particles in highly concentrated SF solution by an *in-situ* synthesis method. Our approach allowed the development of SF scaffolds with good mechanical properties and generation of nano-sized CaP particles in the scaffold. It was reported that the nano-sized particles tend to aggregate and precipitate due to the electrostatic interaction [17]. Our preliminary result also showed that the CaP particles would precipitate at the bottom when aqueous SF solutions of low concentration were used (data not shown). The backscattered SEM images confirmed that the nano-sized CaP presented a homogeneous distribution, without aggregation, in the SF matrix at a microscopic scale (Figure 6). Furthermore, the CaP particles were homogeneously distributed in all the Silk-NanoCaP scaffolds, at a macroscopic scale (Figure 7). It can be concluded that the highly concentrated SF aqueous solution had prevented the aggregation and the precipitation of the nano-size CaP particles in the composite system.

A homogeneous distribution of nano-sized or micro-sized CaP particles in the SF scaffold without aggregation is still very challenging. Liu *et al.* [31] prepared silk/CaP scaffolds by physical mixture of SF and nano-sized HA particles in aqueous phase. But there were evidences that the nano-sized HA particles partially aggregated into micro-sized particles on the surface of the macro-pores in the scaffold. By its turn, Bhumiratana *et al.* [27] produced porous silk/CaP scaffolds by physically mixing the SF and micro-sized HA

particles in organic phase. From the SEM observations, it was found that the HA particles were partially aggregated at the surface of the scaffolds. Zhang *et al.* [17] prepared silk/CaP porous scaffold from SF aqueous solution and micro-sized CaP/silk hybrid particles. The CaP/silk particles distributed homogeneously at a macroscopic scale in the scaffold in their study. From the above mentioned studies, it was found that it is feasible to achieve a homogeneous distribution of the CaP particles in silk fibroin scaffolds, but not at a microscopic scale. In the present study, the combination of an *in-situ* synthesis method and highly concentrated SF aqueous solution was used to solve this challenge. Notably, this combination approach allowed the formation of nano-sized CaP particles and prevented the aggregation and precipitation of the CaP particles (Figure 6a-h). As a consequence, it allowed the homogeneous distribution of the nano-sized CaP particles in the Silk-NanoCaP scaffolds, at both microscopic scale (Figure 6c-h) and macroscopic scales (Figure 7). The prepared Silk-NanoCaP scaffolds showed to comprise both homogeneous porosity and CaP particles distribution across the scaffolds, which is an advantage for tissue engineering scaffolding.

The mechanical performance of a scaffold plays an important role in tissue regeneration, especially when hard tissues are the targets such as in the case of bone. The control of the mechanical properties of the scaffolds aiming at matching the mechanical environment of the host tissues has been a subject of great attention over the last years [16,32,33]. In this sense, we have envisioned the use of high concentration aqueous SF solution as a possible strategy to improve the mechanical properties of the SF scaffolds. Regarding the dry status properties of the Silk-NanoCaP scaffolds, it is possible to state that the addition and increasing content of CaP particles in the SF scaffolds showed no statistically differences for the control, silk/CaP-4, silk/CaP-8 and silk/CaP-16 (Figure 8a-b), indicating that the nucleation and growth of the nano-sized CaP particles within the SF scaffolds did not compromise its dry state mechanical performance in this study. The DMA data indicated that the control, silk/CaP-4, silk/CaP-8 and silk/CaP-16 possessed good elasticity and stable viscosity as their storage modulus increased with the increase of the frequency and their loss factors were between 1.6-0.2 (Figure 9). The storage modulus (wet state) of the Silk-NanoCaP scaffolds presented porosity dependence when the initially incorporated CaP content was comprised between 4 and 16 wt.%. The storage modulus of silk/CaP-8 is comparable to that of the control, while silk/CaP-16 presented a higher value. These results demonstrated that the mechanical performance

of the silk-based scaffolds (wet state) can be maintained or even improved after incorporation certain amount of nano-sized CaP in the salt-leached SF scaffolds.

As compared to the mechanical properties of silk/CaP scaffolds reported from other studies, silk/CaP-16 prepared in this study possessed a compressive modulus of around 19 MPa which is about 5 times higher than the highest one reported by Liu *et al.* [31]. In their study the highest compressive modulus of the silk/HA scaffolds was around 3.2 MPa. By its turn, the compressive modulus of silk/CaP-16 was around 9 times higher to that of the salt-leached SF scaffolds coated with a biomimetic CaP layer developed by Kim *et al.* [15]. The compressive modulus in that study was about 2 MPa. By comparing the compressive strength of silk/CaP-16 (0.62 MPa) to other studies, our value was around 7 times and 3 times higher as compared to those reported by Zhang *et al.* [17] and Kim *et al.* [15], respectively. In their studies, the compressive strengths of the scaffolds were about 80 and 150 kPa, respectively. However, the mechanical properties of scaffolds prepared in this study are inferior as compared to cross-linked silk/CaP scaffolds reported by Collins *et al.* [16]. In their study, the chemical cross-linking was performed by using hexamethylene diisocyanate to endow the scaffolds with mechanical performance comparable to that of cancellous bone, with average compressive modulus of 175 MPa and strengths of 14 MPa. The size of the interconnected pores of those scaffolds was relatively lower (50-100 μm) compared with the one in this study (around 500 μm). The lower pore sizes may also contributed to the outstanding mechanical performance of the silk/CaP scaffolds developed in their study. But this approach is a little risky, the residual of the hexamethylene diisocyanate must be removed completely and the safety of the degradation products should also be investigated.

The hydration behavior and the degradation ratio of the scaffold are also critical to the cell attachment, proliferation and the final outcome of the regenerated tissue. The hydration ratio of the Silk-NanoCaP scaffolds is related with the porosity and structure integrity. From silk/CaP-4 to silk/CaP-16, the hydration degree decreased as the porosity decreased (Figure 10a and Figure 5a). Since the integrity of silk/CaP-25 is lower as compared to the other groups (Figure 3), its ability to retain the water inside the scaffold also decreased (Figure 10a). The hydration degrees of Silk-NanoCaP scaffolds showed in this study were higher than those reported by Liu *et al.* [31]. In their study, the porosities of the silk/CaP scaffolds were between 41-61%, resulting in the lower hydration degrees. All the Silk-NanoCaP scaffolds showed good stability, after 1 month of

immersion in a sodium chloride solution. This result is related with the β -sheet conformation of the SF, which conferred good water stability to the Silk-NanoCaP scaffolds. Actually, the degradation profiles of Silk-NanoCaP scaffolds resemble that reported for the control SF scaffolds after immersion in ISS for 7 days [18]. Despite, the slight weight loss of the Silk-NanoCaP scaffolds can be directly related to the partial dissolution of the poorly crystalline CaP. In fact, small amounts of calcium and phosphate ions were detected in the degradation solution of the Silk-NanoCaP scaffolds by Ion-Coupled Plasma (data not shown), in a preliminary study. The systematic study of the CaP particles degradation profile in the Silk-NanoCaP scaffolds is presently ongoing. The release of calcium and phosphate ions from the Silk-NanoCaP scaffolds is viewed as an advantage as it would promote bone regeneration when applied them for bone tissue engineering, as previously reported by Oliveira *et al.* [2,3], Zhang *et al.* [17], and Bhumiratana *et al.* [27].

In vitro bioactivity test performed in SBF solution has been used to predict the *in vivo* bone bioactivity of biomaterials [20]. If a biomaterial can induce an apatite layer on its surface in SBF solution, it probably can bond to living bone *in vivo*. HA was found to be bioactive both in SBF solution and *in vivo*. The formation of apatite on its surface in SBF solution was due to the dissolution of calcium and phosphate ions from HA [21]. In this study, nano-sized CaP particles were incorporated into the silk fibroin scaffolds. The low crystallinity nature of these CaP particles allowed the dissolution of calcium and phosphate ions in SBF solution and subsequently induced the formation of apatite on the surface of the scaffolds. It was noticed that Silk-NanoCaP scaffolds still presented bioactivity even when incorporated with small amount of nano-sized CaP particles, for instance silk/CaP-4. The bioactive nature of Silk-NanoCaP scaffolds indicates that these scaffolds possess great potential for bone tissue engineering application.

The assessment of the cytotoxicity of scaffolds by using their extract fluid has been investigated in our previous studies [21,22]. Since silk/CaP-16 presented higher mechanical performance (wet state) as compared to other Silk-NanoCaP scaffolds, it was selected along with the control for the cytotoxicity test. The cell viability was over 100% in some data points means that the leachables from the scaffolds can induce higher cell metabolic activity compared with normal culture medium. The leachables from the silk fibroin scaffold probably composed of some small silk fibroin nano-particles, which may act as nutrient source for the cells. Similarly, the leachables from the Silk-NanoCaP

scaffolds which were almost certainly the nano-sized CaP/silk composite. The nano-sized particles released calcium and phosphate ions, as well as the silk fibroin fragments. These factors might be helpful to increase the cell's viability. The cell viability data clearly demonstrated that the silk/CaP-16 and the control presented no cytotoxicity, which means that there is no toxic traces remained in the scaffolds. This data also validates the processing method for the macro/microporous silk and Silk-NanoCaP scaffolds.

5. Conclusions

In this study, macro/microporous Silk-NanoCaP scaffolds were produced, through the *in-situ* synthesis of nano-sized CaP in a high concentration aqueous SF solution (16 wt.%) followed by scaffolding using a salt-leaching/lyophilization approach. The CaP particles consisted of poorly crystalline HA and the SF presented β -sheet conformation. The synergetic effect of the *in-situ* synthesis method and the highly concentrated SF aqueous solution allowed to uniformly distributing the CaP particles in the scaffolds, at both microscopic and macroscopic scales. The combination of salt-leaching/lyophilization approaches allowed the formation of highly interconnected macro-pores, homogeneous porosity distribution, and high interconnectivity in the Silk-NanoCaP scaffolds. The Silk-NanoCaP scaffolds with the theoretical CaP content of 16 wt.% present the highest wet status storage modulus. The porosity and hydration degree of the Silk-NanoCaP scaffolds can be controlled by the amount of CaP particles incorporated. The developed silk and Silk-NanoCaP scaffolds are non-cytotoxic. The Silk-NanoCaP scaffolds developed present promising mechanical properties, architecture and stability, bioactivity and no cytotoxicity, which make them suitable for possible application in bone tissue engineering scaffolding.

6. Future Perspective

By combining the *in-situ* synthesis method with the traditional scaffolding approaches, bioactive nanocomposite-based scaffold with homogeneous distribution of the nano-particles were achieved. The incorporation of nano-sized CaP particles will endow the SF scaffolds with osteoconductivity property. Besides that, much room remains for the development of multi-functional Silk-NanoCaP scaffolds based on this study. The surface

functionality of these scaffolds might be helpful to guide the cells attachment and migration. By using some green chemistries, such as plasma treatment and supercritical fluid processing, the surface chemistry and surface roughness can be tuned and play important role on the cell behavior. Furthermore, the nano-CaP particles also present advantage on the affinity of bisphosphonates on their surface, such as alendronate and zoledronate. Combining the achievement of this study, it could be even better enhance bone regeneration by loading these drugs in the nano-CaP particles during the *in-situ* synthesis procedure and subsequently incorporating into the scaffolds. Other drugs, for instance water soluble dexamethasone or antibiotics, could also be combined in the scaffolds by this manner, to improve the bone repair outcomes.

Acknowledgements

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* = of interest

** = of considerable interest

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Chapter V

In Vitro Evaluation of the Biological Performance of
Macro/Microporous Silk Fibroin and Silk-Nano Calcium
Phosphate Scaffolds

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In Vitro Evaluation of the Biological Performance of Macro/Microporous Silk Fibroin and Silk-Nano Calcium Phosphate Scaffolds

Abstract

Tissue regeneration greatly depends on the biological performance of scaffolds. This study evaluates the biostability, cytocompatibility, and biomechanical properties of previously developed salt-leached macro/microporous silk fibroin (SF) scaffolds (S16) and silk-nano calcium phosphate scaffolds (SC16), both deriving from a 16 wt.% SF aqueous solution. A biostability assay was performed by immersion the scaffolds in a protease solution for different time periods. Human adipose tissue derived stromal cells (hASCs) were cultured onto the scaffolds *in vitro* for two weeks, in order to evaluate the cytocompatibility. The cell viability and proliferation were analyzed by Alamar blue assay and DNA content quantification, respectively. The cell attachment and migration onto the scaffolds were observed by scanning electron microscopy. The extracellular matrix (ECM) production in the scaffolds was studied by histological staining. The mechanical properties of S16 and SC16 after cell culturing were determined by compressive testing. The results showed that the silk-based scaffolds presented desirable biostability. The incorporation of calcium phosphate further improved the scaffolds' biostability during the enzymatic degradation study. S16 and SC16 were non-cytotoxic and supported the viability and proliferation of the hASCs. The microporous structure of the scaffolds was beneficial for the cell adhesion while the macroporous structure of the scaffolds favored the cell migration and proliferation. The histological analysis displayed abundant ECM formed inside the scaffolds after culturing with the hASCs for 7 days. The compressive modulus of the silk based scaffolds significantly increased after culture of the hASCs for two weeks. These results revealed that the S16 and SC16 were of superior biostability

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and cytocompatibility, and it could be promising alternatives for cartilage and bone tissue engineering scaffolding applications, respectively.

1. Introduction

Large defects in bone and cartilage are a common problem in orthopedics [1, 2]. In both cases without proper treatment, the mobility of the patients will be reduced or even impaired [3]. The current treatment procedures in the clinics include the use of autografts and allografts [4]. The lack of sufficient donors and risks of diseases infection are the main disadvantages of these procedures. Regeneration of the damaged tissues by developing three dimensional scaffolds and subsequently engineering the tissues in these scaffolds *in vitro* or *in vivo*, is a promising strategy [5].

Many synthetic or natural occurring biodegradable materials have been explored to generate porous scaffolds for bone or cartilage regeneration, including poly(α -hydroxy acids), poly(ethylene glycol), chitosan, collagen, gelatin, gellan gum and silk fibroin [6-8]. Among these polymers, silk fibroin (SF) derived from *Bombyx mori* cocoon has been showing numerous advantages [9]. It presents tailored mechanical properties and degradation profile depending on its conformation [10]. Moreover, SF can be easily shaped in several architectures such as porous scaffolds, hydrogels, particles, membranes, etc. [9, 11-13]. Regarding bone regeneration, inorganic based, polymeric based or inorganic/organic composite scaffolds have been studied [2, 14]. Inspired by the chemical component in natural bone, preparation of scaffolds constituted by calcium phosphate (CaP) and protein based polymer could be a good strategy [15-17].

Several methods have been explored to generate a porous structure in scaffolds, such as freeze-drying [18, 19], rapid-prototyping [20], gas-foaming [21], supercritical fluid processing [22], salt-leaching [12], microsphere sintering [23], and phase separation[24]. Freeze-drying is able to prepare scaffolds of porous structure, but it is difficult to achieve the homogeneous porosity distribution unless specific treatment is performed [19]. Rapid prototyping can control the pore size and porosity distribution easily, however it is quite limited when concerning very complex shaped defect architectures [20]. On the other hand, while gas-foaming is disadvantageous for the control of the pore size [21], supercritical fluid processing is limited in generating macro-pores [22]. Phase separation

is mainly feasible for synthetic polymers [24]. In order to produce scaffolds of controlled porosity and pore size, salt-leaching is an efficient and low cost approach as compared to the other approaches.

In previous studies, porous SF and silk-nano CaP (Silk-NanoCaP) scaffolds derived from high concentrated aqueous SF solution were produced via salt-leaching approach [25, 26]. These scaffolds were of superior mechanical properties, controllable porosity, and macro/microporous structure. The leachables of these scaffolds were found to be non-cytotoxic and the scaffolds also presented desirable biocompatibility *in vivo* [26, 27]. However, the direct contact cell culture on these scaffolds has not yet been reported. It is important to evaluate the interaction between cells and the SF based scaffolds, as well as the potential of these scaffolds to form engineered tissues *in vitro*. Pores size and porosity of the scaffolds are critical issues for cell attachment and growth [28]. Murphy *et al.* [29] reported that the collagen/glycosaminoglycan scaffolds of large pores size showed the highest cell number during the *in vitro* culture of osteoblasts. Mandal BB *et al.* [30] explored the influences of pore size and porosity of the SF scaffolds on the behavior of fibroblasts. They found that the higher pore size was associated with the higher cell proliferation, but porosity determined maximum on cellular migration and proliferation. Surface properties of the scaffolds also play important role on cellular performance. Abbah SA *et al.* [31] performed the surface modification of polycaprolactone scaffolds in order to enhance the surface hydrophilicity and roughness. Furthermore, Kim SS *et al.* [32] employed the gas forming/particulate leaching approach to improve the surface properties of polymer/hydroxyapatite composite scaffold which induced higher cell growth on these scaffolds. In this study, it aimed to answer how the macropores and the micropores in the SF based scaffolds affect the cellular behavior. Moreover, the influence of the nano CaP particles on the properties and biological performance of the scaffolds was also elucidated.

In this study, the biostability of the developed SF scaffolds were evaluated by enzymatic degradation in protease XIV solution. The microstructure and phase distribution of the scaffolds were examined by scanning electron microscopy (SEM) and micro-computed tomography (micro-CT). The cytocompatibility of the SF scaffolds were analyzed by culturing with human adipose tissue derived stromal cells (hASCs) for up to 14 days. The hASCs are multipotent stem cells and can be differentiated into bone, cartilage, muscle, and adipose lineages [33]. Additionally, they proliferate fast *in vitro* and can be obtained

easily from the liposuction procedure and proliferate fast *in vitro* [34], which justifies their selection for the current study. The viability and proliferation of the hASCs were screened by Alamar blue assay and DNA quantification. The cell attachment and migration in the scaffolds were recorded by SEM. The extracellular formation was studied by haematoxylin and eosin (H&E) staining. The mechanical properties of the scaffolds cultured with the hASCs for two weeks were also tested.

2. Materials and Methods

2.1. Materials and reagents

Bombyx Mori cocoons were purchased from the Portuguese Association of Parents and Friends of Mentally Disabled Citizens (APPACDM, Castelo Branco, Portugal). The other materials and reagents were provided from Sigma-Aldrich (St. Louis, MO, USA) unless mentioned otherwise.

2.2. Preparation of the SF and Silk-NanoCaP scaffolds

At first, the high concentration of aqueous SF solution was prepared as previously reported [25]. Briefly, the cocoons were degummed for one hour in 0.02 mol/L boiling sodium carbonate solution. The obtained SF was then dissolved in 9.3 mol/L lithium bromide solution, followed by transferring into a benzoylated dialysis tubing (MWCO: 2 kDa) and dialysis in distilled water for two days. Afterwards, the SF solutions were concentrated by 20 wt.% poly(ethylene glycol) solution (M_n : 20 kDa). The concentrated SF aqueous solutions were diluted by distilled water to prepare the 16 wt.% SF aqueous solution.

The salt-leached SF scaffolds were prepared by addition of 2 g sodium chloride particles (particle size: 500-1000 μm) into 1 mL 16 wt.% SF solution in a mold made by silicon tubing (diameter: 9 mm). The molds were dried in air for two days and subsequently the sodium chloride was leaching out by placing the molds into distilled water. The SF scaffolds were removed from the tubing and cut into pieces. The skin of the scaffolds was removed by using a stainless steel punch (diameter: 6 mm). The final scaffolds were

obtained by lyophilization in a freeze-drier (CRYODOS-80, Telstar, Barcelona, Spain) after freezing them in a -80°C freezer overnight.

Regarding the produce of the salt-leached Silk-NanoCaP scaffolds, firstly, calcium chloride solution (6 mol/L) and ammonia dibasic phosphate solution (3.6 mol/L) were sequentially introduced into the 16 wt.% aqueous SF solution to generate the milky Silk-NanoCaP suspension [26]. It was hypothesized that the introduced calcium and phosphate ions would form hydroxyapatite, $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$, and the amount of calcium phosphate (CaP) introduced was fixed at 16 wt.% (CaP:Silk). The pH of the suspension was adjusted to around 8.5 by addition of ammonia (30%). After aging overnight, 1 mL suspension was transferred into the silicon mold and subsequently 2 g sodium chloride particles (particle size: 500-1000 μm) were added into the mold. The molds were dried and then the scaffolds were obtained following the same procedures for the preparation of SF scaffolds as mentioned above. The SF scaffolds and the Silk-NanoCaP scaffolds were designated as S16 and SC16, respectively.

2.3. Microstructure and phase distribution analysis of the SF based scaffolds

The morphology of S16 and SC16 were observed by SEM. Before the observation by SEM (Nova NanoSEM 200; FEI, Hillsboro, OR, USA), the specimens were coated with Au/Pd SC502-314B in an evaporator coater (E6700; Quorum Technologies, East Grinstead, UK).

The microstructure and the phase distribution of the scaffolds were evaluated by micro-CT. S16 and SC16 specimens were first scanned in the micro-CT (1072 scanner; SkyScan, Kontich, Belgium) at 40 keV/248 μA and 61 keV/163 μA , respectively. The data were converted into bitmap images by NRecon v1.4.3 software (SkyScan) in a cone-beam model. The three-dimensional (3D) qualitative visualization of the morphology and the varied phases in the scaffolds were conducted by using the CTvol software (SkyScan).

2.4. Enzymatic degradation of the SF based scaffolds

The biostability of S16 and SC16 were analyzed by enzymatic degradation in protease XIV solution. The scaffolds used for the degradation study were of 6 mm in diameter and 2 mm in height. Each specimen was placed into a vial supplemented with 5 mL protease XIV solution (1 U/mL or 4 U/mL). The initial dry weight of each specimen was measured first. When 1U/mL protease solution was used, the scaffolds were degraded for 0.5, 1, 2, 3, 5 and 7 days. In the case of using 4 U/mL protease solution, the samples were analyzed at 3, 6, 12, 24 and 48 hours. All the enzyme solutions were refreshed every 24 hours. At the end of each time point, the samples were removed from the enzyme solution, and then rinsed by distilled water. The remaining mass of the specimen was measured after drying it at 70°C in an oven overnight. The weight loss ratio (%) was calculated using Equation 1, as follows:

$$\text{Weight loss ratio} = \left(\frac{m_i - m_{d,t}}{m_i} \right) \times 100\% \quad (1)$$

Where m_i is the initial dry weight of the sample, and $m_{d,t}$ is the dry weight of the degraded sample at each time point. At least five specimens were used for each group at each time point.

2.5. Cytocompatibility of the SF based scaffolds

2.5.1. Culturing of the hASCs

The hASCs were isolated from the adipose tissue which was obtained from the liposuction procedure [34]. The use of the hASCs was approved by the Ethics Committee of University of Minho. The isolated hASCs were expanded and then stored in liquid nitrogen for long-term use. In this study, the hASCs in passage two (P2) were defrost from the liquid nitrogen and expanded in α -MEM (Gibco[®], Life Technologies, Carlsbad, CA, USA). The α -MEM was supplemented with 10% fetal bovine serum (Life Technologies, Carlsbad, CA, USA), and 1% Antibiotic-Antimycotic liquid prepared with 10,000 units/mL penicillin G sodium, 10,000 μ g/mL streptomycin sulfate, and 25 μ g/mL amphotericin B as Fungizone[®] in 0.85% saline (Life Technologies, Carlsbad, CA, USA).

The cells were cultured in an aseptic condition, at 37°C in an incubator with 5% CO₂ atmosphere (MCO-18AIC (UV), Sanyo, Osaka, Japan). The medium was refreshed every two day until the cells reached around 90% confluence. In the following, the cells were detached from the culture flask by using TrypLE Express (1X) with phenol red (Life Technologies, Carlsbad, CA, USA). The cell number was counted in a cell counter. Afterwards, the cell suspension (Passage 3, P3) was centrifuged at 1200 rpm for 5 minutes (5810R, Eppendorf, Hamburg, Germany). And then, the supernatants were discarded, and the cells were re-suspended and subsequently passaged into new flasks. The cells were expanded until P4 before seeding in the scaffolds.

2.5.2. Seeding and culturing of the hASCs in the SF based scaffolds

All the scaffolds (diameter: 6 mm; height: 2 mm) for cell seeding study were sterilized by ethylene oxide. Before the cell seeding, the scaffolds were degassed and hydrated in α -MEM overnight in the CO₂ incubator. In the following day, the hydrated scaffolds were transferred to a 24-well suspension cell culture plate (Cell star, Greiner Bio-One, Kremsmuenster, Austria). The hASCs of P3 were detached and a new cell suspension (P4) was prepared (cell density: 5 million/mL). Each scaffold was seeded with 200,000 cells on its surface, and then the constructs were kept in the CO₂ incubator. Three hours later, the constructs were moved to a new 24-well suspension culture plate and 2 mL of α -MEM were added for each construct. The culture medium was changed every two or three days.

2.5.3. Viability, proliferation, attachment, and migration of the hASCs in the SF based scaffolds

The viability of the hASCs seeded in the scaffolds was evaluated after cell seeding for 1, 3, 7, 10 and 14 days, by using the Alamar blue reagent (AlamarBlue[®], AbD Serotec, Kidlington, Oxford, UK). Resazurin, the main active component in Alamar blue, is nontoxic and can be converted into resorufin via the reduction reaction of living cells. During the reduction reaction, the color of this reagent will change from blue (resazurin) to bright red (resorufin). Thus, the Alamar blue can be used as an indicator for cell metabolic activity (viability) or cell number [35]. The Alamar blue working solution

containing 10% Alamar blue stock solution and 90% α -MEM was prepared and protected from light. At the end of each time point, the constructs were transferred into a new 48-well cell culture plate which was supplemented with 500 μ L Alamar blue working solution in each well. The plate was kept in dark and incubated for three hours in the CO₂ incubator. Afterwards, 100 μ L supernatant from each construct was transferred into each well of a new 96-well cell culture plate. The constructs were washed by phosphate buffered saline (PBS) solution for three times and then returned to the corresponding well in the original culture plate. The culture medium was changed accordingly. The reacted AlamarBlue[®] was read in a microplate reader (Synergy HT, Bio-Tek, VT, USA) at 570 and 600 nm, respectively. And then, the reduction percentage of AlamarBlue[®] was calculated following the protocol from the manufacturer. Scaffolds without cell seeding were used as controls. Four specimens were used for each group at each time point. Three independent experiments were performed.

The proliferation of the seeded hASCs in the scaffolds was analyzed by the total DNA content in each construct, after culturing for 1, 3, 7, 10 and 14 days. At the end of each time point, the constructs were removed from the medium, followed by rinsing with PBS solution. Afterwards, each construct was transferred into one vial containing 1 mL ultrapure water. The vials were stored at -80°C freezer before the DNA content determination. For the DNA quantification, the constructs were defrosted firstly, and then underwent ultrasonication treatment for 20 minutes to release the DNA from the scaffolds. The double-stranded DNA (dsDNA) was quantified by using a Quant-IT PicoGreen dsDNA Assay Kit 2000 assays (Life Technologies, Carlsbad, CA, USA) according to the instruction from the manufacturer. Briefly, 30 μ L supernatant from each vial was mixed with 70 μ L PicoGreen working solution and 100 μ L Tris-EDTA buffer. The fluorescence intensity of the samples was recorded in the microplate reader (Synergy HT, Bio-Tek, VT, USA), with the excitation wavelength at 485/20 nm and the emission wavelength at 528/20 nm. Standard curve was prepared by using standard dsDNA solutions with different concentrations, in order to quantify of the DNA content in the samples.

The attachment and migration of the hASCs in the scaffolds were observed by SEM, after culturing for 1, 3, 7, 10 and 14 days. At the end of each time point, the constructs were removed from the medium and rinsed by PBS solution, followed by fixing in 10% formalin solution for at least overnight. In order to dehydrate the specimens, the fixed

constructs were immersed in a serial of aqueous ethanol solutions with gradient increased concentration in ethanol (from 30% to 100%). The samples were dried in a flow chamber. And then the surface of the constructs was coated by Au/Pd before observing by SEM.

2.6. Histological analysis

The scaffolds cultured with the hASCs for 3, 7 and 14 days were used for histological analysis by H&E staining. At the end of each time point, the constructs were removed from the culture medium and washed by PBS solution. Afterwards, the constructs were fixed in 10% formalin overnight then immersed in paraffin after dehydration. Slides of 4 μm in thickness were prepared, following the H&E staining and Toluidine blue staining were performed.

2.7. Mechanical properties of the hASCs-seeded SF based scaffolds

The compressive modulus of the scaffolds after culturing with hASCs for two weeks was examined. At the 14th days, the constructs were removed from the culture medium and subsequently rinsed by PBS solution. The specimens were tested in a universal testing machine (Instron 4505, Instron, Norwood, MA, USA), after removing the surface liquid by filter paper. The samples were screened under a compressive rate of 2 mm/minute until reaching 60% strain. The slope of the initial linear domain in the compressive curve was used to determine the elastic modulus of each specimen. Scaffolds kept in culture medium for two weeks but without cell seeding were used as controls. At least six specimens were analyzed for each group.

2.8. Statistical analysis

The data were presented by mean $\pm$ standard deviation (SD). The results were evaluated by one-way analysis of variance (ANOVA). The means of each group were compared by Tukey's test, and $p < 0.05$ was considered statistically significant.

3. Results

The morphology, microstructure, and phase distribution of the scaffolds were studied by SEM and micro-CT (Figure 1). Figure 1a and b shows the morphology of S16 and SC16. It was found that both S16 and SC16 presented macro-pores with size ranged from around 300-700 μm , as well as micro-pores distributed in the walls of the macro-pores with size mainly less than 50 μm . The macro-pores were highly interconnected. The thickness of the trabeculae in the macro-pores is around several hundred micrometers. The micro-CT 3D images showed that both S16 and SC16 were porous and highly interconnected (Figure 1c and d). The CaP phase distributed evenly in SC16 (Figure 1d).

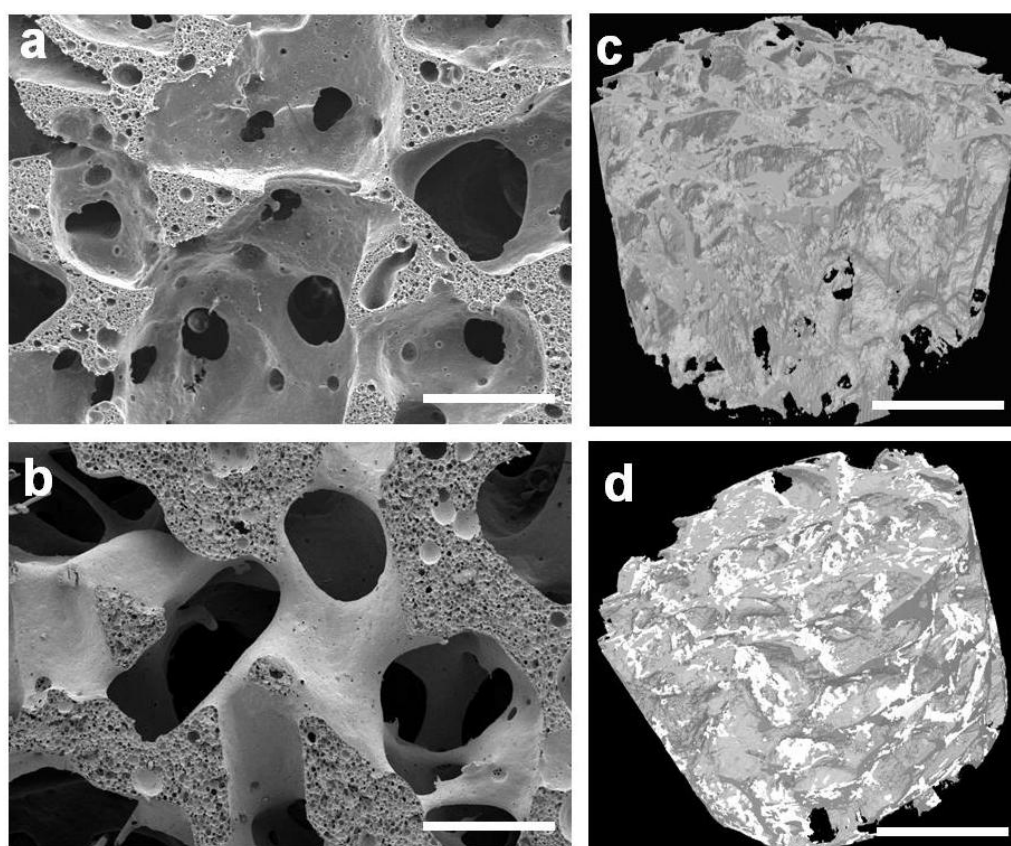


Figure 1. Microstructure and phase distributions of S16 and SC16. (a-b) The SEM images of S16 and SC16, respectively (scale bar: 500 μm). (c-d) The micro-CT three-dimensional images of S16 and SC16, respectively (scale bar: 1 mm). The white domain in (d) indicated the CaP phase, and the gray region was corresponding to the SF matrix.

The biostability of the scaffolds were studied by *in vitro* enzymatic degradation. Figure 2 shows the degradation profiles of S16 and SC16 when immersion in protease XIV

solutions of different concentrations. When 4 U/mL protease XIV solution was used, S16 lost more than 50% mass, while SC16 only showed about 23% weight loss, in the first 12 hours (Figure 2a). After one day, S16 presented around three-quarters weight loss, and SC16 displayed approximately 28% mass reduction (Figure 2a). S16 degraded completely in 48 hours and SC16 only lost less than 35% initial mass (Figure 2a). S16 and SC16 degraded much slower when 1u/mL protease solution was used (Figure 2b). In the first 12 hours, S16 and SC16 showed around 15% and 8% weight loss, respectively. In the end of 24 hours, about 20% and 13% mass reduction was observed for S16 and SC16, respectively. After 2 days, there were roughly 25% and 15% weight loss for S16 and SC16, respectively. After 1 week, S16 and SC16 still maintained around 60% and 80% initial mass, respectively. In both protease concentrations, it was observed that SC16 degraded much slower than S16.

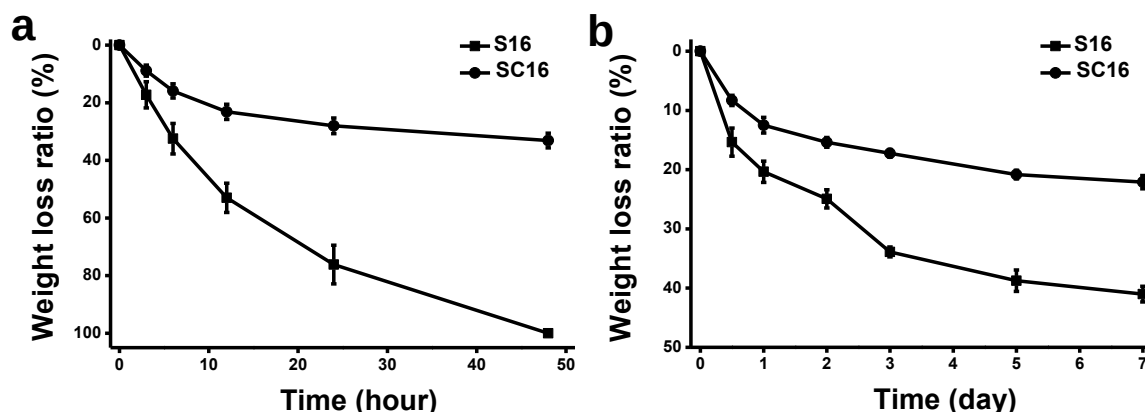


Figure 2. Enzymatic degradation profile of S16 and SC16 screened by immersion the scaffolds in protease XIV solution. (a) The protease solution was 4 U/mL; (b) the protease solution was 1U/mL.

The viability of the hASCs cultured on the scaffolds was studied by Alamar blue assay. Figure 3 shows the viability profile of the hASCs during the 14 days of culture in S16 and SC16. It was found that the viability of hASCs cultured in S16 and S16 increased gradually from the 1st day to the 14th day. There were no significant differences in cell viability between these two groups of scaffolds in the tested time points. During the 14 days, there was a big increase in viability for both groups from day 1 to day 3. Significant improvements in viability were also observed for S16 and SC16, from the 3rd to the 7th day and from the 7th to the 14 day. From the 7th to the 10th day and from the 10th to the 14th day, the viability seemed increase but not significantly.

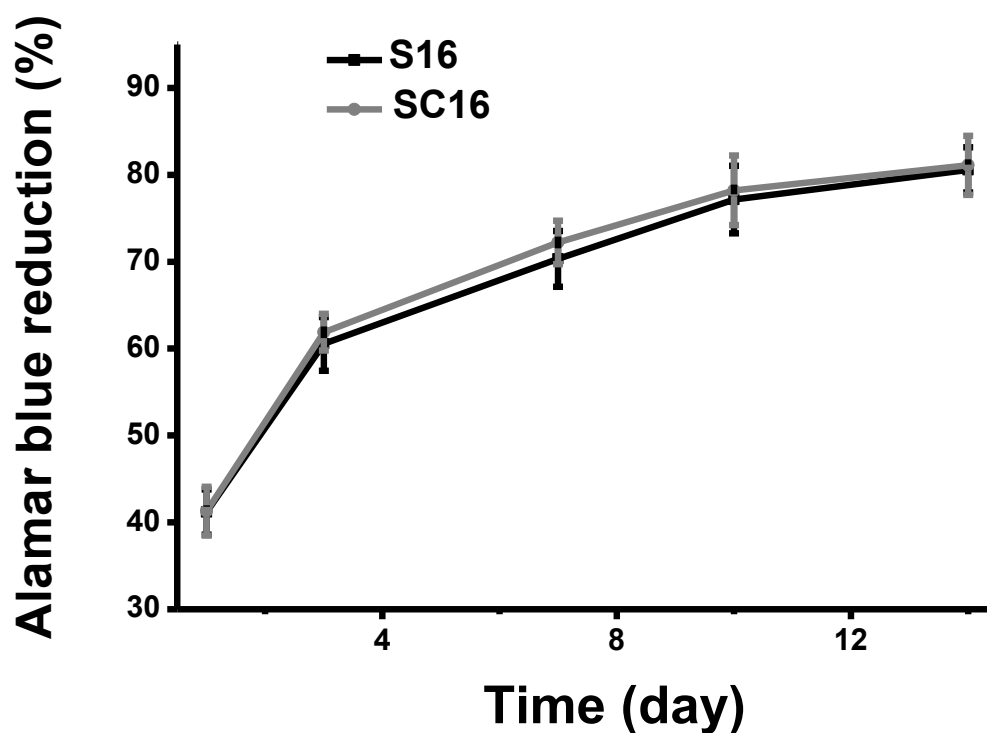


Figure 3. The viability of the hASCs in S16 and SC16 examined by Alamar blue assay.

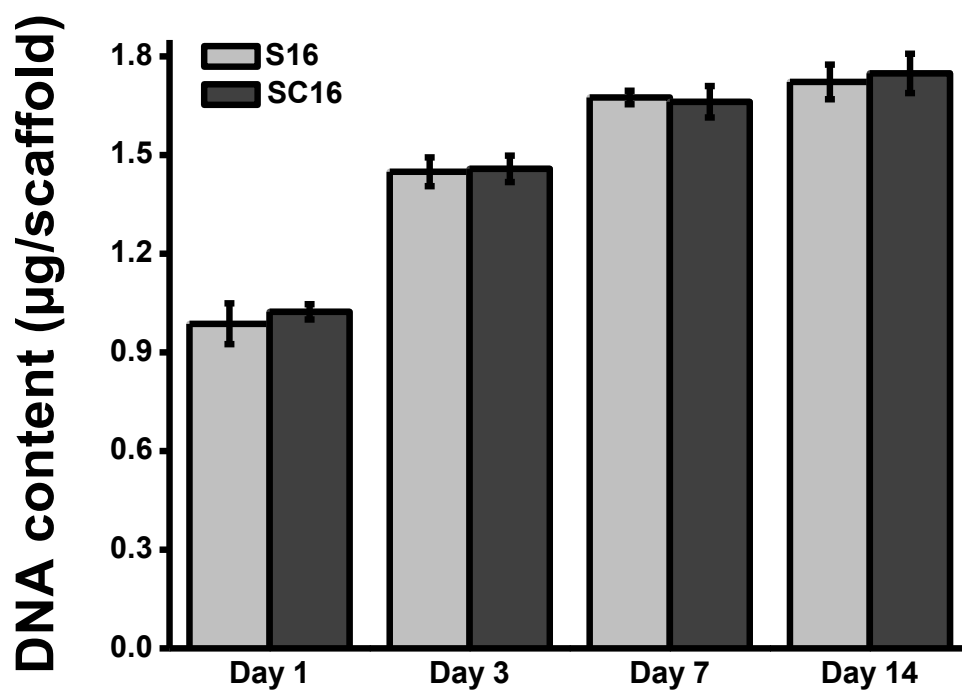


Figure 4. The proliferation of the hASCs in S16 and SC16 evaluated by DNA content quantification.

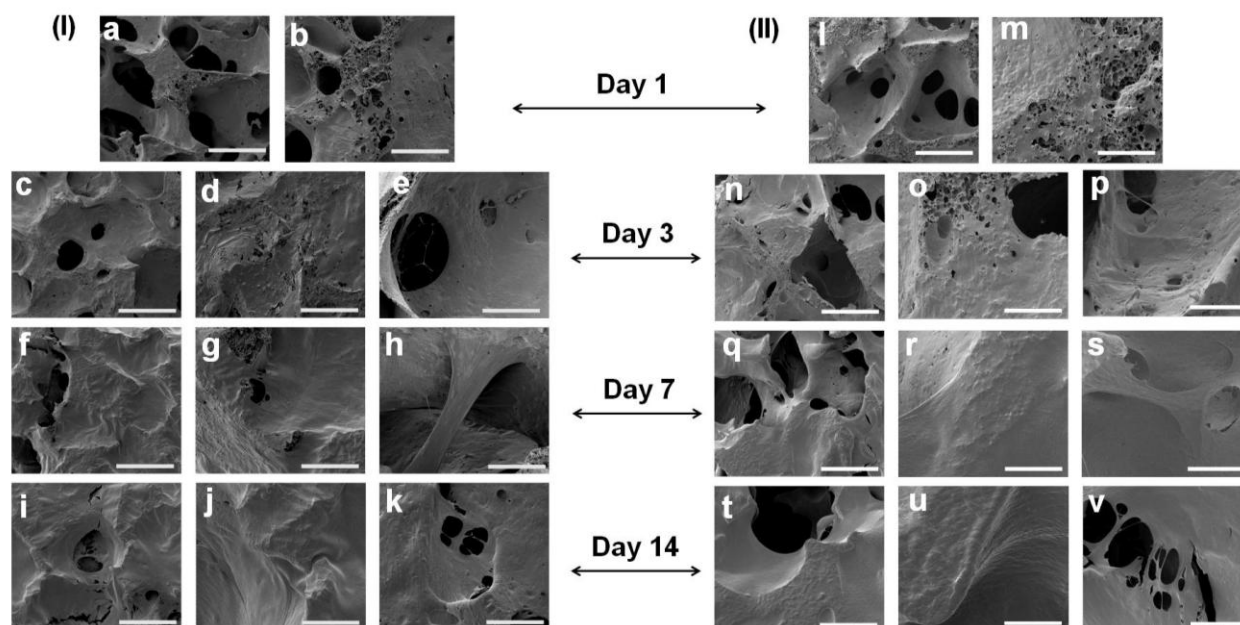


Figure 5. Attachment and migration of the hASCs on (I) S16 and (II) SC16 analyzed by SEM. (a, c, f and i) Overview of cell attachment in S16; (l, n, q and t) overview of cell attachment in SC16 (Scale bar: 500 μ m). (b, d, g and j) Cell attachment in the microporous region of S16; (e, h and k) cell migration in the inside region of S16; (m, o, r and u) cell attachment in the microporous region of SC16; (p, s and v) cell migration in the inside region of SC16 (Scale bar: 100 μ m).

The proliferation of the hASCs on S16 and SC16 were evaluated by DNA content. Figure 4 displayed the DNA content of the hASCs cultured on S16 and SC16 up to 14 days. The DNA content of S16 and SC16 was similar in the tested time points. The DNA contents of both groups showed a dramatic increase from the 1st to the 3rd days. From the 3rd to the 14th day, the DNA contents of S16 and SC16 improved gradually. There were no significant differences in the DNA contents between the 7th and the 14th day.

The adhesion and the growth of the hASCs on S16 and SC16 were studied by SEM, as presented in Figure 5. The cells behavior was similar in S16 and SC16 during the 14 days of cell culture. After seeding for one day, the majority of the SEM images demonstrated that the adhered hASCs began to spread and migrate in the surface of the scaffolds (Figure 5a, l). Interestingly, it was found that the seeded cells preferred to adhere in the microporous regions as compared to the macroporous domain at the beginning (Figure 5b, m). The cells formed small cell sheets in the microporous area and began to spread from the microporous area to the pore wall of the macroporous region. After 3 days the hASCs proliferated well and covered a large amount of the surface area of the scaffolds

(Figure 5c, n). In the microporous region, the cells occupied most of the area, forming large pieces of cell sheet (Figure 5d, o). Furthermore, the cells stretched and formed a slim strip-like structure over the macropores inside the scaffolds (Figure 5e, p). After 7 days, the hASCs formed large pieces of cell sheet and grew on most of the surface area in the scaffolds (Figure 5f, q). In the microporous area, the cells covered nearly all the area in the microporous area of the scaffolds (Figure 5g, r). In the inner region of the scaffolds, the cells formed wide strip-like tissues connecting the void space in the macropores (Figure 5h, s). Two weeks later, the cells grew fully in the surface of the scaffolds (Figure 5i, t), as well as the microporous area (Figure 5j, u). Inside the scaffolds, the cells formed sheet like structure over the macropores (Figure 5k, v).

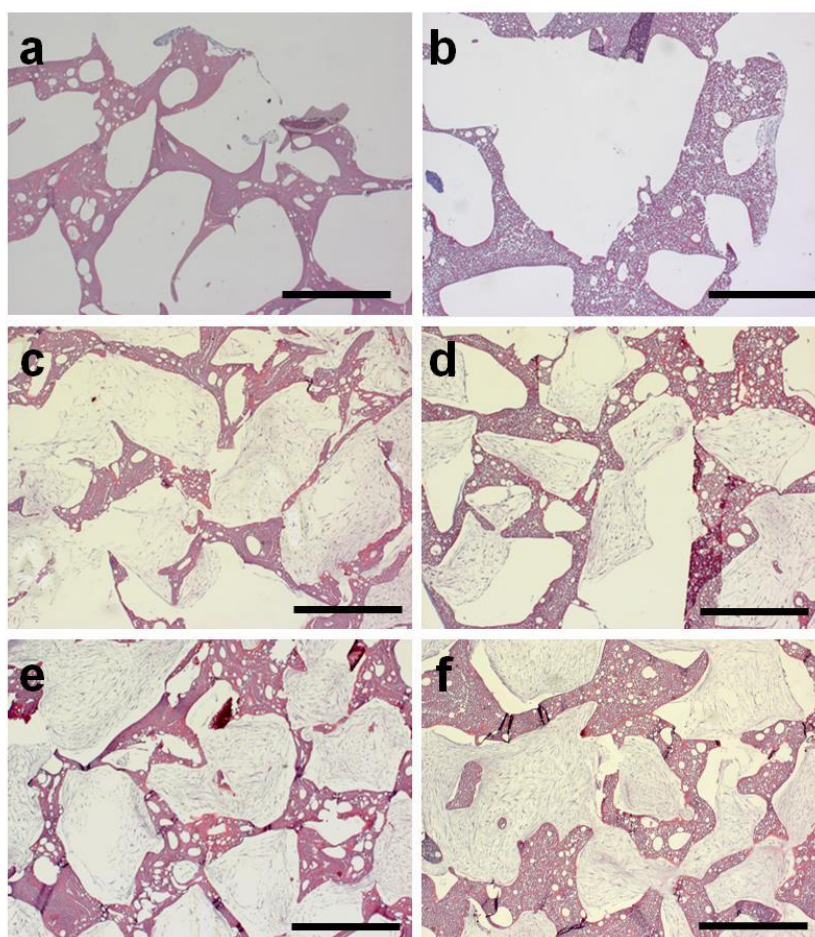


Figure 6. H&E staining of S16 and SC16 cultured with the hASCs. (a, c and e) The hASCs cultured on S16 for 3, 7 and 14 days, respectively; (b, d and f) The hASCs cultured on SC16 for 3, 7 and 14 days, respectively (Scale bar: 500 μ m).

The adhesion, migration, and proliferation of the hASCs in S16 and SC16 were studied by H&E staining. It was showed that there were only a few cells adhered and little

amount of ECM formed in the edge of S16 and SC16, after culturing the hASCs for 3 days (Figure 6a, b). After 7 days, the cells grew into the inner region of the scaffolds and secreted large amount of ECM in the void space of the macropores (Figure 6c, d). At the end of two weeks, the macropores were fully filled with the ECM (Figure 6e, f).

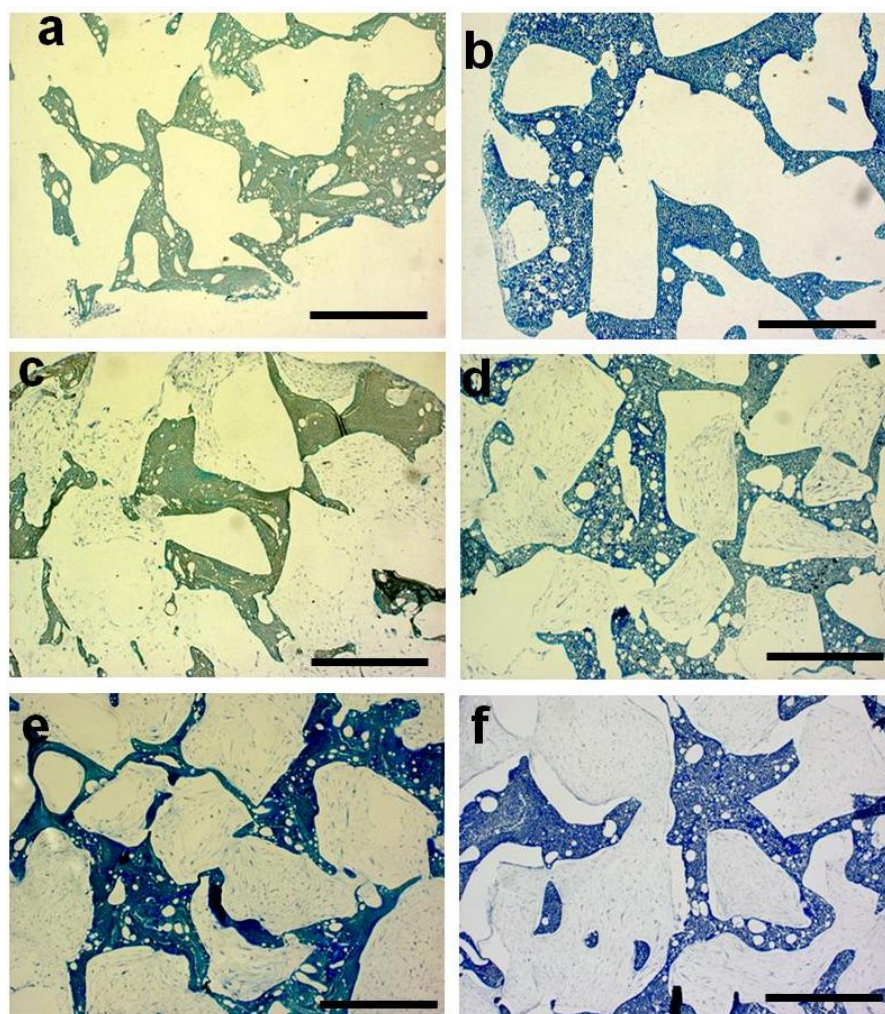


Figure 7. Toluidine blue staining of S16 and SC16 cultured with the hASCs. (a, c and e) The hASCs cultured on S16 for 3, 7 and 14 days, respectively; (b, d and f) The hASCs cultured on SC16 for 3, 7 and 14 days, respectively (Scale bar: 500 μ m).

The Toluidine blue staining (Figure 7) was also used to show the ECM deposition and cell migration in the scaffolds. It demonstrated similar trends as observed in the H&E staining. S16 and SC16 displayed small amount of cells and ECM in the edge of the scaffolds after 3 days culture of the hASCs (Figure 7a, b). More ECM appeared in the macropores of the scaffolds after 1 week (Figure 7c, d). The inner macropores of the

scaffolds were almost occupied by the ECM and cells in 14 days culture of the hASCs (Figure 7e, f).

The mechanical property of the scaffolds after cell culture was screened by the compressive test. Figure 8 showed that the wet state compressive modulus of both S16 and SC16 increased significantly after culturing with the hASCs for two weeks *in vitro*. The modulus improved from 0.41 to 0.69 MPa and from 0.40 to 0.72 MPa for S16 and SC16, respectively. But there were no statistical differences in the modulus of S16 and SC, before and after the cell culture.

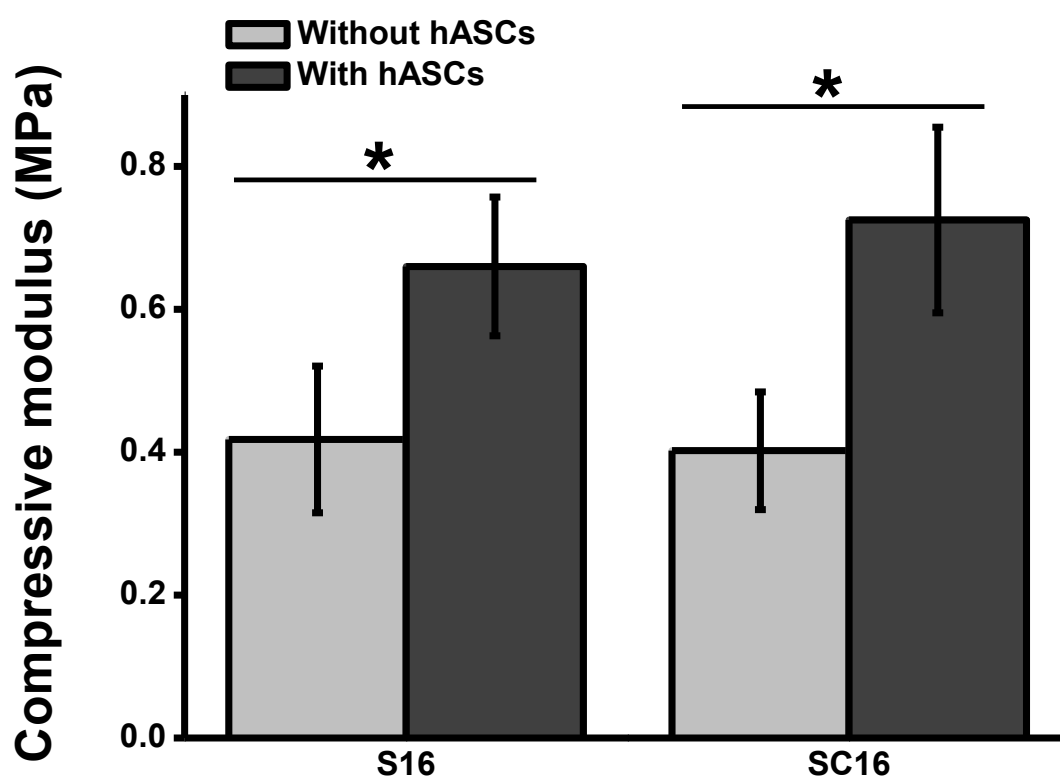


Figure 8. The wet state compressive modulus of S16 and SC16 after culturing with the hASCs for two weeks *in vitro*. * indicated significant differences.

4. Discussion

Due to the superior performance of SF scaffolds in tissue engineering applications, the development of SF scaffolds has attracted great attention [9]. Nazarov *et al.* [21] performed the pioneer study on preparing porous SF scaffolds using salt-leaching method in organic solvent system, as well as the freeze-drying and gas foaming approaches. Kim *et al.* [12] discovered that salt-leaching approach can also be used to generate porous SF scaffolds in aqueous system, which is “greener” compared to the organic solvent system. The previous studies on aqueous derived salt-leached SF scaffolds was limited in using less than 10 wt.% SF aqueous solutions. Recently, our group developed salt-leached SF scaffolds derived from high concentration aqueous SF solutions [25]. Later on, salt-leached Silk-NanoCaP scaffolds were also produced using 16 wt.% aqueous SF solution and *in-situ* synthesis approach [26]. Based on their superior mechanical properties or *in vitro* mineralization performance, S16 and SC16 were chosen for further biological examination in this study.

The SEM and the 3D micro-CT images showed that the produced scaffolds were composed of interconnected pores and macro/microporous structure. This unique macro/microporous structure in silk based scaffolds was only presented in scaffolds derived from high concentration SF aqueous solution [25, 26]. It has been reported that the pore size higher than 100 μm was good for cell migration, and pore size larger than 300 μm was beneficial to the new bone and capillary formation [28]. The pores of large size are important for the exchanges of nutrients and metabolites, and they can also provide enough space for cells migration and proliferate. Previously, Oh SH *et al.* [36] showed that the PCL fibril-like scaffolds of 380-405 μm pore size supported better growth of chondrocytes and osteoblasts, compared to scaffolds of less pore size. Therefore, the macropores in S16 and SC16 are good for the cell migration, proliferation, and *de novo* bone tissue formation. The micropores are helpful to increase the cell seeding efficiency due to their high specific surface area and roughness [29]. Thus, micropores in S16 and SC16 may enhance the cell seeding efficiency. Our previous studies showed that S16 and SC16 were of homogeneous porosity distribution [25, 26]. Considering their structural properties, S16 and SC16 possess great potential as scaffolds for cartilage and bone regeneration, respectively.

The stability of the scaffolds is critical for their application in cell culture and implantation. The salt-leaching procedure promoted the β -sheet formation in S16 and SC16. This conformation endowed good stability to the SF based scaffolds confirmed by long-term immersion in the isotonic saline solution (ISS) [25, 26]. Since the *in vivo* environment is full of varied biological molecules (such as the protease), it is important to know the biostability of these scaffolds by performing enzymatic degradation. In our previous study, SC16 presented higher weight loss compared with S16, during the long-term (one year) degradation in ISS [26]. The fast degradation of SC16 in ISS was due to the dissolution of incorporated CaP. In this study, it was found that the incorporation of CaP significantly decreased the enzymatic degradation ratio of SF (Figure 2). The slow degradation profile of SC16 in protease solution was attributed to the good affinity of the CaP crystals and the SF molecules. There were hydrophilic and negative charged groups in the backbone of the SF molecules, such as carboxyl groups [37, 38]. During the *in-situ* synthesis of CaP in the SF matrix, the introduced calcium ions bounded to the carboxyl groups and subsequently formed molecules complex. These complexes reacted with the phosphate groups and generate the silk/CaP nanocomposite. The addition of sodium chloride promoted the β -sheet transition in SF which further led to the stability of the silk/CaP nanocomposite. Thus, the presence of the CaP crystals in the silk/CaP nanocomposite inhibited the proteolytic effect of protease XIV.

In previous study, the mass loss ratio of the aqueous derived scaffolds decreased when increasing the SF concentration [12]. At day 6, the scaffolds derived from 4, 6 and 8 wt.% SF solution displayed around 80%, 45% and 40% weight loss (0.2 U/mL protease XIV solution, 5 mL/scaffold), respectively [12]. In this study, S16 presented around 40% weight loss at day 7 (1 U/mL protease XIV solution, 5 mL/scaffold) (Figure 2a). It is obvious that S16 was more stable than the aqueous derived SF scaffolds developed previously during the enzymatic degradation. These differences came from the concentration of the aqueous SF solution used for scaffolds preparation. These results confirmed that S16 and SC16 were of higher biostability.

The cytotoxicity of the extracts from S16 and SC16 evaluated previously showed no cytotoxic behavior of L929 cells. In the present study, the cytotoxicity of these scaffolds was examined by direct cell culture on the scaffolds. The viability of the hASCs cultured on the scaffolds was studied by Alamar Blue Assay which is an indicator for cell metabolic activity (viability) or cell number [35]. Our results proved that S16 and SC16

scaffolds were of non-cytotoxicity and promoted the viability of hASCs (Figure 3). The SF based scaffolds also supported the proliferation of the hASCs (Figure 4). The proliferation results were consistent with the viability data. In the first three days, the cells proliferated very fast, and the cell viability also presented a big increase (Figure 3 and 4). In the following days, the cells proliferated gradually and their viability also enhanced progressively. Similar results were reported in previous study on SF based scaffolds derived from low concentration aqueous SF solution. Wang *et al.* [39] found that the viability of human chondrocytes seeded on SF scaffolds dramatically increased during the three weeks *in vitro* cell culture. Another study from Bhardwaj *et al.* [35] also showed that the SF scaffolds supported the viability of rat bone marrow mesenchymal stromal cells (BMSCs) cultured in freeze-dried SF scaffolds for 21 days. In the literature, Kim *et al.* [40] found that the salt-leached SF scaffolds derived from low concentration aqueous SF solution supported the human BMSCs proliferation from one to two weeks. Moreover, lyophilized silk/CaP composite scaffolds were able to support the viability of human bone marrow stromal cells, as reported by Zhang *et al.* [16].

These promising cellular response results were partially attributed to the intrinsic properties of the components in the scaffold, namely SF and CaP. As a large protein, SF composes of 5263 amino acids and most of these amino acids are non-reactive [41]. The SF molecules endow the scaffolds with proper hydrophilicity and a compatible environment for cell attachment and growth. Furthermore, the degradation product of SF is peptide sequences which would not induce severe decrease in the pH as reported in case of poly(lactic acid) based scaffolds [42]. Numerous *in vivo* studies showed that only very mild inflammation response was observed with silk [42]. These properties are attractive for tissue engineering. Based on the promising properties of proteins based biomaterials, synthetic polyester based scaffolds were surface modified with proteins to improve the surface hydrophilicity and compatibility. Liu *et al.* [43] introduced gelatin molecules in the surface of nano-fibrous poly(L-lactic acid) scaffolds, and found that the initial cell adhesion and proliferation were significantly improved. Ma *et al.* [44] performed the grafting of collagen onto the poly(L-lactic acid) scaffold surface, and the chondrocytes spreading and growth were dramatically improve. On the other hand, the nano-sized CaP particles in SC16 were low crystalline hydroxyapatite as reported in our previous study [26]. The chemical composition of these CaP particles is similar to the one of the major inorganic component in bone. The cytocompatibility of CaP based scaffolds has been

proved by previous studies. Oliveira *et al.* [45] developed a porous hydroxyapatite scaffolds which were able to support viability and proliferation of rat bone marrow stromal cells.

Besides the intrinsic properties of the scaffolding materials, the microstructure of the scaffolds is also crucial for cell attachment, migration, and proliferation. In this study, the cell adhesion and migration profiles revealed that both the macropores and the micropores in S16 and SC16 played important roles. Murphy *et al.* [29] showed that the micropores favored the initial cell adhesion. Additionally, the advantageous of rough surface for cell attachment has been addressed by Abbah *et al.* [31]. Our results were in good agreement with the previous studies. In the first stage, the hASCs mainly attached and spread in the rough micropores at the beginning, rather than in the smooth surface of the macropores. After 3 days proliferation, the hASCs migrated to the macropores. As stated previously by Mandal *et al.* [30], the macropores were advantageous for cell migration and proliferation. The macropores not only allow the cells have good access to the nutrients, but also can reduce the cell aggregation along the edge of the scaffolds [41]. In the second stage, the hASCs proliferated quickly and reached the inner region of the SF based scaffolds, which confirmed the merit of the macroporous structure. Even though S16 presented higher porosity compared with SC16 [26], cell proliferation was not affected by this issue. Previously, Bhumiratana *et al.* [46] also reported similar finding that the cell number was not significantly affected by introduction HA in SF scaffolds. The good access of culture medium and easy transport of metabolites through the macropores may contribute to these results.

The surface properties of the composite scaffolds are also important for cellular behavior. Kim SS *et al.* [32] addressed that the exposure of the hydroxyapatite particles in poly(lactide-co-glycolide)/hydroxyapatite composite scaffolds improved the cell growth and mineralization deposition. Cellular performance on SF and CaP composite scaffolds prepared by blending were also studied. Bhumiratana *et al.* [46] found there were no significant differences in cell proliferation between silk and silk/hydroxyapatite scaffolds derived from organic system, after 5 and 10 weeks *in vitro* culture. Zhang *et al.* [16] also reported that the cell viability was similar in the freeze-dried silk and silk/CaP scaffolds. The different cellular behavior between the SF based and synthetic polymer based composite scaffolds may come from their different hydrophilicity and chemical composition. The amino or carboxyl groups on the surface of the SF based scaffolds

provided more attractive sites for cells growth compared to the hydrophobic and inert surface from the polyesters. Since both SF and CaP presented superior biocompatibility, there were no obvious differences in cellular proliferation between SF or SF/CaP scaffolds. In our study, the nano CaP particles were formed inside the SF matrix in the form of nano-size clusters. Therefore, there were no obvious differences in the scaffolds surface tomography between S16 and SC16. The tomography similarity induced the similar cell proliferation profiles in S16 and SC16. This result was in good agreement with a previous study from Oliveira *et al.* [17], on the development of silk/hydroxyapatite bioactive scaffolds using a one-step methodology. In that study, it was not possible to distinguish the ceramic phase from the polymeric phase because the hydroxyapatite particles synthesized by *in-situ* approach were in nano-size.

The histological analysis further validated that the porosity and pore size of the macro/microporous scaffolds were advantageous for cell migration and ECM formation (Figure 6 and Figure 7). Since the macropores were able to provide sufficient nutrient and metabolic products exchanges, the hASCs proliferated very fast and migrated along the macropores into the inner region of the scaffolds after 7 days and then filled the void space of the macropores by the secreted ECM. These results consolidated the SEM observation (Figure 5i and t), and showed the good cytocompatibility of these silk-based scaffolds.

After culture for 14 days, the mechanical properties of the SF based scaffolds increased significantly (Figure 8). These results demonstrated that the scaffolds were stable and maintained the integrity structure during the short term *in vitro* cell culture. It was also found that it supported the cell proliferation and extracellular cellular matrix deposition. The improvement in the compressive modulus of the scaffolds came from the ECM formed inside the scaffolds. As observed by SEM, the cells formed large pieces of cell sheet in the scaffolds, which played a role similar to the fibre reinforcement in the polymer composites (Figure 5). The histological analysis also demonstrated that great amount of ECM formed inside the inner pores of the scaffolds (Figure 6 and 7). These results were in good agreement to the previous study [40]. It was reported that wet state compressive modulus of the salt-leached SF scaffolds (derived from low concentration aqueous SF solution) increased from around 75 kPa to less than 200 kPa, after culturing with human MSCs for 12 weeks [40]. Comparing our observation with this study, the compressive modulus of S16 and SC16 was much higher, before and after cell culture

(Figure 8). Tailoring the mechanical properties of the scaffolds for specific tissue regeneration, such as for bone, cartilage, and osteochondral, is still in great demand [15]. The proposed S16 and SC16 provide a promising alternative for cartilage and bone regeneration, respectively. In the future, further biomechanical analysis is required to deeply evaluate these scaffolds cultured with cells, e.g. the dynamic mechanical analysis (DMA) and equilibrium modulus test [46].

In this study, ECM-analogue materials SF and CaP were used to develop the SF based scaffolds, aiming to mimic the ECM environment for cell growth and tissue formation. These scaffolds were under well control, from the molecular level SF assembly to the macroscopic level porous structure formation. S16 constituted by the structural protein SF possesses great potential for cartilage and meniscus regeneration. Mimicking the component and structure of natural bone tissue, SC16 was suitable for bone tissue engineering. The evaluation of the potential of S16 for cartilage and SC16 for bone regeneration is presently being conducted through *in vitro* cell culture and *in vivo* studies with specific animal models. By combining the advantages of S16 and SC16, a bilayered Silk/Silk-NanoCaP construct is also being proposed as a final integrated solution for osteochondral regeneration.

5. Conclusions

In this study, macro/microporous SF based scaffolds with superior performance were developed. The SF based scaffolds derived from high concentrated aqueous SF solution displayed an improved biostability as compared to previous reported SF scaffolds derived from low concentration aqueous SF solutions. The incorporation of CaP in the SF matrix further improved the stability of the scaffolds during enzymatic degradation. Both S16 and SC16 were non-cytotoxic, and promoted the attachment, viability, proliferation, and migration of the hASCs. The microporous structure favored the adhesion of the hASCs, and the macroporous structure promoted the proliferation and migration of the cells. The culture of the hASCs upgraded the biomechanical properties of these SF based scaffolds. These SF based scaffolds or their combination could be promising candidates for cartilage and bone regeneration, and in an osteochondral tissue engineering strategy.

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Chapter VI

De Novo Bone Formation on Macro/Microporous Silk
and Silk/Nano-Sized Calcium Phosphate Scaffolds

Chapter VI

De Novo Bone Formation on Macro/Microporous Silk and Silk/Nano-Sized Calcium Phosphate Scaffolds

Abstract

Macro/microporous silk/nano-sized calcium phosphate scaffolds (SC16) with bioactive and superior physicochemical properties have been recently developed. In this study, we aim at evaluating the new bone formation ability in rat femur of SC16 *in vivo*, using silk fibroin scaffolds (S16) as control. The CaP distribution profile in the scaffolds was characterized by Micro-Computed Tomography. The CaP phase was distributed homogeneously in SC16. Mineralization was only observed in SC16, and both scaffolds gradually degraded with time. By staining the explants, new bone growth was observed directly on SC16 surface and with higher density than S16. These results demonstrated that SC16 are osteoconductive and can be good candidates for bone tissue engineering as promoted superior *de novo* bone formation.

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1. Introduction

Bone defects derived from trauma or diseases often require grafts to regenerate the function of the impaired bone tissues [1,2]. Currently, autografts and allografts are the dominant treatments for bone defects [1,2]. However, both of them have limitations, such as lack of sufficient supplies and risks of disease transmission [1,2]. To counter such drawbacks, tissue engineered bone can be a promising alternative strategy for bone regeneration [3,4]. Porous biodegradable scaffolds play a crucial role towards this goal [5]. Several ceramic-based or polymeric biomaterials have been investigated as scaffold materials, such as hydroxyapatite, tricalcium phosphate [6-9], collagen, chitosan and silk fibroin [10-13]. However, it has been recognized that the ideal scaffolds for bone regeneration are those with osteoconductive or osteoinductive properties, as well as with good mechanical performance [7,14,15]. Calcium phosphate based biomaterials are osteoconductive, yet their mechanical properties are compromised by their fragile nature [16]. With this in mind, composite based scaffolds composed of a calcium phosphate (CaP) phase and a polymeric phase have been studied intensively [17-20].

Silk fibroin (SF) derived from *Bombyx Mori* or other species have been used as versatile degradable biomaterials for years [21-26]. Several methods have been used to prepare silk scaffolds with controlled structure and mechanical properties [11,12,27-32]. The compatibility of silk based biomaterials with different kinds of cells have also been studied [15,23-26,33-35]. Scaffolds composed of SF and CaP have been explored in a few studies [16,18,19,36], but the homogeneous distribution of the inorganic phase within the SF matrix remains challenging [19]. In order to overcome this problem, we have developed a novel strategy which uses an *in-situ* synthesis method to produce SF/nano-sized CaP composites, and subsequently generates the macro/micro porous scaffolds by salt-leaching/freeze-drying [37]. The final scaffolds have a homogeneous distribution of sub-micron CaP particles. These macro/micro porous silk/nano-sized calcium phosphate (Silk-NanoCaP) scaffolds have bioactivity and good mechanical properties. Systematic *in vitro* mineralization and *in-vitro* long-term stability of the scaffolds was assessed in this study. In parallel, the *in vivo* new bone formation ability of these scaffolds was evaluated by implantation in rat femur defects.

2. Materials and Methods

2.1. Materials and reagents

Bombyx Mori cocoons were purchased from the Portuguese Association of Parents and Friends of Mentally Disabled Citizens (APPACDM, Portugal). Commercial sodium chloride particles were obtained from the local market (Portugal). The silicone tubing was purchased from Deltalab (Barcelona, Spain). All the other materials or reagents were from Sigma-Aldrich (MO, USA) unless addressed otherwise.

2.2. Scaffold preparation

SF extraction and scaffold preparations followed our previously reported methods [12,37]. Briefly, 0.02 mol/L boiling sodium carbonate solution was used to removed sericin from the cocoons for 1 hour. And then, the purified SF was dissolved in a 9.3 mol/L lithium bromide solution for 1 hour at 70°C, followed by dialyzing in distilled water for 48 hours using a benzoylated dialysis tubing (MWCO: 2000). The concentrated SF solution was obtained by dialyzing the SF solution against a 20 wt.% poly(ethylene glycol) solution. The final SF content in the solution was determined by drying the SF solution at 70°C overnight. The SF solution was kept at temperature below 6°C before use. Normally the SF solution was stored for less than 24 hours before use.

Regarding the Silk-NanoCaP scaffold preparation, 6 mol/L calcium chloride and 3.6 mol/L ammonia dibasic phosphate solutions were sequentially added into the 16 wt.% SF solution. The final calcium to phosphate atomic ratio in the silk solution was fixed at 1.67, equaling that in $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ (hydroxyapatite) [37]. The mixture of the calcium chloride and ammonia dibasic phosphate solutions will form the CaP particles in an aqueous environment with an appropriate pH value. The CaP particles will transform into hydroxyapatite by aging the suspension under basic conditions. The viscous silk solution (16 wt.%) prevents the aggregation of the formed CaP particles and thus a homogeneous dispersion of the CaP particles was achieved. The CaP content was 16 wt.% of the SF mass, with the assumption that all the introduced calcium and phosphate ions reacted completely and transformed into hydroxyapatite. The pH value of the obtained milky suspension was adjusted to 8.5. The mixture was gently stirred for 30 minutes and subsequently aged for 24 hours. Then, 2 g sodium chloride particles (500-1000 μm) were

added into 1 mL Silk-NanoCaP suspension in a silicone tube and the tube was dried in air for two days. The final Silk-NanoCaP scaffolds were prepared by leaching out the sodium chloride particles in distilled water followed by lyophilization of the scaffolds. The salt-leached SF scaffolds were prepared by addition of the sodium chloride particles into the 16 wt.% SF solution and the remaining steps were the same as mentioned above. The salt-leached Silk-NanoCaP scaffolds and SF scaffolds were abbreviated as SC16 and S16. We have chosen SC16 and S16 for our current studies in bone regeneration on the basis of their previously tested mechanical properties and *in vitro* mineralization studies [37]. CaP particles were prepared as the procedure mentioned above, but the silk solution was replaced by distilled water. The prepared particles were washed after aging, followed by freeze-drying.

2.3. Physicochemical characterization of the scaffolds

2.3.1. Scanning electron microscopy (SEM)

The scaffolds were coated with a layer of Au/Pd SC502-314B in an evaporator coater (E 6700, Quorum/Polaron, East Grinstead, UK) before morphology observation by Scanning Electron Microscopy (NanoSEM-FEI Nova 200, OR, USA). The distribution of CaP particles into the SF matrix was examined in a backscattered SEM model without any coating on the powder from the SC16. Three specimens were tested for each group of scaffolds.

2.3.2. X-ray diffraction (XRD)

The crystallinity states of silk fibroin and CaP in the scaffolds were determined in an X-ray diffractometer (Philips PW 1710; Philips, Amsterdam, Netherlands) with Cu-K α radiation ($\lambda=0.154056$ nm). The data was recorded from 0-60° 2 θ values, with step width and counting time set at 0.02° and 2 second/step, respectively. Three specimens were analyzed for each group.

2.3.3. Fourier transform infrared spectroscopy (FTIR)

The SF conformation and scaffold composition information were evaluated by Attenuated total reflectance (ATR) model in a FTIR (IRPrestige-21; Shimadzu, Kyoto, Japan), equipped with a Germanium crystal. Each specimen was scanned 48 times with a resolution of 4 cm⁻¹. Triplicate samples were used for each group of scaffolds. CaP control was also analyzed.

2.3.4. Compressive test

The compressive modulus of the scaffolds were recorded in a Universal Testing Machine (Instron 4505; Instron, Norwood, MA, USA), with a compressive speed of 2 mm/minute. The modulus was determined from the slope of the linear domain in the stress-strain curve. At least five samples were examined for S16 or SC16.

2.3.5. Micro-computed tomography (Micro-CT) analysis

The porosity, CaP distribution profile in the scaffolds, and total CaP content (vol.%) in the scaffolds were analyzed by Micro-CT (1072 scanner; SkyScan, Kontich, Belgium) [37,38]. For the porosity determination, S16 and SC16 were scanned at 40 keV/248 μA and 61 keV and 163 μA, respectively. For the CaP distribution and CaP content, the scaffolds were both scanned at 61 keV and 163 μA. The data sets were processed into bitmap images in NRecon v1.4.3 software (SkyScan, Netherland) in a cone-beam model. For the porosity calculation, the images were transferred into binary images using a grey value ranged from 40-255. Regarding the CaP content analysis, grey values comprised between 120 and 255 were used. The CaP content and distribution in the scaffolds were analyzed vertically. At least three specimens were evaluated for each formulation.

2.3.6. *In vitro* mineralization

The mineralization of the scaffolds was performed by immersion S16 and SC16 in a simulated body fluid (SBF) solution at 37°C for 1, 3, 7 and 14 days [9,37,38]. At the end of each time point, the samples were removed and the surfaces were observed by SEM as mentioned above. The Energy Dispersive X-Ray Detector (EDX, NanoSEM-FEI Nova

200, OR, USA) was used to analyze the surface elements of the scaffolds after *in vitro* mineralization. An area of 5 x 5 µm was selected to scan each specimen. Three samples were used for each group at each time point.

2.3.7. Long-term hydration and degradation evaluations

The long-term hydration degree and degradation profile were studied by immersion the scaffolds in 0.154 mol/L sodium chloride Isotonic Saline Solution (ISS, pH 7.4) at 37°C a water bath (GFL 1086; GFL, Burgwedel, Germany), at dynamic condition (60 rpm) [12,37]. The wet weight of each specimen was measured immediately after removing the samples at the end of 1, 3, 6, 9 and 12 months. The dry weight was recorded after drying the samples at 60°C for 24 hours. The hydration degree and the weight loss ratio were obtained by the following equations 1 and 2 (Eq.1 and Eq.2).

$$\text{Hydration degree} = \left(\frac{m_{w,t} - m_i}{m_i} \right) \times 100\% \quad (1)$$

$$\text{Weight loss ratio} = \left(\frac{m_i - m_{d,t}}{m_i} \right) \times 100\% \quad (2)$$

In Eq.1, m_i is the initial weight of the specimen before hydration, and $m_{w,t}$ is the wet weight of the specimens at time t after being removed from the ISS in the end of each time point, and $m_{d,t}$ is the dry weight of the specimen been degraded for a certain period of time and after drying. Five samples were measured for each group at each time point.

2.4. *In vivo* implantation

2.4.1. Implantation Procedure

Young male Wistar rats (n=6 per group) with an a body weight of 125 to 150 g were purchased from Charles River (Senneville, Quebec, Canada), housed in light- and temperature-controlled rooms, and fed a standard diet. Bone defects were drilled bilaterally in each distal femur, proximal to the epiphyseal plate, of every rat [39]. The

defects were made using a low speed drill (2.3 mm in diameter) with copious saline irrigation. The defects were made until it reached the bone marrow domain. Previously prepared scaffolds were then press fit into the defects. Before the implantation, the wet scaffolds were cut into 3 mm in height and punched into 4 mm in diameter, followed by lyophilization and subsequently sterilized by ethylene oxide. The diameter of the lyophilized scaffold was around 3.3 mm. Thus the diameters of the defect and the scaffolds were very close. The maintenance and use of animals were in accordance to the Ethics Committee of University of Minho.

2.4.2. Histological Processing

Animals were sacrificed after 3 weeks and the femurs were removed. The femurs (n=5 per group) were fixed in neutral formalin, decalcified in a 1:1 mixture of 45% formic acid and 20% sodium citrate, dehydrated and embedded in paraffin. Five-micrometer-thick serial sections perpendicular to the long axis of the implant were cut with a Spencer 820 microtome (Spencer 820, American Optical Company, NY, USA). Sections were then stained with Masson's Trichrome stain to selectively stain muscle, collagen fibers, fibrin, and erythrocytes respectively. A green color is attributed to collagen in the newly formed bone.

2.4.3. Histomorphometry

Bone histomorphometry was evaluated via IMAGE J (National Institutes of Health, Bethesda, MD). The images of the Masson's Trichrome slides (area for each slide: 0.45 mm*0.35 mm) of each explants were first converted to gray-value images. And then proper threshold values were selected for each image in order to best match the new bone area in original images [13]. The new bone area in each slide image was calculated by the software. Slides from 4 explants were used for each group, and at least 10 slides were evaluated per explant.

2.5. Statistical analysis

The data were presented as an average and its standard deviation. The compressive modulus, porosity, CaP content, and new bone area were assessed by a One-way Analysis of Variance (ANOVA). The average values of each group were compared by Tukey's test, and $p < 0.05$ was considered statistical significance.

3. Results and Discussion

3.1. Conformation and chemical composition

It is generally recognized that there are mainly three conformations in native and regenerated silk fibroin, namely random coil (amorphous), silk-I (also called type II β turn) and silk-II (antiparallel β -sheet) [40]. The amorphous state usually exists in diluted regenerated aqueous silk fibroin solution, silk-I is generated from amorphous silk solution by a well-controlled water annealing procedure, and silk-II can also be induced from the amorphous silk solution by many stimuli (such as temperature, pH value, organic solvent, or saline) [26,41]. Among these three conformations, silk-II is the most stable state and has been taken advantage of in formulating silk-based matrices for tissue engineering and regenerative medicine [21,22]. Regarding the scaffold preparation, Kim *et al.* found that the addition of sodium chloride particles in aqueous silk solution could induce β -sheet formation, and this finding led to the formation of salt-leached silk scaffold derived from aqueous solution [27]. Recently, we have produced silk fibroin scaffolds from high concentration aqueous silk fibroin solution [12]. Salt-leached Silk-NanoCaP scaffolds were also produced using this process [37].

The conformation of silk fibroin has been studied by different technologies, such as X-ray diffraction (XRD), ^{13}C cross polarization-magic angle spinning nuclear magnetic resonance and FTIR [40,42]. The most typical XRD peak of β -sheet structure in silk fibroin is at 20.8° [40]. Hydroxyapatite presents characteristic XRD peaks at around 32° and 39° [7]. In figure 1, it was found that both S16 and SC16 had main peaks at $\sim 20.5^\circ$, confirming the Silk-II structure in both scaffolds. Furthermore, the XRD patterns also had peaks at $\sim 32^\circ$ and $\sim 39^\circ$, which are feature peaks of hydroxyapatite. The low intensity of these two peaks may come from the low crystallinity of the formed CaP and also the low content of CaP in the silk matrix. The lower crystallinity of the CaP phase is related with the preparation procedure [7]. ATR-FTIR has been used as a reliable and facile way to

reflect any subtle conformation change of silk fibroin, normally in the absorbance areas of amide-I and amide-II [43]. Figure 2 contains the ATR-FTIR spectra of S16 and SC16, and spectra of CaP control was showed in Supplementary Figure 1 (Figure S1). Both scaffolds have broad absorbances between 1480 cm^{-1} - 1590 cm^{-1} and 1590 cm^{-1} - 1700 cm^{-1} , with peaks located at 1520 cm^{-1} and 1627 cm^{-1} . These two absorbance ranges are assigned to amide-I and amide-II, respectively [43]. Both peaks (1520 cm^{-1} and 1627 cm^{-1}) indicated the β -sheet conformation in the silk matrix [27,43]. Similar to the CaP control spectra (Figure S1), SC16 had a distinct absorbance between 1000 cm^{-1} - 1100 cm^{-1} (peak position was around 1030 cm^{-1}), which was the vibration from a PO_4^{3-} group in the CaP [9,37]. The XRD patterns and the ATR-FTIR spectra proved that the CaP was incorporated into the scaffolds, and the scaffolds were composed of silk fibroin in β -sheet conformation and CaP of low crystallinity. The β -sheet conformation of silk matrix is crucial for the mechanical properties and structural stability of the produced scaffolds.

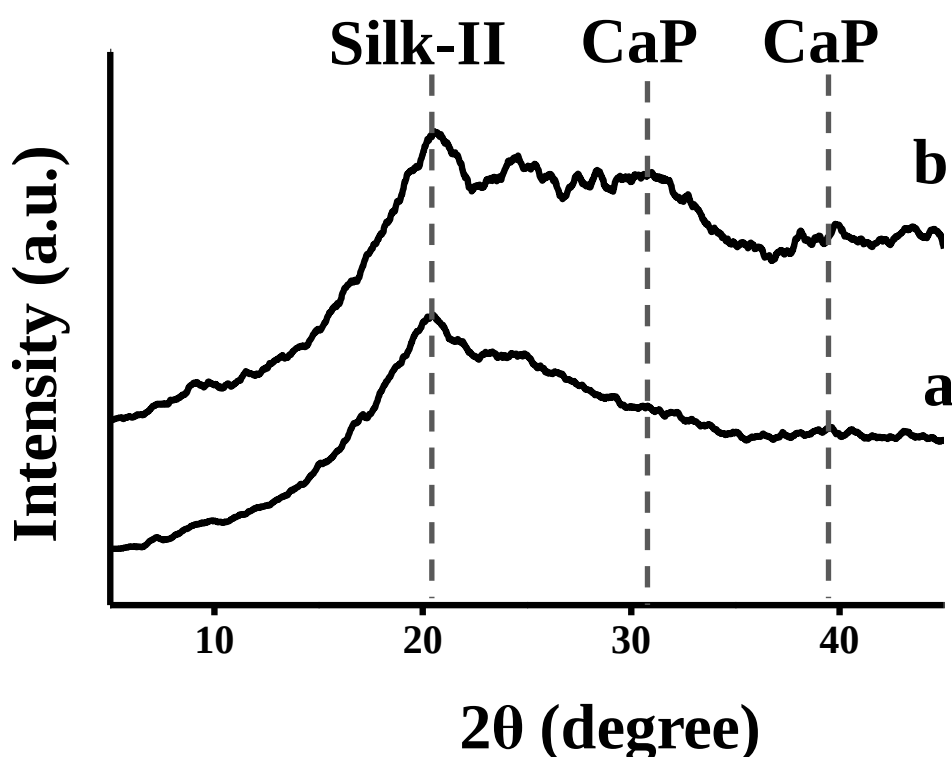


Figure 1. XRD patterns of the salt-leached silk fibroin based scaffolds. (a) S16 and (b) SC16.

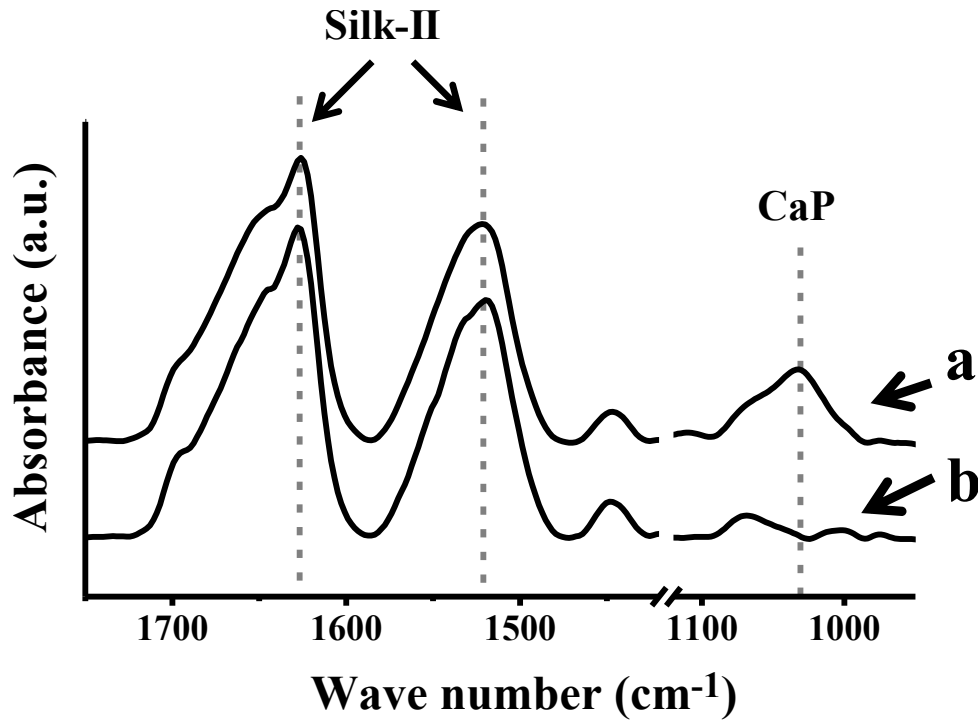


Figure 2. ATR-FTIR spectra of the salt-leached silk fibroin based scaffolds. (a) S16 and (b) SC16.

3.2. Structure, CaP distribution, and mechanical properties

For bone tissue engineering, the pore size and porosity of the scaffolds are important [1,5,44]. The ideal scaffold should have adequate porosity and proper pore size in order to facilitate the new bone in-growth, vessel invasion, cell migration, and nutrients/metabolic products transportation [2]. Many methods have been used to generate porous scaffolds for bone tissue engineering, including rapid prototyping [1], salt leaching [27], and supercritical fluid evaporation. Based on our previous work [12], salt leaching was selected for this study and produced porous structures in both S16 and SC16 (Figure 3a, b). From the SEM images, both scaffolds presented a macro/microporous structure (Figure 3d, e), with the macropore size ranging between 500 and 1000 μm . Based on the micro-CT analysis data shown in Table 1, the porosities of S16 and SC16 were 79.8% and 63.6%, respectively. SC16 displayed an inferior mean pore size and a higher trabecular thickness as compared with S16. The structure information indicated that CaP does affects the structure of the scaffolds, but the porosity of the SC16 is still higher than 63% and thus still adequate for bone tissue regeneration [5].

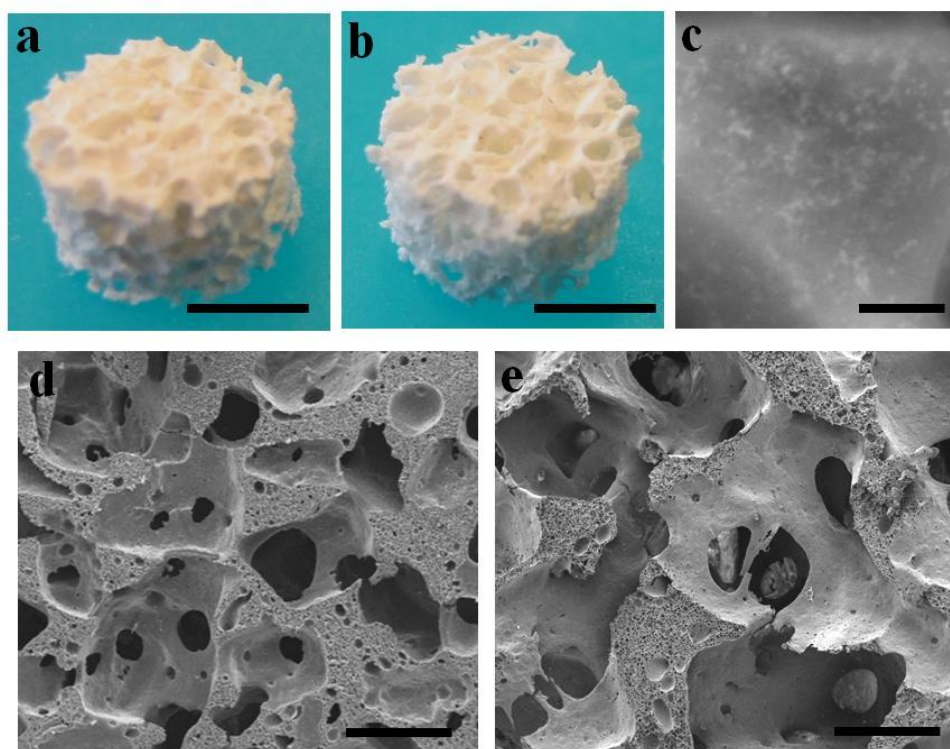


Figure 3. Morphologies of the salt-leached silk fibroin based scaffolds and the nano-CaP particle distribution in the scaffold. (a, b) Macroscopic photos of S16 and SC16, respectively (Scale bar: 3 mm); (c) backscattered SEM image of SC16, the white spots are nano-CaP particles and the gray domain is silk matrix (scale bar: 3 μm); (d, e) SEM images of S16 and SC16, respectively (scale bar: 500 μm).

The homogeneous distribution of CaP particles in the scaffolds is challenging to produce [19]. By physically blending nano-sized CaP particles in the scaffolds, it is difficult to maintain their original size. The particles usually aggregate and subsequently lead to inhomogeneous dispersions. Herein, an *in situ* synthesis method was used to introduce CaP into the silk scaffolds. The *in situ* formed nano-sized CaP particle was homogeneously distributed in the silk matrix (Figure 3c) at a microscopic level. And the size of the CaP particles was less than 200 nm. From our previous study, it was found that around 87% of the introduced CaP was maintained in the scaffolds after leaching out the sodium chloride particles. Based on the micro-CT analysis, the CaP occupied ~9.2 vol.% in the scaffolds. As seen in Figure 4, the CaP distribution was quite even along the SC16. By the procedure used herein for micro-CT analysis, there was zero percentage of CaP detected in S16 (data not shown). Considering the resolution of micro-CT, the volume percentage obtained cannot completely reflect the CaP volume percentage, but it still can give us an idea of the CaP distribution in the scaffolds at a macroscopic level. By

comparing with previous work, the SC16 prepared in this study presented better CaP distribution.

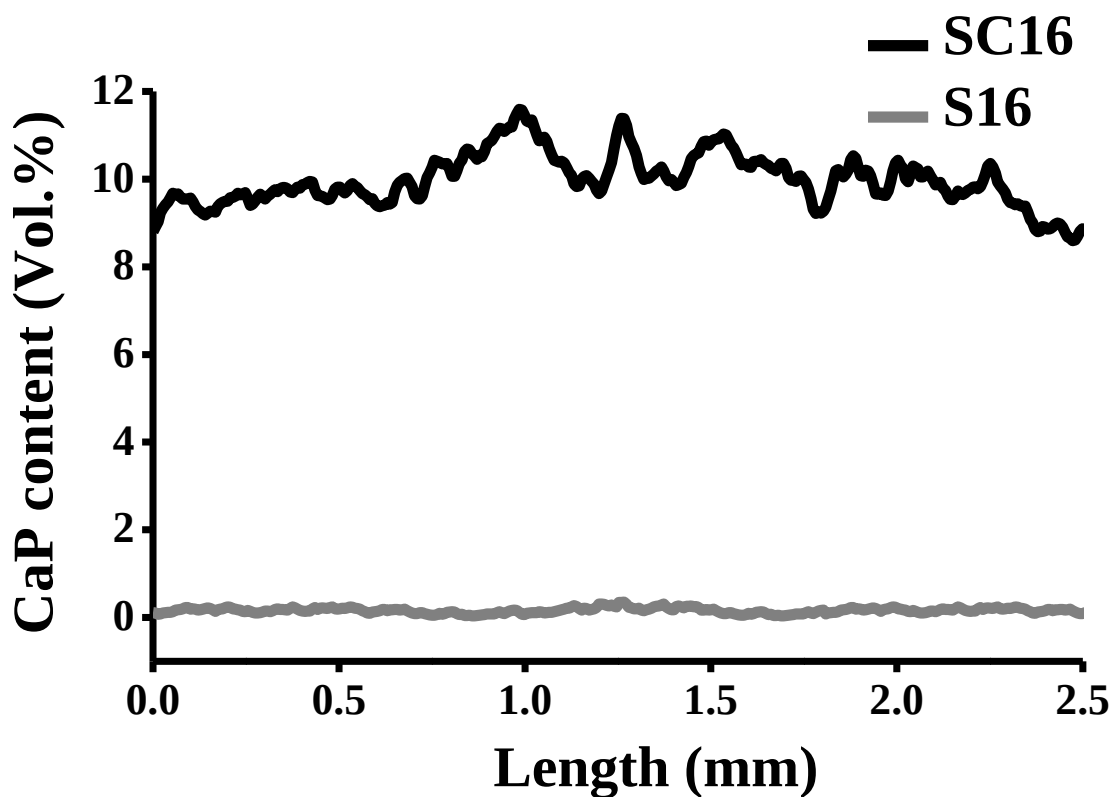


Figure 4. Calcium phosphate distribution in the SC16 as determined by micro-CT.

The mechanical properties of the scaffolds are quite important since they certainly will be under load once implanted [1]. Attention has been paid to strengthen the silk based scaffolds for bone tissue engineering, such as by means of chemical crosslinking, fibre filling, or particle reinforcement [20,29,31]. S16 and SC16 present similar compressive modulus as seen in Table (1), which indicated that the introduction of CaP did not compromise the scaffold strength. In the previous study, we tested the dynamic mechanical properties of the scaffolds in a wet state, and the SC16 had a slightly higher storage modulus as compared to that for S16. The mechanical properties of these scaffold produced in this study were superior to the scaffolds in previous studies using freeze drying or salt-leaching approaches [18,19,27]. The good mechanical properties of S16 and SC16 came from the β -sheet conformation of the silk matrix in the scaffolds and also from the high concentration aqueous silk solution.

Table 1. Structural and mechanical properties of the silk and Silk-NanoCaP scaffolds

Groups	Mean porosity (%) ^a	Mean pore size (μm) ^a	Mean trabecular thickness (μm) ^a	Compressive modulus (MPa) ^b	CaP content (vol.%) ^c
S16^d	79.8±0.3	285.1±52.3	69.8±1.2	15.1±1.7	0
SC16^e	63.6±2.4	251.0±15.0	90.1±1.4	19.0±5.8	9.2±0.4

^athe mean porosity, mean pore size, and mean trabecular thickness were obtained from the Micro-CT analysis.

^bthe compressive test was performed in a dry state.

^cthe CaP content was determined by Micro-CT analysis.

^dSalt leached silk fibroin scaffolds derived from 16 wt.% aqueous silk fibroin solution.

^eSalt leached silk fibroin/nano-CaP scaffolds derived from 16 wt.% aqueous silk fibroin solution and contains theoretical 16 wt.% CaP (CaP:silk, in wt.) in the silk matrix.

3.3. *In vitro* mineralization and long-term stability

The bioactivity test in SBF solution is a commonly used method to predict the osteoconductive properties of materials [9,38]. There are numerous successful examples using this method to evaluate the *in vivo* bone-bonding ability of materials, such as hydroxyapatite and bioactive glass [46]. Using this method we found that SC16 rapidly induce apatite crystal formation on their surface on the first day (Figure 5a). This finding is confirmed by the EDX spectra (Figure 5e). The number of crystal clusters increased at day 3 (Figure 5b). At day 7, the crystals almost covered the entire surface of SC16 (Figure 5c). After 2 weeks, the surface was completely covered by the crystal (Figure 5d), with improved elemental signal of calcium and phosphate (Figure 5f). No apatite crystal formation was observed on the S16 surface after two weeks (Figure 5g-h). These results confirmed that SC16 possessed good bioactivity and induce apatite crystal formation in a short time. This finding also validated that the *in-situ* synthesis of nano-sized CaP particles in silk matrix can endow the final porous scaffolds with bioactivity.

The long-term stability of the scaffolds attracts much concern since the implanted scaffolds need to stay *in vivo* from weeks to months, or even longer. Figure 6a shows that both scaffolds maintained their hydration degree from 1 month up to 12 months. Regarding weight loss, S16 and SC16 both lost their weight in a slow manner during the test period. After one year, SC16 lost around 15% of their original mass, and SC16 had ~10% loss (Figure 6b). Thus, the silk based scaffolds were quite stable in a hydrated

condition and were stable enough for long-term implantation. The stability of the silk scaffolds can be attributed to the β -sheet conformation in silk fibroin.

In our previous study, it was confirmed that the silk-based scaffolds were non-cytotoxic by culturing the cells in the scaffolds' extractions [37]. Based on their bioactivity and long-term stability, SC16 merit evaluation of their behavior during *in vivo* bone regeneration.

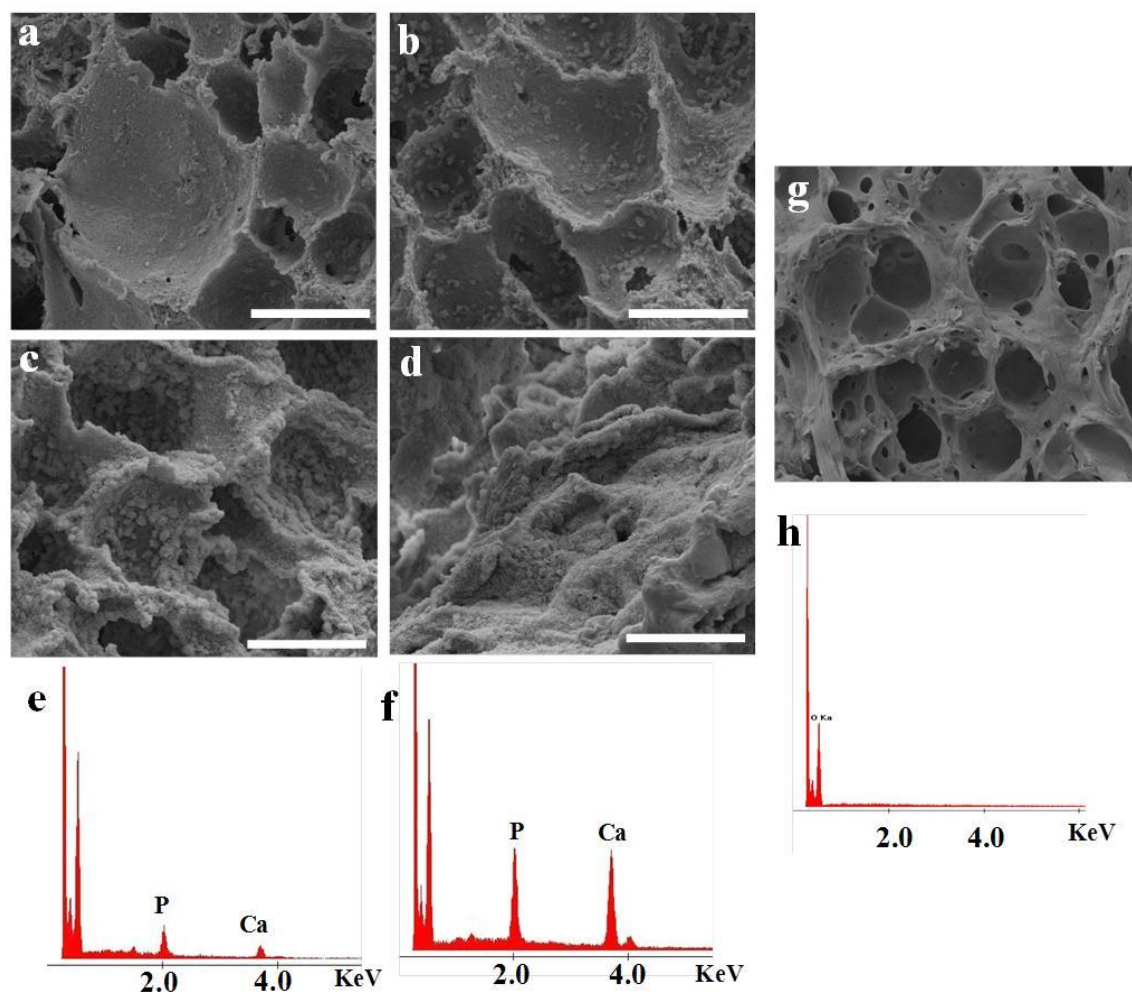


Figure 5. Mineralization of SC16 and S16. (a-d) SEM images of SC16 after immersion in SBF solution for 1, 3, 7 and 14 days at 37°C, respectively (scale bar: 10 μ m); (e, f) EDX spectra of (a, d), respectively (Scan area: 10 μ m x 10 μ m); (g, h) SEM image and EDX spectra of S16 after immersion in SBF solution for 14 days at 37°C (Scale bar: 10 μ m).

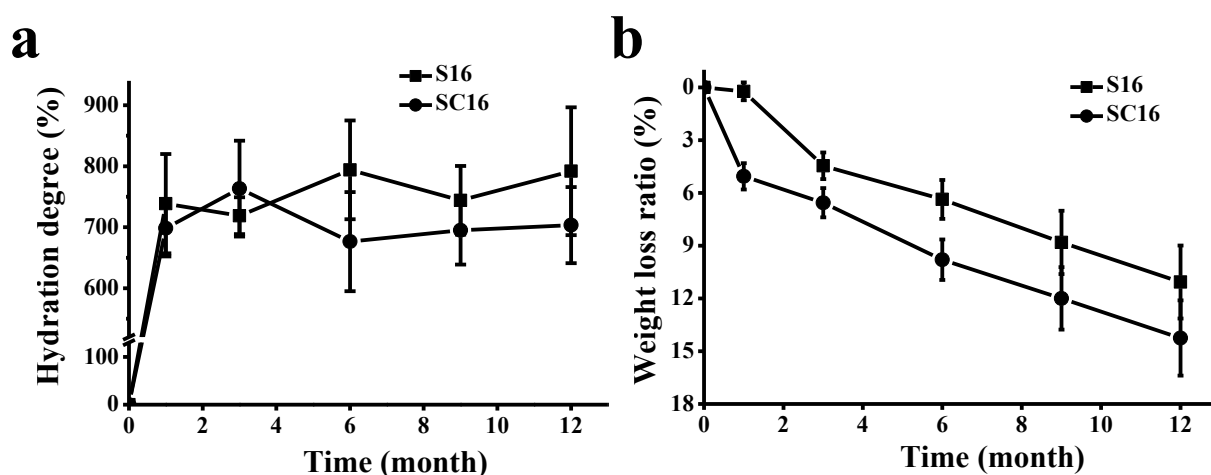


Figure 6. (a) Long-term hydration degree and (b) weight loss ratio of the salt-leached silk fibroin based scaffolds.

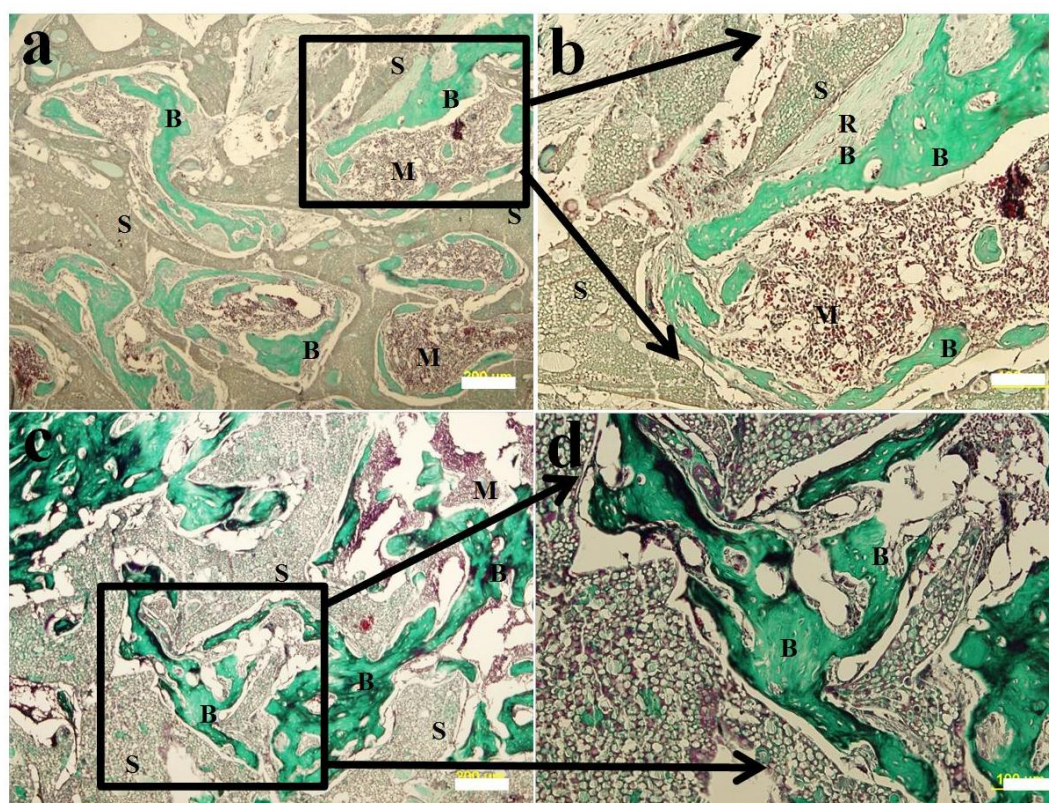


Figure 7. Masson's trichrome staining of the salt-leached silk fibroin based scaffolds after implantation in rat femur defect for 3 weeks. (a, b) S16; (c, d) SC16; (b, d) are enlarged images from (a, c), respectively. Among the images, "S", "B", "M" and "R" correspond to scaffold residuals, formed new bone, bone marrow, and rapid forming new bone. Scale bar: 200 μ m for (a, c) and 100 μ m for (b, d).

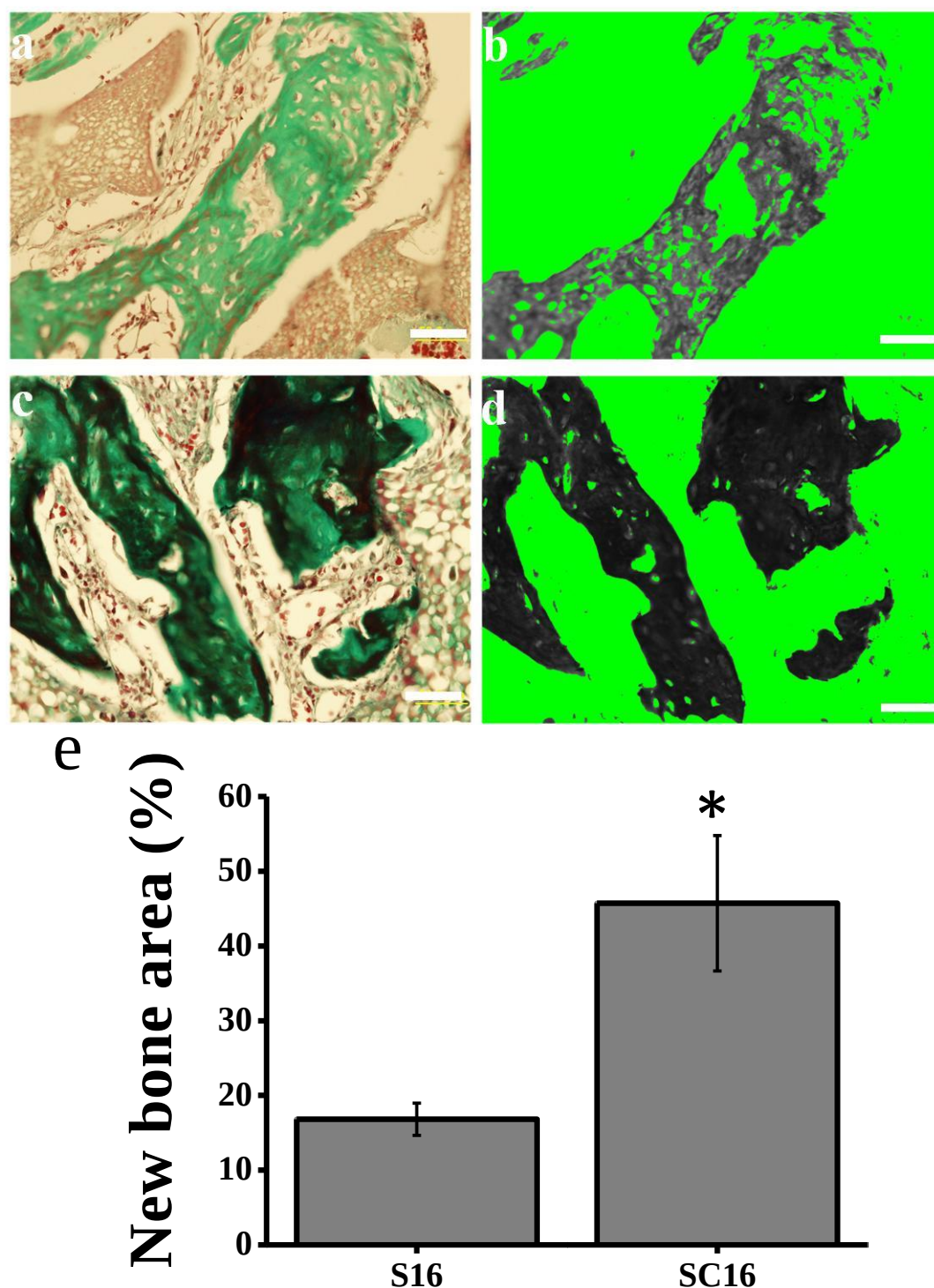


Figure 8. Bone histomorphometry of the S16 and SC16 explants by means of using the software WCIF IMAGE J. (a, b) were representative Trichrome images of S16 and SC16, respectively. (c, d) were processed image from (a, b) for bone histomorphometry analysis, respectively. (e) Calculation of the new bone area in the Masson's Trichrome images (Area for each slide: 0.45 mm*0.35 mm) after image processing. Four explants were used for each group, and at least 10 slides were evaluated per explants. Scale bar: 50 μ m. * indicates significant difference ($p < 0.05$).

3.4. *In vivo* new bone formation

The *in vitro* bone tissue formation has been studied by culturing stem cells in silk and hydroxyapatite scaffolds [16]. *In vivo* implantation was also tested in mice using freeze-dried silk/CaP scaffolds [19]. Based on these interesting studies, CaP can improve bone tissue formation. Here, we report the *in vivo* behaviour of the Silk-NanoCaP scaffolds. Figure 7 shows a typical image of the implanted scaffolds section stained with the Masson's Trichrome. After three weeks of implantation, no chronic inflammation was observed nor was a fibrous capsule detected for both scaffolding materials (Figure 7). Only some scattered multinucleated giant cells (MGCs) were found in S16 (Figure 7b). Moreover, bone growth was observed within the porous structure of both scaffolds (Figure 7a, c). Comparing the two groups of scaffolds, SC16 presented a more intense staining of collagen as compared to S16, which indicated that the new bone was more mature in SC16 as compared to that observed in S16. Another difference observed in both groups was the presence of soft tissue in the bone/scaffold interface in the S16 group. This apparent soft tissue did not present the typical morphology of fibrous tissue. Instead, as it can be observed in Figure 7b, there is some continuity with the surrounding bone. For the silk/CaP group this fibrous tissue was not detected, and in fact bone seemed to grow directly on the surface of the scaffolds (Figure 7d). Furthermore, the bone histomorphometric analysis of the) SC16 group had a much higher new bone area (~45%) in the Masson's Trichrome slide images than that from S16 group as shown in Figure 8e. From Figure 8a-d, it was found that bone area in the processed images (black area in Figure 8c, d) matched very well with that in the original images (Figure 8a, b).

Our results indicate that both tested scaffolds were biocompatible as no obvious inflammation or fibrous encapsulation was observed. Additionally, the macroporous structure and high porosity of both S16 and SC16 supported new bone formation and in-growths. The fibrous tissue observed for the S16 group was not identified as inflammatory tissue, as it resembles what has been previously described as rapidly forming bone by Salgado *et al.* [39]. This tissue most likely represents an early bone matrix similar to that seen in advancing fronts of intramembraneous bone formation. As previously mentioned, this tissue was not found in the SC16 group, which may indicate that for this group bone is being synthesized and remodeled at a higher rate when compared to the S16 group. Furthermore, this group also had bone growing directly on its surface, which indicated that this scaffold has osteoconductive properties.

Impressively, the SC16 group exhibited an outstanding ability to induce new bone formation after 3 weeks of implantation, compared with the S16 group. Therefore, SC16 induced better new bone formation and is more osteoconductive than the S16 group. These data are also consistent with the *in vitro* mineralization results.

4. Conclusions

In this study, the novel salt-leached silk/nano-sized calcium phosphate scaffolds presented a homogeneous CaP distribution and a rapid bioactive response *in vitro*. During long-term degradation, both the silk and silk/nano-sized calcium phosphate scaffolds had an adequate biostability in terms of hydration degree along with a slow weight loss. After 3 weeks implantation, both scaffold types supported new bone in-growth and no acute inflammatory response was observed. The silk-based scaffolds were shown to be osteoconductive since they supported new bone formation on their surfaces. Furthermore, silk/nano-sized calcium phosphate scaffolds induced significantly higher amount new bone formation as compared to that observed for silk scaffolds. The silk/nano-sized calcium phosphate scaffolds are good candidates for bone tissue engineering.

Acknowledgements

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Supplementary Data

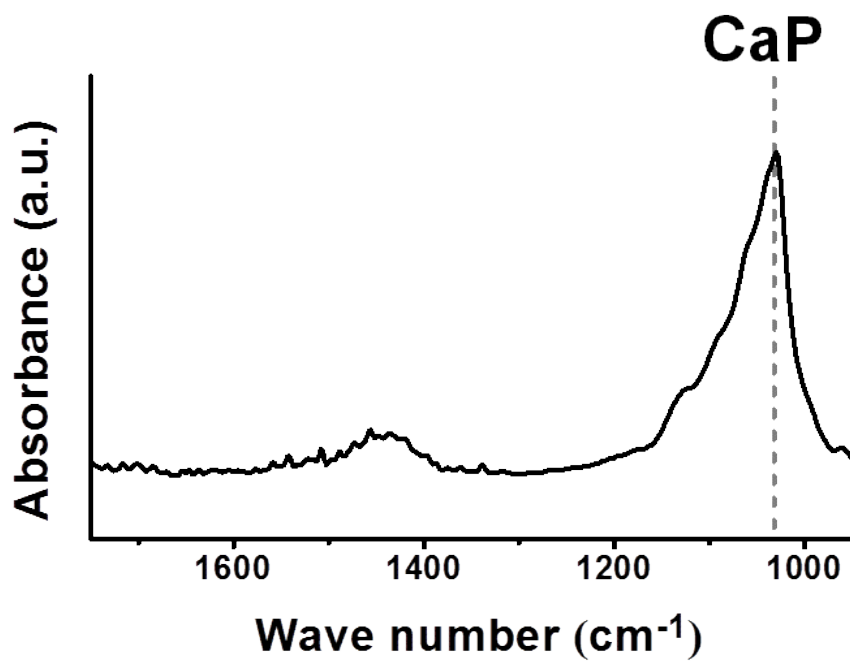


Figure S1. ATR-FTIR spectra of the CaP control.

Chapter VII

Bilayered Silk/Silk-NanoCaP Scaffolds for Osteochondral
Tissue Engineering: *In Vitro* and *In Vivo* Assessment of
Biological Performance

Chapter VII

Bilayered Silk/Silk-NanoCaP Scaffolds for Osteochondral Tissue Engineering: *In Vitro* and *In Vivo* Assessment of Biological Performance

Abstract

Scaffolds that can mimic the composition of osteochondral tissues and properly integrate in subchondral bone are crucial for osteochondral defect (OCD) regeneration. This study proposes novel bilayered silk/silk-nano calcium phosphate (Silk/Silk-NanoCaP) scaffolds for osteochondral tissue engineering. Micro-CT examination showed homogeneous porosity distribution in both layers. Mechanical analysis revealed that the scaffold presents compressive moduli of 16 and 0.4 MPa in dry and wet state, respectively. Under dynamic mechanical analysis the scaffolds displayed an outstanding integrity and elasticity. When immersed in a simulated body fluid solution, mineralization was confined to the Silk-NanoCaP layer. Rabbit bone marrow mesenchymal stromal cells (RBMSCs) were cultured onto the scaffolds, and good adhesion and proliferation were observed. Osteogenesis was also evaluated *in vitro*. The Silk-NanoCaP layer showed a higher alkaline phosphatase level than the silk layer in osteogenic conditions. *In vivo* subcutaneous implantation in the back of rabbits demonstrated abundant tissue infiltration and weak inflammation, after 4 weeks. In a critical size OCD model, the scaffolds firmly integrated into the host tissue, and supported the cartilage regeneration in the silk layer and promoted *de novo* bone ingrowths in the Silk-NanoCaP layer, after 4 weeks. These bilayered scaffolds can therefore be promising candidates for OCD regeneration.

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1. Introduction

Osteochondral defect (OCD) is a common problem in the joint. It includes defects both in the articular cartilage and the underlying subchondral bone [1, 2]. Cartilage defects are normally irreversible, and will likely induce the OCD. Diseases arising from the subchondral bone can also cause OCD, such as osteochondritis dissecans and osteonecrosis [3]. Osteochondral fracture induced by trauma constitutes one of the reasons for OCD. Knee is the most common location for an OCD, but these defects can also be found in the ankle, specific in the talus [4, 5]. OCD will induce persistent symptoms of pain and limited motion of the joint, accompanying by swelling and stiffness. Every year, the healthcare cost for OCD is about \$95 billion in United States alone [6].

Currently, there are several techniques used in clinics to treat OCD, including arthroscopic debridement, micro-fracture, osteochondral (OC) autograft transplantation, and autologous chondrocyte implantation (ACI) [7]. These approaches are not ideal, since they were palliative or induced donor site morbidity. OC tissue engineering emerged as a promising alternative strategy for OCD regeneration [8-11]. The OC tissue is located in an environment of high pressure and shear stress. The strength of the implanted scaffold should be strong enough to bear the load and maintain the dimensional integrity during tissue regeneration. Otherwise the surrounding and the repaired tissues may collapse and induce abnormal regeneration. For a long period, the treatment of OCD has been focusing on the cartilage surface only, and thus regeneration of subchondral bone has not been addressed properly [3]. However, it has been reported that the cartilage did not spontaneously repair without the support from healthy subchondral bone [12]. Therefore, the rehabilitation of the subchondral bone should be performed simultaneously as the reconstruction of the cartilage layer.

Since the integrated cartilage and subchondral bone tissues presented distinct properties, the development of bilayered scaffolds and introduction of chemical/biologic cues in specific layer of the scaffold for OCD regeneration has been considered as a desirable strategy [13-16]. Growth factors, such as insulin-like growth factor or transforming growth factor- β 1, have been introduced into the cartilage layer of the bilayered scaffolds, and enhanced cartilage repair was achieved [15, 16]. Bilayered scaffolds with spatially controlled dual growth factors or genes release system have also been developed for OCD regeneration, and both the repair of cartilage and subchondral

bone layers were observed [17, 18]. On the other hand, the incorporation of osteoconductive materials (for instance, nano-hydroxyapatite particles) into the subchondral layer of the bilayered scaffolds was able to promote the fast subchondral bone formation [19, 20]. Some commercial bilayered scaffolds have been applied in clinics in an acellular strategy, such as TruFit® and MaioRegen® [21-23]. Kon *et al.* [21] performed a pilot clinical trial on human OCD using biomimetic multilayer collagen/nano-hydroxyapatite scaffolds. The clinical scores showed that this scaffold by itself promoted the bone and cartilage tissue restoration.

Regeneration of OCD by bilayered scaffold with acellular strategy is an attractive approach, since it is a one-step procedure and cost reduction. Nevertheless, the improvement of the mechanical properties/stability of the bilayered scaffold and optimization of the way for incorporation the bioactive factors in the scaffolds are still big challenges [17, 24, 25]. Other problems are related to the good interface between the different layers, and the best choice of the appropriate biomaterial [7, 26].

Natural biopolymers have been used for OCD or other tissues regeneration and presented superior *in vitro* and *in vivo* compatibility [7, 13, 27, 28]. The common disadvantage of the natural biomaterials is their weak mechanical properties. Among the natural polymer family, silk fibroin (SF) exhibits outstanding mechanical properties [29], thus it has been finding different applications in tissue engineering [30-34]. Calcium phosphate (CaP) based materials have been showing outstanding osteoconductivity in bone regeneration [35, 36]. However, the pure CaP scaffolds are not with sufficient elasticity and development of polymer/CaP composite scaffolds is a promising strategy to overcome this drawback [20, 21]. Previously, salt-leached SF scaffolds with superior mechanical strength were produced [37]. Following, silk-nano calcium phosphate (Silk-NanoCaP) scaffolds with homogeneous CaP distribution were obtained [38]. The Silk-NanoCaP scaffolds were able to promote *in vivo* new bone formation [39].

In this study, a mechanically robust bilayered scaffold composed of a silk layer and a Silk-NanoCaP layer was developed for OCD regeneration. The chemical composition and microstructure of the scaffold were evaluated by Fourier transform infrared spectroscopy and micro-computed tomography, respectively. The mechanical properties were analyzed by compressive test and dynamic mechanical analysis. The CaP distribution in the interface region was determined by an energy dispersive X-ray

detector. *In vitro* mineralization was performed by immersion the scaffolds in a simulated body fluid solution. The biostability of the scaffold was examined by enzymatic degradation. *In vitro* studies were conducted to investigate the viability and proliferation of the rabbit bone marrow mesenchymal stromal cells (RBMSCs) in the scaffolds up to 7 and 14 days, respectively. *In vitro* osteogenic differentiation of the RBMSCs in the scaffold was also studied. The *in vivo* biocompatibility was evaluated in white New Zealand rabbit model. The bilayered scaffolds were both implanted subcutaneously and in an osteochondral critical size defect of the rabbit knee joint.

2. Materials and Methods

2.1. Materials and reagents

Bombyx Mori cocoons were supplied by the Portuguese Association of Parents and Friends of Mentally Disabled Citizens (APPACDM, Castelo Branco, Portugal). The other materials and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless mentioned otherwise.

2.2. Preparation of the bilayered scaffolds

SF was purified by boiling the cocoons in 0.02 mol/L sodium carbonate solution for 1 hour, in order to remove the sericin [37]. Then, SF was dissolved in 9.3 mol/L lithium bromide solution at 70°C for 1 hour, followed by transferring the solution into a benzoylated dialysis tubing (MWCO: 2000) and dialysis in distilled water for 48 hours. The concentration of the SF solution was performed by dialysis the SF solution against a 20 wt.% poly(ethylene glycol) solution. The concentrated SF solution was collected and stored in low temperature (4-8°C) before further use. The weight percentage of the SF solution was determined by drying the SF solution at 70°C overnight.

Regarding the preparation of the bilayered scaffolds, Silk-NanoCaP scaffolds were prepared firstly as previously reported [38]. Briefly, calcium chloride solution (6 mol/L) and ammonia dibasic phosphate solution (3.6 mol/L) of the same volume were sequentially added into the 16 wt.% SF solution, and the pH of the mixture was adjusted to around 8.5 by addition of ammonia (30%). It was hypothesized that the introduced

calcium and phosphate ions would form hydroxyapatite- $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$, and the amount of CaP introduced was fixed at 16 wt.% (CaP:Silk). Then, 1 mL of this suspension was then added into a mold (inner diameter: 9 mm) made by silicon tubing, followed by addition of 2 g of sodium chloride particles (500-1000 μm). The mold was dried for 2 days, and then immersed in distilled water overnight. In the following day, the Silk-NanoCaP scaffolds were cut into pieces after removal from the molds. Each piece of the scaffolds was placed into the bottom of a new silicon mold and 300 μL of 16 wt.% silk solution was added onto the top of Silk-NanoCaP scaffolds. Then, 600 mg of sodium chloride particles (500-1000 μm) were added to the suspension in the mold [38]. After drying for 2 days, the scaffolds were extracted in distilled water to remove the sodium chloride and by-products. Afterwards, the length of the bilayered scaffold was tailored to achieve specific lengths for the Silk-NanoCaP layer and the silk layer. The skin of the scaffold was removed by a stainless steel punch (diameter: 6 mm). The final scaffolds were obtained by lyophilization in a freeze drier (CRYODOS-80; Telstar, Barcelona, Spain) after freezing the scaffolds at -80°C for at least 3 hours. As controls, pure silk scaffolds and Silk-NanoCaP scaffolds were also prepared by using 16 wt.% silk solution and introducing 16 wt.% CaP content, respectively. The pure silk scaffolds, the Silk-NanoCaP scaffolds, and the bilayered scaffolds were abbreviated as S16, SC16, and Bilayered, respectively.

2.3. Physicochemical characterization of the bilayered scaffolds

2.3.1. Chemical analysis of the bilayered scaffolds

The chemical composition and structural conformation of the bilayered scaffolds were analyzed by a Fourier transform infrared spectroscopy (FTIR) under an attenuated total reflectance (ATR) model (IRPrestige-21, Shimadzu, Kyoto, Japan) [39]. Each layer of the bilayered scaffolds was respectively scanned by contacting the sample with the germanium crystal. The scanning number was fixed at 48 times with a resolution of 4 cm^{-1} . The spectrum of the atmosphere was used as the background for all the specimens. A minimum three specimens were used for each layer.

The CaP content in the Silk-NanoCaP layer was evaluated by a thermal gravimetric analysis (TGA). The organic phase was degraded by heating the specimen in air

atmosphere from 50°C to 700°C, with an increase rate of 20°C/minute in the TGA instrument (TGA Q500; TA Instruments, DE, USA). The Ca/P atomic ratio of the ash obtained after the TGA assay was studied by an energy dispersive X-ray detector (EDX). At least three specimens were used for both assays.

2.3.2. Microstructure evaluation of the bilayered scaffolds

The morphology of the scaffold was observed by a scanning electron microscopy (SEM) (Nova NanoSEM 200; FEI, Hillsboro, OR, USA). Before the observation, the scaffolds were coated with one layer of Au/Pd (SC502-314B) in a coater (E6700; Quorum Technologies, East Grinstead, UK). Elemental analysis was performed in four zones around the interface area by EDX affiliated in the SEM. Three independent areas were selected in each zone, and each scanned area was 100 µm x 100 µm.

Micro-computed tomography (micro-CT) was used to qualitatively and quantitatively evaluate the porosity and the CaP distribution profile in the bilayered scaffolds. The scanning of the scaffolds was conducted under 61 keV and 163 µA in the micro-CT (1072 scanner; SkyScan, Kontich, Belgium). Both the diameter and the height of the scaffolds were 8 mm (Silk layer: 3 mm in height; Silk-NanoCaP layer: 5 mm in height). The integration time was fixed at 1.3 seconds and the pixel resolution was 9.4 µm. For each scanning, around 400 projections were achieved after a rotation of 180° with 0.45° step width. The data sets were processed in a cone-beam model using a standard software (NRcon v1.4.3, Skyscan), and subsequently around 750 serials bitmap images with 1024 x 1024 pixels was generated for each specimen. The qualitative visualization of the three dimensional morphology and the different phase in the bilayered scaffolds were performed by using the CTvox software (Skyscan). In order to achieve the porosity and CaP content distribution profiles in the bilayered scaffolds, the generated bitmap images were processed in standardized software (CT Analyser, version 1.5., Skyscan). The images in each dataset were firstly transferred into binary images by using grey values (dynamic threshold). For the porosity calculation and the CaP content determination, dynamic threshold was set from 45 to 255 and 120 to 255, respectively. Five scaffolds were used for the qualitative and quantitative microstructure evaluation.

2.3.3. Mechanical tests of the scaffolds

The compressive test of the bilayered scaffolds was performed in a universal testing machine (Instron 4505; Instron, Norwood, MA, USA), under a compressive rate of 2 mm/minute until reaching 60% strain. The slope of the initial linear domain in the compressive curve was used to determine the elastic modulus of each specimen. The diameter and the height of the scaffolds were 6 and 5 mm, respectively (Silk layer: 2 mm in height; Silk-NanoCaP layer: 3 mm in height). The samples were tested both at dry and wet states in an unconfined compression model. The dry state test was run at room temperature. For the test in wet state, the samples were first hydrated in phosphate buffered saline (PBS) solution overnight at 37°C. Before the test, the absorbed liquid in the specimen was removed by a tissue, and subsequently the compressive test was performed immediately. S16 and SC16 were used as controls (5 mm in height, 6 mm in diameter). For each test, six specimens of each group were screened.

The dynamic mechanical analysis (DMA) was also conducted to study the viscoelastic properties of the bilayered scaffolds. The sizes of the scaffolds were the same as for the compressive test. Before the test, the scaffolds were kept in PBS solution overnight at 37°C. And then, the specimen was fixed to the DMA apparatus and immersed in PBS solution in the chamber of the DMA instrument (TRITEC8000B DMA; Triton Technology, Lincolnshire, UK). The measurement was performed under 37°C, with a frequency sweep from 0.1 to 25 Hz. The strain amplitude was set at 50 μm for all the tests. S16 and SC16 were used as controls. Five samples of each group were tested.

2.4. *In vitro* degradation and mineralization ability

2.4.1. Hydration degree and enzymatic degradation studies

The initial dry weight of the specimen was measured. And then the hydration degree of the bilayered scaffolds was studied by immersion the scaffolds 0.154 mol/L sodium chloride isotonic saline solution (pH 7.4) overnight at 37°C. The scaffolds of the same sizes as for the compressive test were used for this test. The wet weight of the specimen was recorded in a balance after removing the surface liquid by tissue. The hydration degree was calculated using Equation 1:

$$\text{Hydration degree} = \left(\frac{m_w - m_i}{m_i} \right) \times 100\% \quad (1)$$

Where m_w is the wet weight of the specimen, and the m_i is the initial dry weight of the specimen before immersion. S16 and SC16 were tested as controls. Five samples were measured for each group.

The stability of the bilayered scaffolds was evaluated by enzymatic degradation test. Protease XIV solution of 1 mg/L was prepared by dissolving the enzyme in PBS solution. The initial dry weight of the scaffold was measured, and then the scaffolds were hydrated in PBS solution at 37°C for 3 hours, followed by immersion in 5 mL of protease solution. The scaffolds were the same size as the ones for the compressive test. The enzyme solution was changed every 24 hours. The specimens were removed from the degradation solution at the end of 0.5, 1, 2, 3, 5 and 7 days. The dry weight of the degraded specimen was measured after drying the sample at 70°C overnight. The weight loss ratio was obtained using Equation 2:

$$\text{Weight loss ratio} = \left[\frac{m_i - m_{d,t}}{m_i} \right] \times 100\% \quad (2)$$

Where m_i is the initial dry weight of the sample, and $m_{d,t}$ is the dry weight of the degraded sample at each time point. S16 and SC16 were used as controls. Five specimens per group were used for each time point.

2.4.2. *In vitro* mineralization of the bilayered scaffolds

The *in vitro* mineralization study was carried out by immersion of the bilayered scaffolds in a simulated body fluid (SBF) solution at 37°C for 1, 3, 7 and 14 days. The SBF solution was prepared as previously mentioned [40]. The size of the scaffolds was the same as for the compressive test. At the end of each time point, the specimens were removed

from the SBF solution, rinsed in distilled water, followed by freeze-drying. The surfaces of each layer in the bilayered scaffolds were observed by SEM after coating with Au/Pd. Samples without Au/Pd coating were used for elemental analysis by EDX. Three independent areas were selected in each layer, and each scanned area was 100 μm x 100 μm . At least three specimens were analyzed for each time point.

2.5. *In vitro* cell studies

2.5.1. Isolation, expansion, and seeding of the RBMSCs

The RBMSCs were isolated from male New Zealand White rabbits (Charles River, Senneville, Quebec, Canada). The maintenance and usage of animals were approved by the Ethics Committee of University of Minho. The 9 weeks old rabbits were sacrificed by injection of overdose anesthetic. All the procedures were performed under aseptic condition. The femurs were first separated from the hind legs, followed by removing the epiphysis heads and subsequently flushing out the bone marrow plug by using alpha-minimum essential medium (α -MEM) (Gibco®; Life Technologies, Carlsbad, CA, USA). The α -MEM was supplemented with 10% fetal bovine serum (Life Technologies, Carlsbad, CA, USA), and 1% Antibiotic-Antimycotic liquid prepared with 10,000 units/mL penicillin G sodium, 10,000 $\mu\text{g/mL}$ streptomycin sulfate, and 25 $\mu\text{g/mL}$ amphotericin B as Fungizone(R) in 0.85% saline (Life Technologies, Carlsbad, CA, USA). The isolated RBMSCs (Passage 0, P0) from one femur were cultured in one T150 cm^2 cell culture flask and expanded in 40 mL α -MEM at 37°C in an incubator with 5% CO_2 atmosphere (MCO-18AIC (UV), Sanyo, Osaka, Japan). The medium were changed for the first time after 4 days, and then changed every two day until the cells reached around 90% confluence. And then the cells were detached from the flask by using TrypLE Express (1X) with phenol red (Life Technologies, Carlsbad, CA, USA) and the cell number were counted in a cell counter. In the following, the cell suspension (Passage 1, P1) was centrifuged at 1200 rpm for 5 minutes (5810R; Eppendorf, Hamburg, Germany). Afterwards, the supernatants were removed, and the cells were re-suspended with new culture medium and subsequently passaged into new flasks. The cells were expanded until passage 2 before seeding in the scaffolds. All the scaffolds were sterilized by ethylene oxide.

For the cell seeding, bilayered scaffolds of 6 mm in diameter and 5 mm in height were used (Silk layer: 2 mm in height; Silk-NanoCaP layer: 3 mm in height). S16 and SC16 (6 mm in diameter and 2 mm in height) were seeded with cells and used as controls for osteogenic differentiation. Before the cell seeding, the scaffolds were hydrated in α -MEM overnight in the CO₂ incubator. Afterwards, the scaffolds were removed from the medium and placed into a 24-well suspension cell culture plate (Cell star; Greiner Bio-One, Kremsmuenster, Austria). RBMSCs of passage 2 were detached from the flasks and a new cell suspension with cell density of 5 million/mL were prepared (P3). The cells were seeded onto the surface of the scaffolds, and then the scaffolds with cells were kept in the CO₂ incubator. After 3 hours, the constructs were transferred to a new 24-well suspension culture plate and each constructs were supplemented with 2 mL of α -MEM. The culture medium was refreshed every two or three days.

2.5.2. Viability, attachment, proliferation, and differentiation of the RBMSCs on the scaffolds

For the cell viability assay, 100,000 cells were seeded onto the bilayered scaffolds. The live/dead of the seeded cells was analyzed by Calcein AM and Propidium Iodide (Molecular Probes®; Life Technologies, Carlsbad, CA, USA) staining after culturing for 3 days. At first, each construct was washed by PBS solution, and then transferred into 1 mL PBS solution supplemented with 1 μ g Calcein AM and 2 μ g Propidium Iodide, for 10 minutes. The samples were observed in a transmitted and reflected light microscope with apotome 2 (Axio Imager Z1m; Zeiss, Jena, Germany) after rinsing by PBS solution twice. By using the accompanying software Zen, a Z-stack function was used to combine images at different depth into one final image. The quantitative cell viability of the constructs were screened by a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay (MTS) using the CellTiter 96® AQueous One Solution Cell Proliferation Assay Kit (Promega, Fitchburg, WI, USA), after culture for 1, 3 and 7 days. The working solution was prepared by mixing the MTS with serum-free culture medium (without phenol red) in a ration of 1:5, and fresh working solution was prepared before the test at each time point. At the end of each time point, the constructs were removed from the culture medium, washed by PBS solution, and then placed into 1 mL working solution in a 48-well cell culture plate and kept in the incubator for 3 hours.

Afterwards, the supernatant from each well was transferred into a 96-well cell culture plate (100 μ L/well) and read in a microplate reader (Synergy HT; Bio-Tek, VT, USA) at 490 nm. The scaffolds without cells were used as control. Three independent experiments were performed for the cell viability assay, and at least three samples were analyzed for each time point in one experiment.

For the cell proliferation and osteogenic differentiation assay, 200,000 cells were seeded onto the bilayered scaffolds. After seeding overnight, the constructs were cultured in basal medium (α -MEM) and osteogenesis medium, respectively. The osteogenic medium was based on the α -MEM, and supplemented with 10 mmol/L beta-glycerophosphate, 50 μ g/mL ascorbic acid (Wako Pure Chemicals, Tokyo, Japan), and 10^{-8} mol/L dexamethasone. The constructs were harvested after culturing for 7 and 14 days. At the end of each time point, each construct was removed from the medium and rinsed by PBS solution. After rinse, the silk layer and the Silk-NanoCaP layer were separated by a blade, and each part was placed into 1 mL ultrapure water in a 1.5 mL centrifuge tube. The tubes were stored at -80°C freezer for at least 3 hours before the following assays. S16 and SC16 were seeded with 100,000 cells per scaffold. Before the DNA quantification, the constructs were defrosted and underwent ultrasound treatment for 20 minutes to release the DNA from the scaffolds. The quantification of the double-stranded DNA (dsDNA) was performed by using a Quant-IT PicoGreen dsDNA Assay Kit 2000 assays (Life Technologies, Carlsbad, CA, USA) according to the instruction of the manufacturer. Briefly, 30 μ L supernatant from each sample was mixed with 70 μ L PicoGreen solution and 100 μ L Tris-EDTA buffer. The fluorescence intensities of the samples were recorded in the microplate reader at an excitation wavelength of 485/20 nm and at an emission wavelength of 528/20 nm (Synergy HT, Bio-Tek, VT, USA). Standard dsDNA solutions were prepared and their fluorescence intensities were tested, in order to make standard curve for quantification of the DNA in the samples. The same lysates for DNA assay were also used for alkaline phosphatase (ALP) activity quantification. For this assay, 20 μ L supernatant was mixed with 60 μ L 0.2% (wt./vol.) *p*-nitrophenyl phosphate disodium solution (*p*NPP) and incubated at 37°C for 1 hour. The *p*NPP was dissolved in 1 mol/L diethanolamine buffer solution (pH 9.8, adjusted by hydrochloric acid). During the incubation, the *p*NPP was hydrolyzed by the ALP and the yellow *p*-nitrophenol (*p*NP) was formed. The reaction was stopped by the addition of 80 μ L 2 mol/L sodium hydroxide solution into each well. The absorbance of each well at 405

nm was read in the microplate reader (Synergy HT; Bio-Tek, VT, USA). The standard solutions were prepared with the 10 mmol/L *p*NP solution. And the absorbance of these standard solutions was read in order to prepare the standard curve. The ALP activity from each sample was reflected by the amount of the formed *p*NP. The ALP activity of the samples was normalized by their corresponding DNA contents. The DNA contents or ALP activities of the bilayered scaffolds were obtained by combined the DNA contents or ALP activities of the corresponding silk layer and Silk-NanoCaP layer. The proliferation and differentiation studies were repeated twice, with at least three specimens for each time point in one study.

The cells' attachment on the scaffolds in both basal and osteogenic conditions was observed by SEM, after culturing for 7 days. Before the observation, the constructs were harvested from the medium and rinsed by PBS solution, followed by immersion in 10% formalin solution for at least 1 day. The fixed constructs were dehydrated by immersion in a serial of aqueous ethanol solution, with gradient increased concentration in ethanol (from 30% to 100%). The surface of the constructs were coated by Au/Pd and observed by SEM.

2.6. *In vivo* implantation of the bilayered scaffolds

The bilayered scaffolds of 6 mm in diameter and 8 mm in height (Silk layer: 3 mm; Silk-NanoCaP: 5 mm) were used for the subcutaneous implantation in male New Zealand White rabbits (Charles River, Senneville, Quebec, Canada). Additionally, the bilayered scaffolds of 5 mm in diameter and 5 mm in height (Silk layer: 2 mm; Silk-NanoCaP: 3 mm) were implanted in the osteochondral defects in the knee of the New Zealand White rabbits. All the rabbits for the *in vivo* studies were male and of 9-11 weeks old, with average weight 2.4 Kg at the implantation time. The maintenance and usage of animals were approved by the Ethics Committee of University of Minho. The scaffolds were sterilized by ETO and all the procedures were performed in an aseptic condition.

For the subcutaneous implantation, six bilayered scaffolds were implanted into three rabbits (2 pieces/rabbit). Each rabbit was anesthetized by intravenous injection of 1.375 mL mixture of Imalgene (Ketamina, 75 mg/Kg) and Domitor (Medetomidina 1 mg/Kg). The hair of the rabbit was cut at the implantation area, followed by washing with 70%

ethanol and iodine. In each rabbit, two skin incisions were made below the ears in the back (one in the left and the other in the right), each around 2 cm length. The scaffolds were subcutaneously implanted into each pocket. And the skin was sutured by using bioresorbable silk suture. After 4 weeks, the rabbits were euthanized by injection of overdose anesthesia and the implanted scaffolds were retrieved. The explants were fixed in 10% formalin, and then dehydrated through graded ethanol, and finally embedded in paraffin. Sections were prepared by cutting the specimen into sections of 5 μm thick using a microtome (Spencer 820, American Optical Company, NY, USA). The obtained sections were stained with Haemotoxylin and Eosin (H&E). The dehydrated explants were also coated with Au/Pd and observed by SEM.

Regarding the implantation in critical size osteochondral defects (4.5 mm in diameter and 5 mm in depth), 9 bilayered scaffolds were implanted into 3 rabbits (3 pieces/rabbit). The anesthesia of the rabbits was administered intravenously with a mixture of Imalgene (Ketamina, 75 mg/Kg) and Domitor (Medetomidina 1 mg/Kg), with 1.375 mL/animal. The hair of the rabbit was cut at the implantation area, followed by washing with 70% ethanol and iodine. The rabbits were anesthetized and the hair in the knee joints of the hind legs was cut. The skin was washing with 70% ethanol and iodine. And then the knee joints were exposed through a medial parapatellar longitudinal incision. Two osteochondral defects (4.5 mm in diameter and 5 mm in depth) were created in each femur using a Brace manual drill, one located between the lateral and the medial condyle, the other was in the opposite site of the patellar. The bilayered scaffolds were implanted into the defects by press fit. The skin was sutured. In each rabbit, one of the defects was empty and used as control. Four weeks post-operation, the rabbits were euthanized with an overdose of pentobarbital sodium, and the knees were excised. Three explants were fixed by 10% formalin and then immersed in paraffin after dehydration. Slides were prepared and H&E and Masson's Trichrome staining were performed.

2.7. Micro-CT analysis of the explants

Three explants were used for micro-CT observation in wet state, under 100 keV and 98 μA . The explants were loaded by a parafilm during the scanning to avoid the evaporation of liquid. The integration time was fixed at 1.3 second and the pixel resolution was 19.13 μm . The specimens were first scanned and the data sets were processed as mentioned

above (Section 2.3.2). The 3D micro-CT images of the explants were obtained by using the CTvox software (Skyscan). In order to calculate the porosity and CaP content in the interested regions, the data set of each specimen was re-arranged by standard software (Dataviewer, Skyscan). The porosity and CaP contents of the defect controls and the defects implanted with scaffolds were analyzed in standardized software (CT Analyser, version 1.5, Skyscan), and the thresholds used were the same as mentioned in Section 2.3.2. In each specimen, a cylinder model region (Height: 4 mm; Diameter: 4 mm) was used for the evaluation of porosity and CaP distribution. For the quantification calculation of the porosity or the CaP content, the top 2 mm region in the cylinder model region was considered as cartilage domain in defect controls or as silk layer in defects implanted with scaffolds, and the down 2 mm region was considered as subchondral bone domain in defect controls or as Silk-NanoCaP layer in defects implanted with scaffolds.

2.8. Statistical analysis

The data were presented by mean $\pm$ standard deviation (SD). The results were evaluated by one-way analysis of variance (ANOVA). The means of each group were compared by Tukey's test, and $p < 0.05$ was considered statistically significant.

3. Results

3.1. Chemical composition and structural conformation of the bilayered scaffolds

The SF conformation and chemical composition in the bilayered scaffolds were studied by ATR-FTIR. As showed in Figure 1, the SF in both layers displayed the same strong absorbance peaks at 1627 cm^{-1} and 1520 cm^{-1} , which were characteristic peaks for β -sheet conformation [30]. The Silk-NanoCaP layer presented strong peak at 1031 cm^{-1} , which is the characteristic vibration absorbance of PO_4^{3-} in the CaP [41]. The size of the nano-CaP particles was analyzed by backscattered SEM. The inserted SEM image showed that the CaP particles were distributed evenly in the silk matrix, and presented a size around 200 nm. The TGA analysis showed that the CaP mass ratio in the Silk-NanoCaP layer was around $13.81 \pm 0.63\%$ (CaP:Silk, by wt.), and the Ca/P ratio of the ash was $1.65 \pm 0.4\%$.

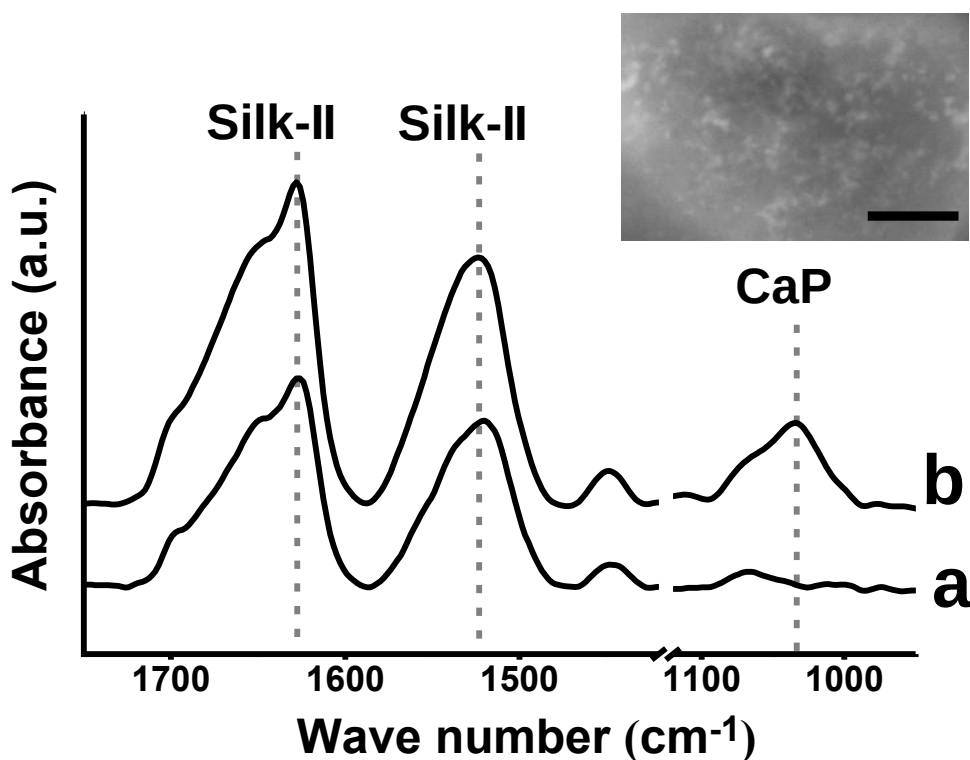


Figure 1. Attenuated total reflectance Fourier transform infrared spectra (ATR-FTIR) of (a) the silk layer and (b) the Silk-NanoCaP layer in the bilayered scaffolds. The inserted is the backscattered SEM image of the Silk-NanoCaP layer, showing the nano-sized CaP particles (white domain) distribution in the silk matrix (Scale bar: 3 μm).

3.2. Microstructure and CaP distribution of the bilayered scaffolds

Figure 2 shows the macroscopic image of the bilayered scaffolds. It was found that the scaffold presented macro/microporous and interconnective structure in both layers. The two layers were well integrated by a continuous interface region. The pore size of the macropores in each layer was around 300 to 700 μm, and the micropores located in the trabeculi of the macropores with size less than 50 μm (Figure 2b). The interface region was of less than 500 μm thickness and located flatly between the two layers (Figure 2b). The EDX scanning from the Silk-NanoCaP layer to the silk layer showed that the calcium ions were only limited in the Silk-NanoCaP layer and the thin interface area (Figure 2c). In the interface region, the intensity of the calcium ion signal in the side close to Silk-NanoCaP layer was higher than the one in the side of silk layer.

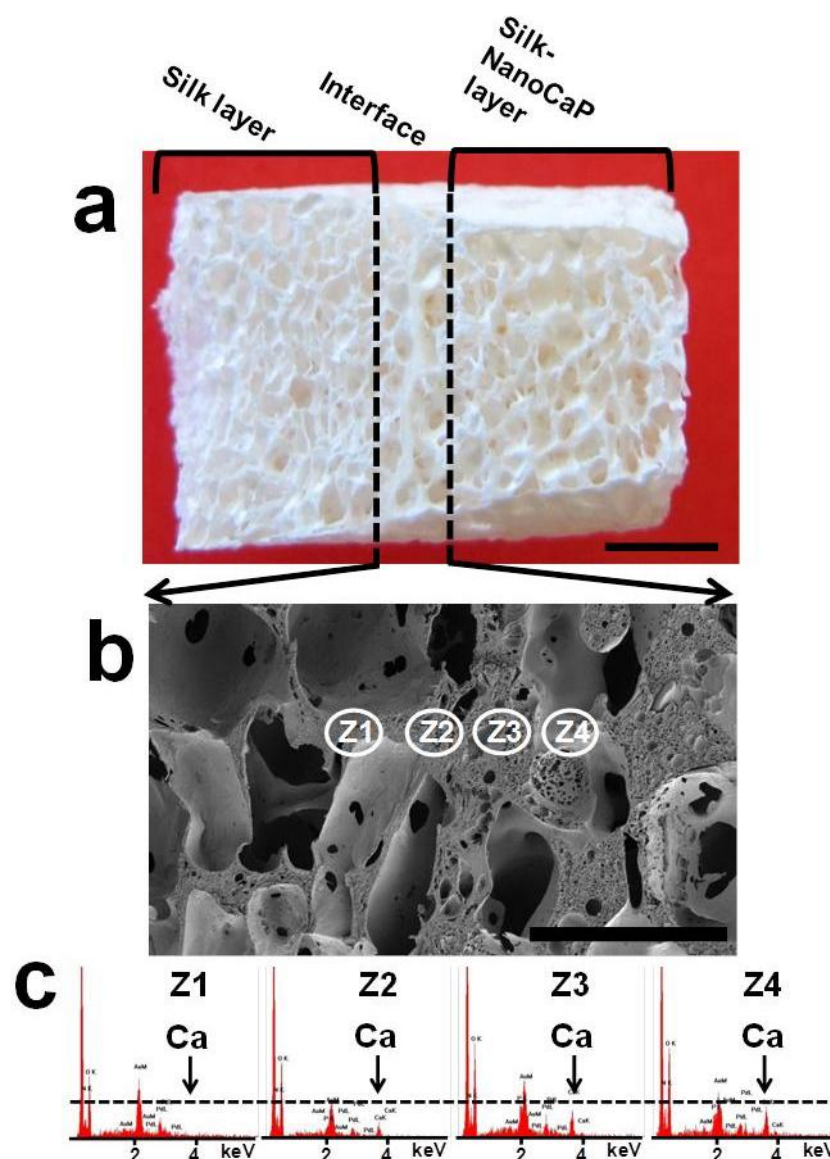


Figure 2. The interface of the bilayered scaffolds. (a) Macroscopic image of the bilayered scaffolds (scale bar: 3 mm). (b) SEM image of the interface region in the bilayered scaffold (Scale bar: 500 μ m). Z1, Z2, Z3 and Z4 indicated different regions from the silk layer to the Silk-NanoCaP layer, around the interface area. (c) The elemental analysis of calcium ions in Z1, Z2, Z3 and Z4 regions by energy dispersive X-ray detector (EDX).

The qualitative and quantitative distributions of the porosity and the CaP in the bilayered scaffolds were assessed by micro-CT. Table 1 demonstrates that both layers presented high porosity and interconnectivity, and the CaP was kept only in the Silk-NanoCaP layer. The three-dimensional images showed two distinct phases in the bilayered scaffold (Figure 3a). The CaP (blue domain) resided only in the Silk-NanoCaP layer, without infiltration into the silk layer. Both layers displayed high interconnectivity and porosities

(Figure 3a). By changing the threshold, it was found that the CaP distribution was homogeneous in the Silk-NanoCaP layer (Figure 3b). The two-dimensional images of each layer also confirmed the interconnectivity and porous structure in each layer (Figure 3c and d). The porosity distribution profile revealed that the porosity was homogeneously distributed in each layer, and lower porosity was observed in the Silk-NanoCaP layer (Figure 3e). The porosity showed a sharp decrease in the interface domain which was around 0.5 mm in thickness. The CaP distributed evenly in the Silk-NanoCaP layer (Figure 3f). It was noticed that the CaP content decreased gradually in the thin interface region and there was no CaP in the silk layer (Figure 3f).

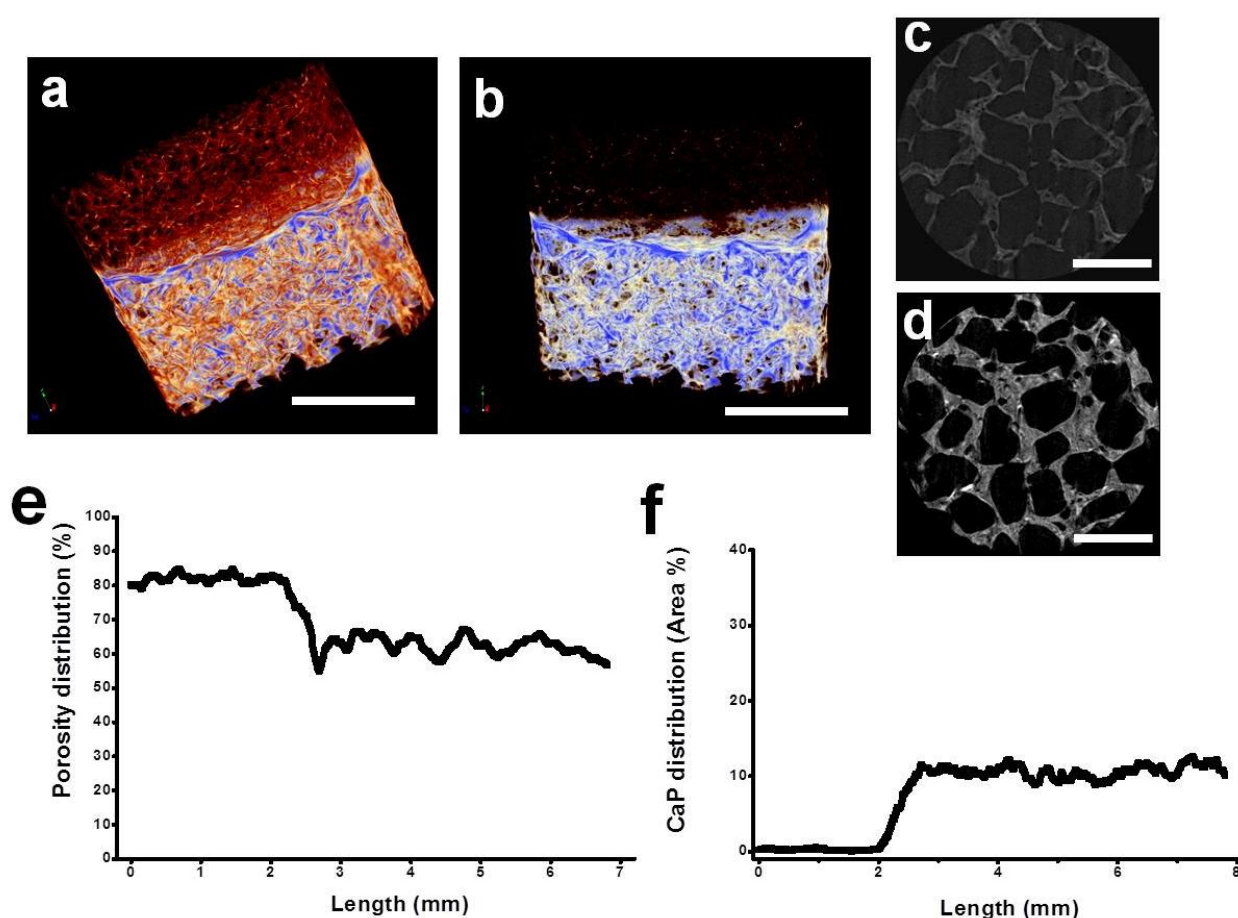


Figure 3. Micro-CT analysis of the bilayered scaffolds. (a) Three-dimensional (3D) micro-CT image of the silk matrix (brown) and the CaP distribution (blue), and (b) 3D micro-CT image of the pure CaP distribution in the bilayered scaffold (Scale bar: 4 mm). (c) Two-dimensional (2D) micro-CT image of the silk layer, and (d) 2D micro-CT image of the Silk-NanoCaP layer (Scale bar: 1 mm). (e) Quantitative analysis of the porosity distribution, and (f) quantitative analysis of the CaP distribution in the bilayered scaffolds.

Table 1. Quantitative micro-CT analysis of the bilayered scaffolds

Layer	Mean Porosity (%)	Mean interconnectivity (%)	Mean CaP content (vol.%)
Silk layer	82.02±1.15	91.13±2.32	0
Silk-NanoCaP layer	62.27±2.61	70.03±4.62	9.60±0.81

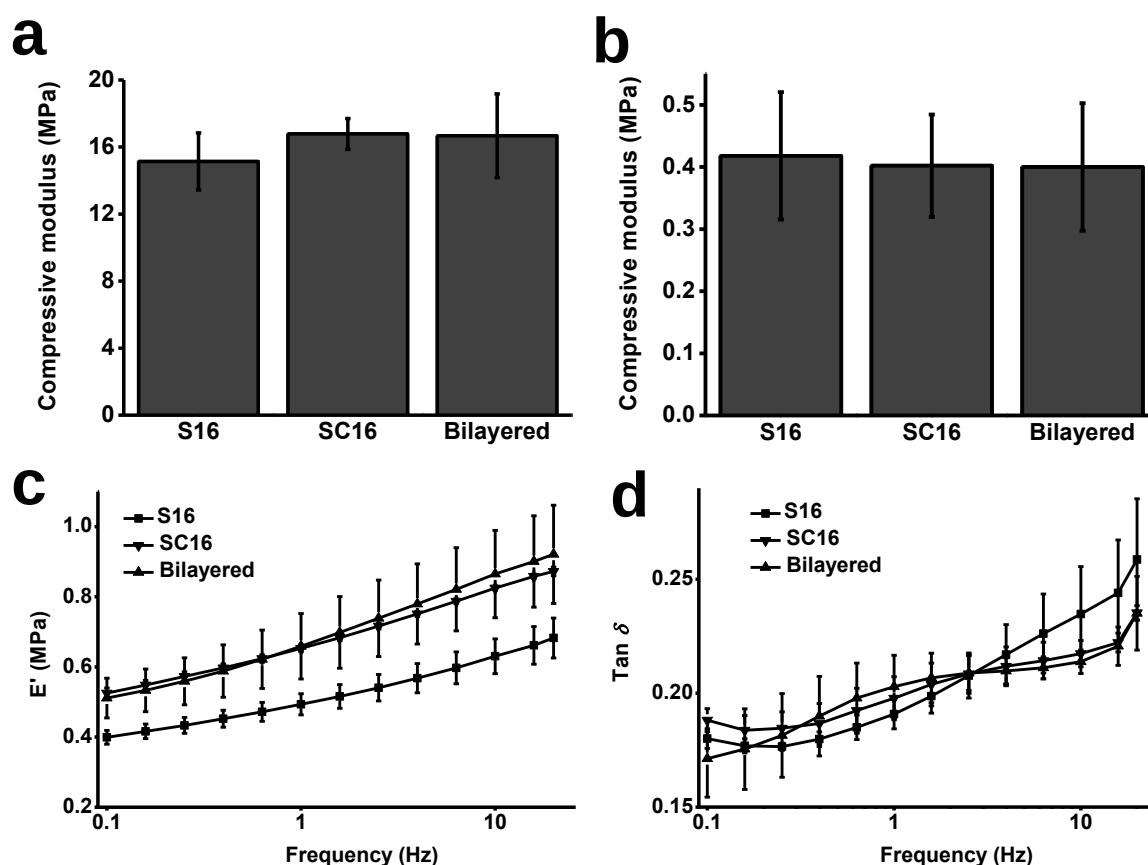


Figure 4. Mechanical analysis of the bilayered scaffolds. (a) Dry status and (b) wet status compressive modulus of the bilayered scaffolds and the controls. (c) Storage modulus (E') and (d) loss moduli ($\tan \delta$) of the bilayered scaffolds and the controls.

3.3. Mechanical properties of the bilayered scaffolds

As showed in Figure 4a, the dry state compressive modulus of the bilayered scaffold was around 16 MPa, and no significant differences were observed from the ones of S16 and SC16. The scaffolds were also tested in hydrated condition to simulate the *in vivo* environment. The wet state modulus of the bilayered scaffolds was around 0.4 MPa,

similar to the ones of the controls (Figure 4b). The dynamic viscoelastic properties of the scaffolds were evaluated by DMA. It was found that the storage modulus of the bilayered scaffolds increased from around 0.5 MPa to 0.8 MPa, as the frequency increasing from 0.1 Hz to 20 Hz (Figure 5c). In the tested frequency range, the storage modulus values of the bilayered scaffolds were similar to the ones of SC16 and higher than the ones of S16. All the three group scaffolds demonstrated similar and low level loss factor values in the tested frequency. The loss factor ($\tan \delta$) of the bilayered scaffolds slightly increased from around 0.17 to 0.23 when increasing the frequency from 0.1 Hz to 20 Hz.

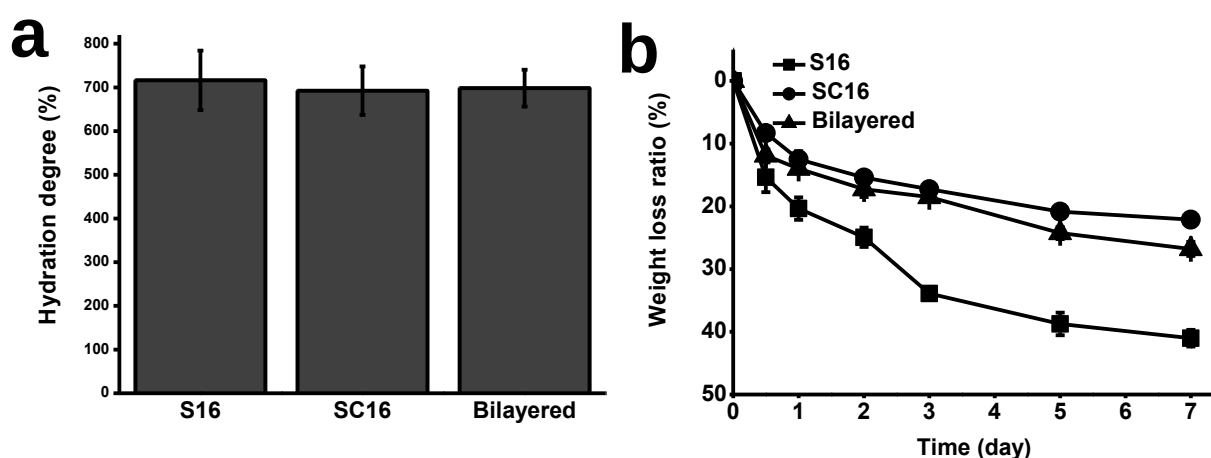


Figure 5. (a) Hydration degree and (b) enzymatic degradation profile of the bilayered scaffolds and controls.

3.4. Hydration and degradation properties of the bilayered scaffolds

The hydration degree data showed that the absorbed amount of isotonic saline solution by the bilayered scaffold was up to seven times of its original mass (Figure 5a). The hydration degrees were similar among the bilayered scaffolds and the controls. In this study, the enzymatic degradation profiles of the scaffolds were analyzed by using protease XIV. It was found that S16 degraded faster than the bilayered scaffolds and SC16 (Figure 5b). In the first 12 hours, the bilayered scaffolds lost around 12% mass, and S16 and SC16 lost about 15% and 7% mass, respectively. After 7 days degradation, the bilayered scaffolds presented approximate 26% weight loss, and S16 and SC16 showed around 41% and 21% weight loss, respectively.

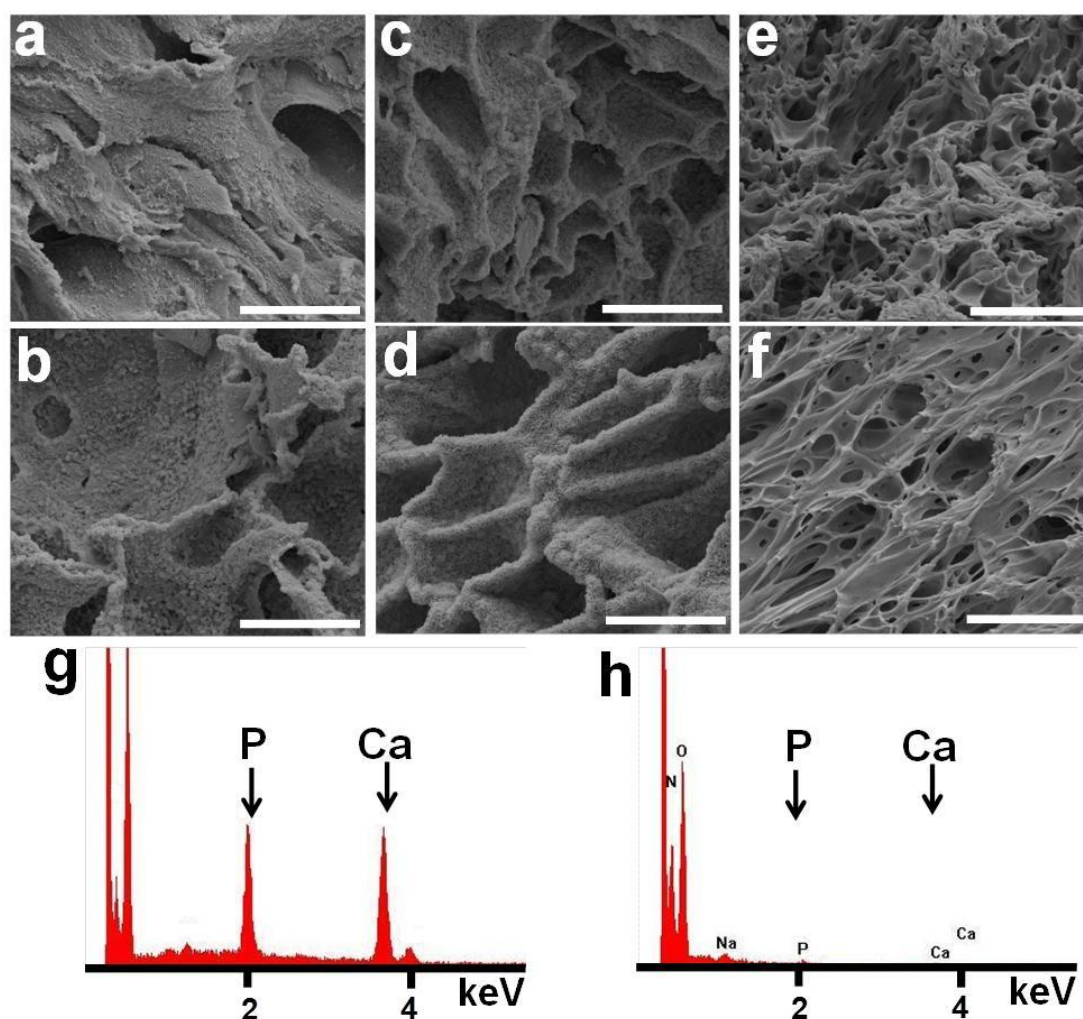


Figure 6. *In vitro* mineralization of the bilayered scaffolds by immersion in SBF solution. (a-d) SEM images of the Silk-NanoCaP layer after immersion in SBF solution for 1, 3, 7 and 14 days, respectively; (e, f) SEM images of the silk layer after immersion in SBF solution for 7 and 14 days, respectively (Scale bar: 10 μ m). (g, h) EDX analysis of the Silk-NanoCaP layer and silk layer after immersion in SBF solution for 14 days, respectively.

3.5. *In vitro* mineralization

The *in vitro* mineralization behavior of the bilayered scaffolds was presented in Figure 6. After immersion in SBF solution for only 1 day, a few amount of crystals already formed in the surface of the Silk-NanoCaP layer (Figure 4a). The crystals became evident after 3 days, and fully covering the surface of the pore walls in the Silk-NanoCaP layer, after 7 and 14 days (Figure 6b, c and d). The worm- or flake-like crystals formed the bigger

cauliflower-like clusters, typically associated to apatite crystals. The silk layer was not able to induce any apatite formation after 7 and 14 days immersion in SBF solution (Figure 6e, f). EDX analysis showed that strong phosphate and calcium ionic signals were observed in the Silk-NanoCaP layer (Figure 6g). There were no obvious calcium and phosphate ions detected in the silk layer (Figure 6h).

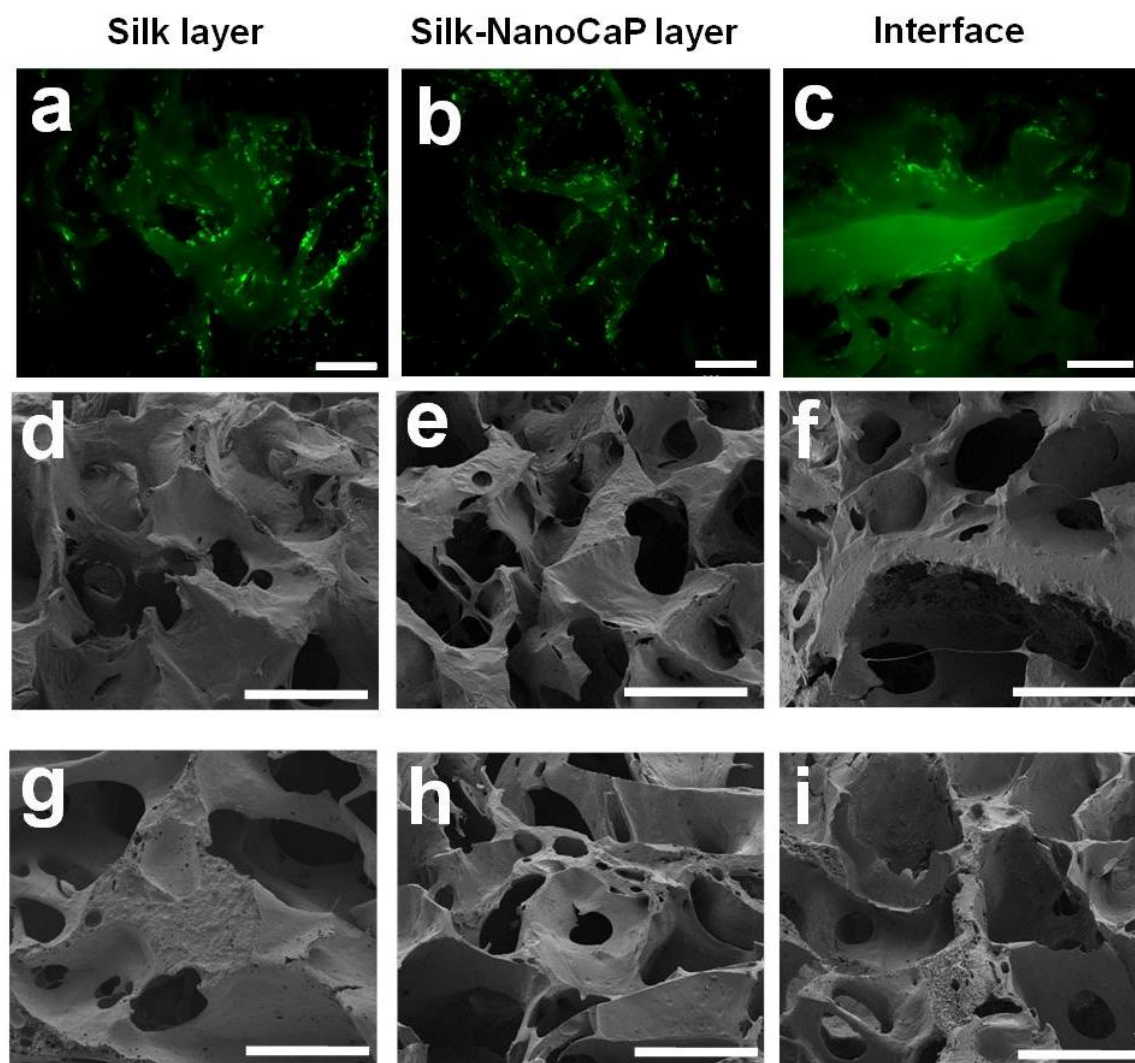


Figure 7. The live/dead staining and attachment of rabbit bone marrow mesenchymal stromal cells (RBMSCs) in the bilayered scaffolds. (a-c) Calcein AM/propidium iodide staining (live/dead) of the RBMSCs in the silk layer, the Silk-NanoCaP layer, and the interface of the bilayered scaffolds after culturing for 3 days, respectively (Scale bar: 400 μ m). Green indicated the living cells, and red showed the dead cells. (d-f) SEM images of the cell attachment in the silk layer, the Silk-NanoCaP layer, and the interface of the bilayered scaffolds after culturing for 7 days in basal condition, respectively (Scale bar: 500 μ m). (g-i) SEM images of the cell attachment in the silk layer, the Silk-NanoCaP layer and the interface of the bilayered scaffolds after culturing for 7 days in osteogenic condition, respectively (Scale bar: 400 μ m).

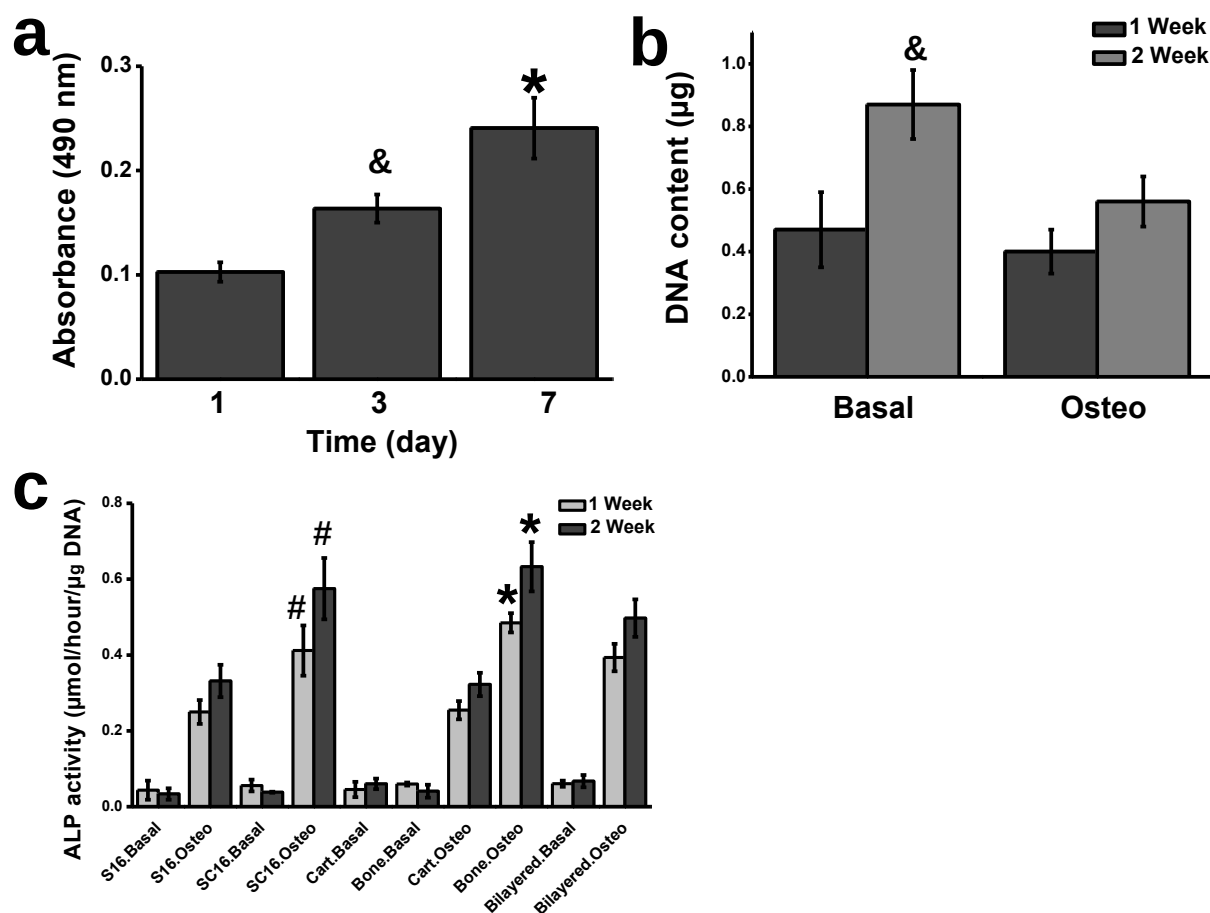


Figure 8. The viability, proliferation, and differentiation of RBMSCs in the bilayered scaffolds. (a) The MTS analysis of the RBMSCs cultured in the bilayered scaffolds for 1, 3 and 7 days. (b) The DNA content of the RBMSCs cultured in the bilayered scaffolds for 7 and 14 days, at both basal and osteogenic conditions. Basal: Basal condition; Osteo: Osteogenic condition. & indicated significant differences compared with DNA content from osteogenic condition. (c) The osteogenesis differentiation of the RBMSCs cultured in the bilayered scaffolds and the controls for 7 and 14 days. S16.Basal and S16.Osteo: S16 with RBMSCs cultured in basal and osteogenic conditions, respectively; SC16.Basal and SC16.Osteo: SC16 with RBMSCs cultured in basal and osteogenic conditions, respectively; Cart.Basal and Cart.Osteo: Silk layer of the bilayered scaffolds with RBMSCs cultured in basal and osteogenic conditions, respectively; Bone.Basal and Bone.Osteo: Silk-NanoCaP layer of the bilayered scaffolds with RBMSCs cultured in basal and osteogenic conditions, respectively; Bilayered.Basal and Bilayered.Osteo: Bilayered scaffolds with RBMSCs cultured in basal and osteogenic conditions, respectively. # indicated significant differences compared with ALP activity from S16 group in osteogenic condition. * indicated significant differences compared with values from the silk layer in osteogenic condition.

3.6. Attachment, viability, and proliferation of the RBMSCs on the bilayered scaffolds

The RBMSCs were seeded into the bilayered scaffolds. The live/dead assay showed that there were a lot of living cells attached on the surface of the scaffolds (Figure 7a-c), after seeding for 3 days. The cells dispersed evenly in the silk and Silk-NanoCaP layers,

presented spreading morphology, and contacted to each other. Some cells also growth on the interface area. The cell attachment was also observed by SEM after culturing for 7 days, in both basal (Figure 7d-f) and osteogenic conditions (Figure 7g-i). It was found that, the surface of the silk layer, the Silk-NanoCaP layer, and the interface were fully covered by the cells and the extracellular matrix, in both basal and osteogenic conditions. The cells not only adhered in the surface of the scaffolds, they also grew inside the scaffolds.

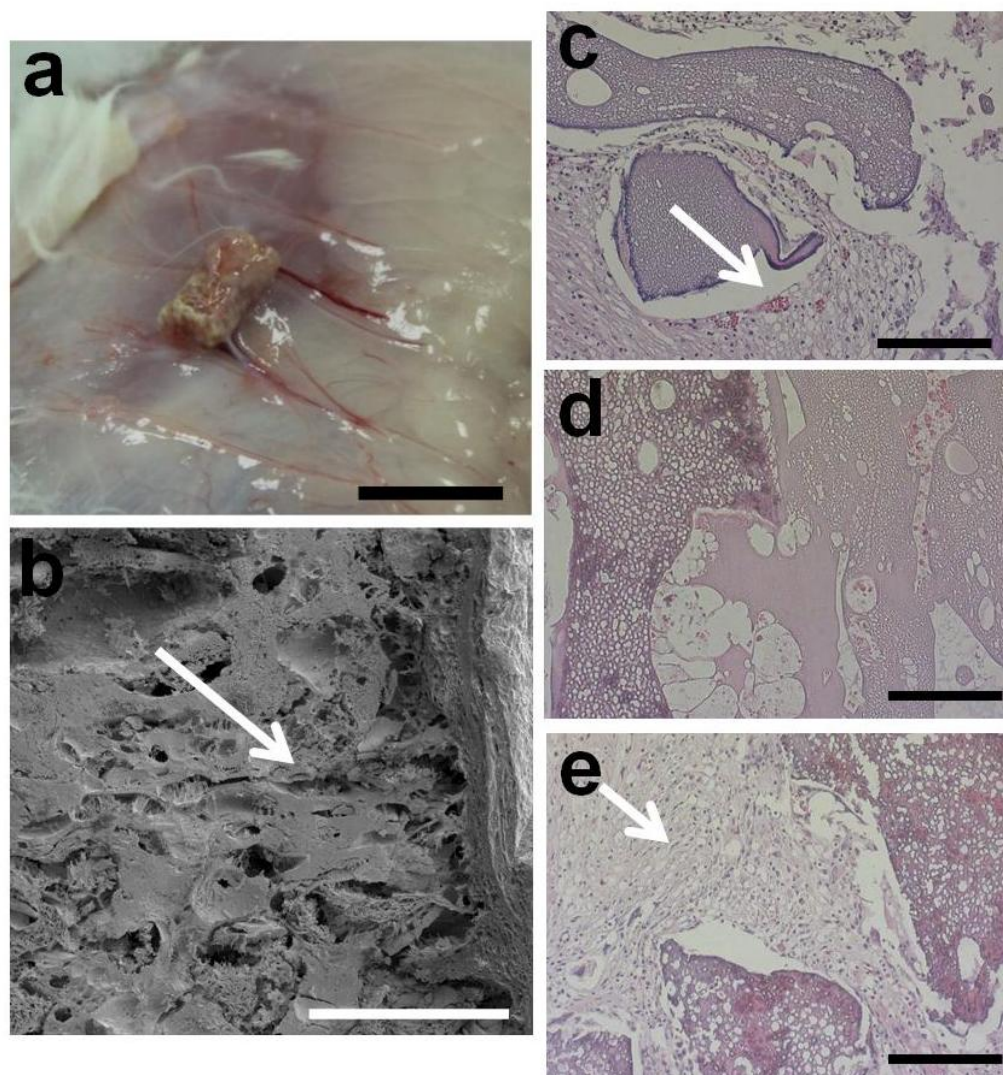


Figure 9. Subcutaneous implantation of the bilayered scaffolds in rabbit for 4 weeks. (a) Macroscopic image of the explants after implantation for 4 weeks (Scale bar: 1 cm). (b) SEM image of the explants after implantation for 4 weeks (Scale bar: 1 mm), the arrow indicated the interface. (c-e) the haematoxylin and eosin (H&E) staining of the silk layer, interface, and Silk-NanoCaP layer in the explants after implantation for 4 weeks, respectively (Scale bar: 200 μ m). Arrow in (c) indicated vessels, and arrow in (e) indicated fibroblasts.

The quantitative analysis of the cell viability was performed by MTS assay (Figure 8a). It was noticed that the MTS absorbance significantly increased during the culture time period. The cell proliferation was screened by DNA content analysis. It was seen that the DNA content of the cells in the bilayered scaffolds significantly increased from day 7 to day 14, in both the basal and osteogenic conditions (Figure 8b). At day 14, the DNA content of the bilayered scaffold in basal condition was higher than the one in osteogenic media (Figure 8b).

3.7. The osteogenic differentiation of the RBMSCs in the bilayered scaffolds

The ALP activity from the cells seeded in the bilayered scaffolds and the controls were normalized by their DNA content, respectively (Figure 8c). It was found the ALP activity in all the groups increased from day 7 to day 14, in osteogenic conditions. In basal condition, the ALP activity showed no differences during the culture time. In osteogenic condition, the ALP activity of the Silk-NanoCaP layer was significantly higher than the one of the silk layer in both tested time points. The same trend was observed in the controls. The ALP activity of SC16 was higher than the one of S16 in day 7 and day 14, when cultured in osteogenic condition.

3.8. Subcutaneous implantation of the bilayered scaffolds

The *in vivo* compatibility of the bilayered scaffolds was assessed by subcutaneous implantation in rabbit. Figure 9a showed that the bilayered scaffolds were still integrated after 4 weeks of implantation. A layer of connective tissue adhered on the whole surface of the scaffolds, and no signs of infection or acute inflammation were observed. The SEM images of the explants displayed that the connective tissues not only tightly integrated to the implants, but also fully filled the inner pores of the bilayered scaffolds (Figure 9b). The H&E staining image of the bilayered scaffolds showed that the connective tissues filtrated into the pores of the scaffolds (Figure 9c-e). There were some vessels formed inside the scaffolds (Figure 9c). Only a few macrophages were observed in the inner part of the scaffolds. There were also some fibroblasts presented in the Silk-NanoCaP layer.

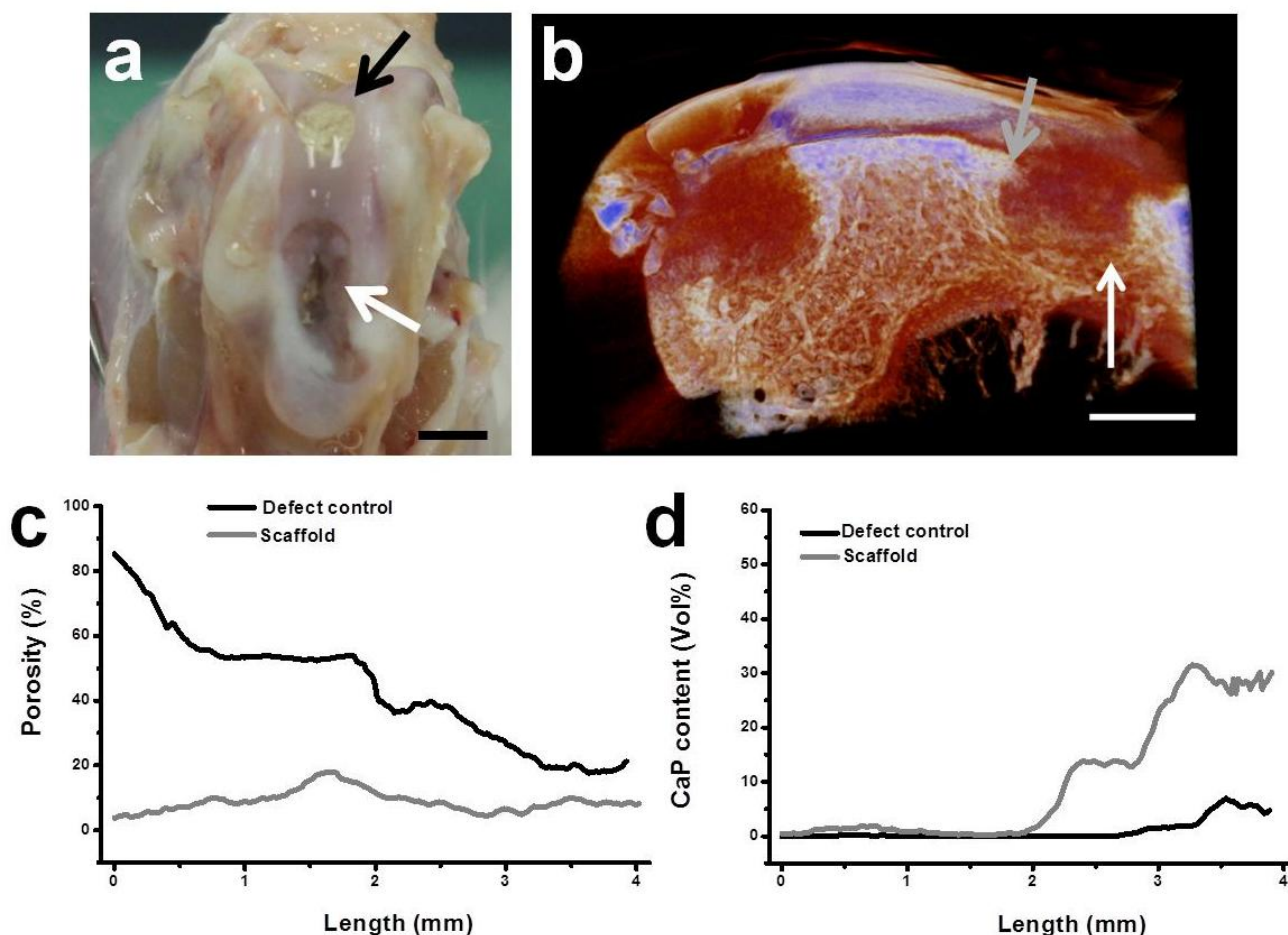


Figure 10. Macroscopic image and micro-CT analysis of the explants after implantation in rabbit OCD for 4 weeks. (a) Macroscopic image of the explants; (b) micro-CT 3D image of the explants; (c) the porosity distribution of the defect control and the defect implanted with the bilayered scaffold; (d) CaP content distribution of the defect control and the defect implanted with the bilayered scaffold. (a) Scale bar: 5 mm; the black arrow indicated the implanted scaffold, and the white arrow indicated the defect control. (b) Scale bar: 4 mm; the grey arrow indicated neocartilage, and the white arrow indicated new subchondral bone formation.

3.9. Regeneration of rabbit knee OCDs by the bilayered scaffolds

The OC regeneration potential of the bilayered scaffolds was studied by implantation of these scaffolds in rabbit OCD for 4 weeks. The macroscopic images of the explants demonstrated that the scaffolds were integrated well with the host tissue (Figure 10a). The scaffolds implanted displayed no obvious mass loss. There were no apparent signals of infection or inflammation of the implants. The defect controls were not regenerated and formed a big void with apparent adjacent tissue collapse. The micro-CT analysis of the explants illustrated that the defect filled with the bilayered scaffold presented less void

space and more regular morphology compared with the defect control (Figure 10b). Besides that, both the ingrowths of the subchondral bone in the bottom domain and the regeneration of cartilage in the surface area of the implant were observed. The defect control showed no cartilage regeneration and few subchondral bone formation. The porosity distribution showed that the defect control was empty in the top region and filled by the tissues in the bottom region, while the defects with implants showed lower than 20% porosity in the region analyzed (Figure 10c). The defect control only showed very small amount of subchondral bone regeneration in the bottom, but the defect with implant presented large amount of CaP content in the Silk-NanoCaP layer (Figure 10d). The quantitative results of CaP content and porosity of different regions were presented in Table 2. The defect controls showed much higher void space than the defects with implants. It was observed that the CaP content in the Silk-NanoCaP layer was around 20% higher than the one from the silk layer.

The explants were further evaluated by H&E and Masson Trichrome staining (Figure 11). It was observed no acute inflammation in all the explants. The defects with scaffolds showed no collapse of the adjacent tissues. The scaffolds presented stable and integrated structure, and firmly integrated with the host tissues. In the silk layer, the new cartilage was formed and gradually spread from the edge to the middle of the defects (Figure 11a, b). The top surface of the scaffolds showed no collapse, and matched the height of the normal cartilage. In the bottom domain, obvious new subchondral bone growth into the Silk-NanoCaP layer was observed (Figure 11a, b). The infiltration of the subchondral bone was limited to the Silk-NanoCaP layer. From the cross-sectional staining, it was observed low level deformation. In addition, de novo bone infiltration was observed in subchondral part, which shows a good integration of the scaffolds in the host tissue (Figure 11c, d). The defect control showed no regeneration of the cartilage layer and the subchondral bone (Figure 11e, f).

4. Discussion

It has been reported that the SF would undergo a conformation transition from random coil to β -sheet when addition of sodium chloride particles into the SF solution [30]. Recently, high strength SF scaffolds were developed by using more than 10 wt.% concentrated aqueous SF solution and salt-leaching approach [37]. Based on that study,

nanocomposite scaffolds composed of SF and nano calcium phosphate (Silk-NanoCaP) were also proposed by using an *in-situ* synthesis method [38]. Considering the superior properties of these scaffolds and the stratified structure of the OC tissue, a bilayered scaffold containing a silk layer and a Silk-NanoCaP layer was prepared for OCD regeneration.

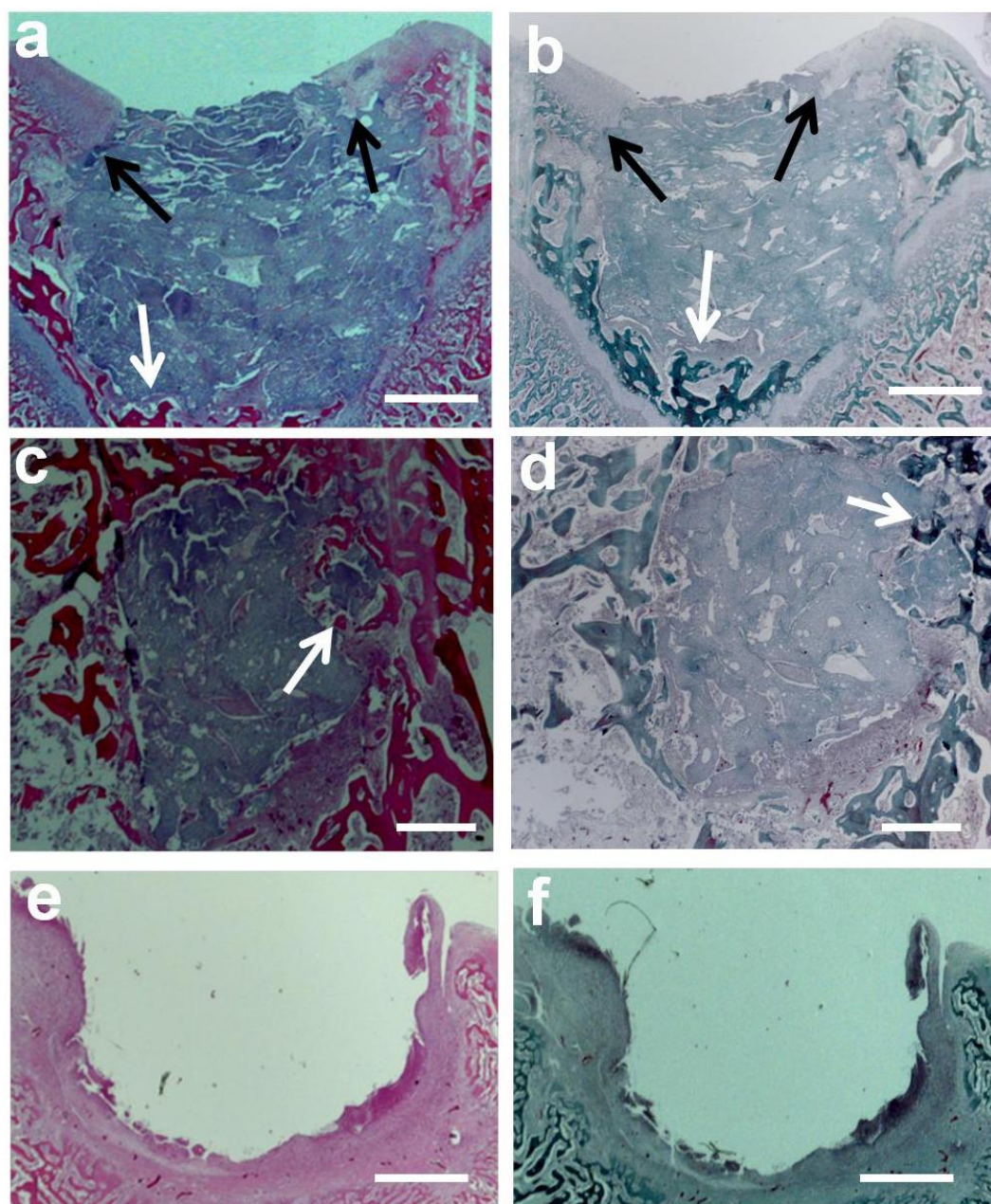


Figure 11. The histological analysis of the explants. (a, b) H&E staining and Masson's trichrome staining of the longitudinal section of the explants, respectively. (c, d) H&E staining and Masson's trichrome staining of the cross-section of the explants in the Silk-NanoCaP layer, respectively. (e, f) H&E staining and Masson's trichrome staining of the longitudinal section of the defect, respectively. The black arrow indicated neocartilage formation in the silk layer, and the white arrow indicated new subchondral bone formation inside the Silk-NanoCaP layer of the bilayered scaffolds. Scale bar: 1 mm.

Table 2. Porosity and CaP content of the explants

Region	Mean Porosity (%)	Mean CaP content (vol.%)
Cartilage layer of the defect control	58.80±9.35	0
Subchondral bone layer of the defect control	31.80±8.95	2.57±2.31
Silk layer of the explant	9.98±4.11	0.88±0.49
Silk-NanoCaP layer of the explant	8.05±1.99	22.67±7.12

Table 3. Compressive modulus of three-dimensional porous natural polymer scaffolds

Scaffold materials	Compressive modulus (KPa), dry state	Compressive modulus (KPa), wet state	Porosity (%)	Reference
Silk ^a	1,300±40	75	92±1.3	[30], [52],
Silk ^b	1,000±75	<10	98±1.0	[53], [54]
Silk ^c		~40	~93	[55]
Chitosan ^c		~35	~80	[55]
Collagen I ^d	-	6.31±0.33	75±8	[56]
Hyaluronic acid ^d		1.33±0.20	80±01	[56]
Gelatin ^e	801±108		97.51	[57]
Gelatin ^f	80±8		~97	[57]
Silk/CaP ^g	16,700±4,500	400±102	Table 1	This study

^aSalt-leached silk fibroin scaffolds derived from 8% aqueous silk solution. The dry and wet state modulus was from [30] and [52], respectively.

^bSalt-leached silk fibroin scaffolds derived from 17% silk solution dissolved in hexafluoroisopropanol. The dry and wet state equilibrium modulus was from [53] and [54], respectively.

^cFreeze-dried scaffold derived from 2% aqueous solution.

^dFreeze-dried scaffold derived 1% aqueous solution.

^eScaffold prepared by a combination of thermally induced phase separation and porogen leaching technique.

^fCommercial gelatin scaffold- Gelfoam®.

^gScaffolds prepared by a combination of salt-leaching/freezing-drying approach.

The ATR-FTIR spectra (Figure 1) demonstrated that the SF in both layers was of β -sheet conformation and this conformation would greatly affect the mechanical and biostability properties of the bilayered scaffolds. Figure 1 also confirmed that the CaP was successfully incorporated into the scaffolds, and was limited to the Silk-NanoCaP layer. The main challenge for preparation of a nanocomposite is to achieve an even distribution of particles in the system. The *in-situ* synthesis approach proposed in the present study allowed us to obtain a homogeneous distribution of the CaP nanoparticles. Comparing with the data in previous study on SC16, the CaP content and Ca/P ratio of the Silk-NanoCaP layer in the bilayered scaffold was quite similar to the ones of SC16 [38]. These results proved that the bilayered scaffolds maintained the chemical properties of the single layer scaffolds.

Tissue regeneration requires that scaffolds presents a porous and interconnected structure, as well as proper pore size and distribution [42]. The macroscopic image (Figure 2a) and the 3D micro-CT image (Figure 3a) confirmed the highly porous and interconnected structure in the bilayered scaffolds. More importantly, the salt-leaching method followed by freeze-drying allowed the achievement of a homogeneous porosity distribution in each layer of the scaffolds (Figure 3e). It has been reported that pore size larger than 300 μm was favorable for cell proliferation, nutrients exchanged, and new tissue formation in bone regeneration; and the micropores related with the surface roughness are good for cell attachment [42]. The macro/microporous structure in the subchondral layer is good for cell attachment and bone tissue filtration. By comparing with previous studies, the porosities and the interconnectivities of the silk layer and the Silk-NanoCaP layer were similar to the ones of S16 and SC16 (Table 1), respectively [37-39]. Furthermore, the CaP content (vol.%) of the Silk-NanoCaP layer present similar value to the one from the single layer SC16 [38]. Combining the chemical composition analysis and structure evaluation of the bilayered scaffolds, it was clearly evident that the silk layer and the Silk-NanoCaP layer maintained the properties of the S16 and SC16, respectively.

For preparation of the nanocomposite, the homogeneous dispersion of the nano-sized particles in another phase is a big challenge since the nanoparticles tend to aggregate. Previously, Liu *et al.* [43] physically combined the nano-sized hydroxyapatite particles and silk to prepare porous scaffold. Aggregation of hydroxyapatite was observed. When using hydroxyapatite particles of micro size, the particles may not be incorporated

homogeneously in the system [44]. The *in-situ* synthesis approach was able to prepare nano-sized apatite in the silk matrix. Oliveira *et al.* [45] prepared silk/hydroxyapatite nano-hybrid scaffold by dissolving silk in calcium chloride/ethanol/water system and subsequent addition of disodium hydrogen phosphate. The nano-sized hydroxyapatite dispersed finely in the silk matrix. Fan *et al.* [46] also prepared nano-hydroxyapatite/silk composite in ethanol calcium chloride/ethanol/water system in 75°C, and they found that rod-like hydroxyapatite crystals with diameter of around 20-30 nm and length of about 200-500 nm were formed. In the present work, using the *in-situ* synthesis method and combining the high concentration aqueous silk solution and the salt-leaching approach, nano-sized CaP particles were evenly distributed in the SF matrix in both microscopic (Figure 1, inserted) and macroscopic levels (Figure 3a, b).

Based on their adequate performance for bone regeneration [35], it is believed that CaP based materials also favor the subchondral bone regeneration. But, it was also reported that CaP can induce the hypertrophy of chondrocytes [47]. It is important to precisely control the distribution of the CaP in the bilayered scaffolds, to avoid its migration to the layer free of CaP (addressed to cartilage). EDX analysis of the interface (Figure 2b, c) demonstrated that the introduced CaP was clearly limited to the Silk-NanoCaP layer. Micro-CT analysis also corroborated SEM/EDX analysis (Figure 3a, b and f), and showing that the thickness of the interface between the two layers was around 0.5 mm (Figure 2b).

The mechanical property of the scaffolds is one of the main issues when addressing bone and cartilage tissue regeneration [48]. Improving the mechanical properties of the scaffolds is a big challenge for skeletal related tissue engineering. Some studies have been performed to produce silk based scaffolds of high strength for bone tissue engineering [49, 50]. The typical Young's modulus of articular cartilage was reported to be around 1 MPa [51]. The bilayered scaffolds presented a wet state compressive modulus around 0.4 MPa. Despite the different testing approach, this strength is comparable to the one of human articular cartilage (Figure 4b). The compressive modulus of the bilayered scaffolds was similar to the one of the controls (Figure 5a and b). This revealed that the silk layer and Silk-NanoCaP layer integrated very well and did not undermine the overall mechanical properties of the bilayered scaffolds. The differences in modulus between dry and wet states results from the hydrophilic domain in the SF molecular chains, such as the amorphous region in the backbones and the C- or

N-terminus, as demonstrated by [29]. Table 3 listed the mechanical properties of previously reported natural polymer scaffolds. By comparing with previous studies on silk based scaffolds derived from aqueous system or organic solvent system, the herein proposed bilayered scaffolds present superior compressive modulus [30, 52-55]. Furthermore, the modulus of the bilayered scaffolds was much higher than the ones of polysaccharide- and protein-based scaffolds (Table 3), such as chitosan [55], collagen [56], gelatin [57], and hyaluronic acid [56]. Besides the influence of β -sheet conformation, the improved mechanical properties result mainly from the high concentration of SF solution used for scaffold preparation which also led to the relative low porosity (Table 1 and 3). The scaffolds implanted in the body would undergo dynamic loading, thus it is crucial to know their dynamic mechanical properties in a physiologic condition. The storage modulus increased with increasing frequency (Figure 5c), indicating that the silk based scaffolds present good elastic properties. Furthermore, it is demonstrated that the binding strength of the two layers was excellent, as the bilayered scaffolds kept their integrity under high frequency loading. In the tested frequency range, the storage modulus values of the bilayered scaffolds were similar to the ones of SC16 and higher than the ones of S16. This may be induced by the good integration of the two layers. The low loss factor showed that these scaffolds were of low viscosity and high elasticity.

Hydration properties of the scaffold are important for cell attachment and tissue infiltration. They are also important for the mineralization process in case of the Silk-NanoCaP layer. The high hydration degree of the bilayered scaffolds results from the high porosity of the scaffolds and the hydrophilic groups in the backbone of SF (Figure 6a). From the SEM and micro-CT analysis, it was found that the scaffolds were highly porous and showed more than 80% and higher than 60% porosity in the silk layer and the Silk-NanoCaP layer, respectively. Additionally, there are short and hydrophilic chains distributed in the GAGAGS repetitive hydrophobic domains in the heavy chain, in the SF molecular chains [29]. These hydrophilic chains together with the C- and N-terminus also contributed to the high hydration degrees (Figure 6a). It was also noticed that the dimensional changes of the scaffolds were less than 5% after hydration, which is good for the implantation.

In vitro mineralization assay is a method to screen the materials potential for bone regeneration [40]. The results demonstrated that the amount of CaP present in the Silk-NanoCaP layer induced the precipitation of an apatite layer after 1 day of immersion

(Figure 6a). Moreover, the results showed that only the Silk-NanoCaP layer was able to promote apatite formation (Figure 6). This result means that the CaP in the Silk-NanoCaP layer was stable enough not to create a severe ionic unbalance in the SBF solution at the surface of the silk layer, which could induce additional mineralization in that region. SF has certain amount of hydrophilic groups in the backbone and the terminus which are helpful to induce the formation of stable CaP crystals. SF had been used to regulate the mineralization of hydroxyapatite in wet state by Kong *et al.* [58]. The authors found that there were strong interaction between calcium ions and the carboxyl groups in SF which greatly contributed to the formation of the nano hydroxyapatite crystals. In the present study, the calcium ions were first introduced into the SF solution. The formed complexes interacted with phosphate groups and subsequently generated the nucleis for CaP crystal growth. Later on, it is expected that the formation of the β -sheet conformation in the SF molecular chains further increased the affinity bewteen the CaP crystals and the SF matrix. Thus, the formed CaP crystals were stable in the Silk-NanoCaP layer even after the salt-leaching procedure. These results showed that the Silk-NanoCaP layer in the bilayered scaffolds can be helpful for subchondral bone integration and regeneration.

In an ideal tissue engineering approach the scaffolds should degrade in the body after implantation. In previous studies, it was showed that the salt-leaching scaffolds gradually lost weight when immersion in isotonic solution for one year [39]. SC16 displayed a slightly higher weight loss profile as compared with S16, due to the dissolution of CaP. When implanted, the scaffolds would contact with the body fluid which is rich in enzymes. The study of the enzymatic degradation of the scaffolds is of great importance to predict the *in vivo* stability of the scaffolds. The degradation results showed that SF scaffolds degraded faster than the SF scaffolds incorporated with nano-CaP (Figure 6b). The bilayered scaffold, containing both silk layer and Silk-NanoCaP layer, presented a degradation ratio between the observed for S16 and SC16.

In order to achieve good tissue regeneration outcomes, it is necessary to evaluate the cytotoxicity of the scaffolds by seeding cells onto the scaffolds and observing the cells viability, attachment, proliferation, and differentiation behaviors. Bone marrow stromal cells are multipotent somatic stem cells and can be differentiated into osteoblasts, chondrocytes, and adipocytes [16]. It has been studied as cell source for OCD regeneration [16]. In this study, RBMSCs were seeded onto the bilayered Silk/Silk-

NanoCaP scaffolds. The live/dead staining images and the SEM images indicated that the bilayered scaffolds were non-cytotoxic, and supported the cell viability, adhesion, and migration (Figure 7). MTS assay can reflect the cells' metabolic activity, thus it is commonly used to evaluate the materials cytotoxicity. It was noticed that the cells presented increased metabolic activity from day 1 to day 7 (Figure 8a). The bilayered scaffolds also supported the RBMSCs proliferation (Figure 8b). The *in vitro* studies indicated that the bilayered scaffolds were cytocompatible. The outstanding performance of the bilayered scaffolds for cell seeding was related with the intrinsic properties of the SF and CaP, as well as the porous structure of the scaffolds. SF fiber has been used as a suture for the wounds for a long time [59]. It is compatible with human tissues. As a protein based biomaterial, the degraded by-product of SF is amino acids which are also compatible to the body and will not induce severe inflammation. SF has been prepared into membrane and scaffolds for cell culture or implantation, and the outcomes were satisfied [29]. CaP based materials, presenting similar chemical properties to the inorganic phase in bone, thus have been developed into implantation materials for bone regeneration [60]. The salt-leaching/freeze-drying approach endowed the bilayered scaffolds with high porosity and interconnectivity which are important for supporting cell ingrowths, migration, proliferation, and nutrients transportation.

The ability of the scaffolds for supporting the RBMSCs differentiation was evaluated. ALP is an important marker of osteogenesis differentiation. The RBMSCs culture in osteogenic condition secreted higher level of ALP compared to those cultured in basal condition (Figure 8c). This result was attributed to the standard osteogenic culture condition. Interestingly, the Silk-NanoCaP layer induced a higher ALP activity as compared to the silk layer, when the bilayered scaffolds were cultured in osteogenic medium (Figure 8c). This indicated that the incorporated CaP facilitated the RBMSCs toward osteogenic differentiation. The observation from the S16 and SC16 also confirmed this conclusion. Our observation is in line with the previous study on CaP/Silk hybrid scaffolds [61]. In that study, the CaP/Silk scaffolds induced higher ALP activity compared to the one of the silk scaffold. The ALP activities of the silk layer and the Silk-NanoCaP layer were similar to the ones of S16 and SC16, respectively (Figure 8c). This result confirmed that the silk layer was not affected by the Silk-NanoCaP layer during the RBMSCs osteogenic differentiation, and the Silk-NanoCaP layer was suitable for subchondral bone regeneration.

The compatibility between the bilayered scaffolds and the *in vivo* tissues was first analyzed by subcutaneous implantation. Due to the crystalline structure of the SF, the bilayered scaffolds maintained their integrity and kept their shapes. This result also revealed that the scaffolds retained good mechanical strength *in vivo*, which is in good agreement with the *in vitro* wet compressive modulus analysis. These scaffolds can support the tissue ingrowths and angiogenesis, presented good biocompatibility *in vivo*, and only induced minimum foreign body reaction (Figure 9). The high porosity and interconnected structure (Figure 2 and 3) contributed to the tissue ingrowth and angiogenesis of the bilayered scaffolds. Similarly, previous study on SF fiber reinforced SF scaffolds showed inflammatory cells surrounded all the scaffolds at the first week and fewer inflammatory cells at the fourth week, in a subcutaneous mice model [49].

To evaluate the potential of the bilayered scaffolds for OCD regeneration, rabbit OCD was used as a model in this study. The OCD without treatment (control) showed no regeneration (Figure 10a), which were related with the specific avascular structure in the cartilage and the low metabolic activity of chondrocytes. More importantly, the empty defects induced the collapse of the neighboring tissues, owing to the changes of the homeostasis in the joint. When implanted with the bilayered scaffolds, the defects did not enlarge and no collapse of neighboring tissue was observed. The implanted scaffolds showed firm integration with host tissue and desirable stability after 4 weeks of implantation (Figure 10a). Previously, it was reported that the collagen/nano-hydroxyapatite scaffolds (MaioRegen®) would swell when contacted the blood, inducing the fixing problem [21]. In this study, the fixing of the scaffolds was easy and the scaffolds maintained their dimensions during the implantation time periods. These results indicated that the bilayered scaffolds were able to bear the mechanical loading in the OC environment and did not swell when contacted with body fluid, which is of good clinical relevance. Besides the good stability observed for the bilayered scaffolds, these scaffolds also promoted the subchondral bone regeneration (Figure 10d and Table 2). Based on the scanning condition used for the explants during the micro-CT analysis, the CaP content detected in the Silk-NanoCaP layer mainly came from the newly formed subchondral bone. In a clinical trial, it was reported that the failure in the reconstruction of patellar came from the slow regeneration of subchondral bone [22]. The promising results in this study showed that the implantation of the bilayered scaffolds in a rabbit knee osteochondral critical size defect can induce fast subchondral bone integration and

healing. The fast formation of new subchondral bone is critical to fix the implant in the defect site, as well as to provide mechanical support to the regeneration of cartilage layer. In the empty defect, only little amount of subchondral bone was formed (Figure 10d).

Since no acute inflammatory reaction was observed, the histological analysis further confirmed the *in vivo* biocompatibility of the bilayered scaffolds as shown in Figure 11. Together with the encouraging *in vitro* performance and the promising subcutaneous implantation data (Figure 8 and 9), it can be stated that the bilayered scaffolds presented adequate properties for OCD regeneration. A proper scaffold for OCD regeneration should possess mechanical strength compatible to the dynamic mechanical environment in OC. Otherwise the scaffolds will lead to abnormal tissue regeneration, due to the unmatched mechanical homeostasis and deformation of the scaffolds. The histological analysis results revealed that the bilayered scaffolds were able to bear the mechanical loading in the OC environment (Figure 11a, b). This observation is consistent with the results obtained in the wet state compressive test and the DMA analysis (Figure 4b and c). The histological staining confirmed that the OCD with scaffolds showed no collapse, and new cartilage formation in the silk layer and new subchondral bone formations in the Silk-NanoCaP layer were observed (Figure 11a, b). Obvious subchondral bone formation was further approved by the cross sectional staining (Figure 11c, d).

The aim of applying the bilayered scaffolds for OCD repair is to spatially control the regeneration of cartilage and subchondral bone [13]. The present study results clearly showed that the bilayered scaffolds were of outstanding biocompatibility when implantation in the OCD. The developed bilayered scaffolds proposed herein supported the cartilage regeneration in the top silk layer, and promoted subchondral bone ingrowths in the bottom Silk-NanoCaP layer. Importantly, the bilayered scaffold were able to replace the role of the normal OC tissue in the defect site, in terms of undertaking the dynamic mechanical loading during the new OC tissue regeneration.

Although the long-term study is necessary to evaluate the final outcome of the cartilage and subchondral bone regeneration, the preliminary *in vivo* data of the bilayered scaffolds together with their other desirable performance confirmed that the Silk/Silk-NanoCaP bilayered scaffolds are suitable for OCD regeneration.

5. Conclusions

This study proposed novel porous bilayered scaffolds, built up by fully integrating a SF layer and a Silk-NanoCaP layer for OCD regeneration. The *in situ* synthesis route allowed controlling the size of hydroxyapatite particles in the bone-like layer. These scaffolds presented superior mechanical properties and suitable stability due to the β -sheet conformation in the SF and the high concentration SF aqueous solution for scaffold preparation. Spatially controllable porosity and CaP distribution and confinement were also obtained in these bilayered scaffolds. Apatite formation was induced after immersion in SBF solution, clearly confined to the Silk-NanoCaP layer. This layer promoted higher ALP activity when seeded with RBMSCs and cultured in osteogenic condition as compared to the silk layer. The scaffolds supported cells' attachment, viability, and proliferation when cultured with RBMSCs *in vitro*. Furthermore, these scaffolds allowed tissue ingrowth and induced very weak foreign body reaction when subcutaneously implanted in rabbit for 4 weeks. When implanted in the knee critical OCD in rabbit for 4 weeks, the bilayered scaffolds were integrated well with the host tissues and induced no acute inflammation. These scaffolds were able to match the mechanical environment of the OCD and maintain their stability. Moreover, the bilayered scaffolds supported the cartilage regeneration in the top silk layer. Promisingly, large amount of subchondral bone ingrowths was achieved only in the bottom Silk-NanoCaP layer. These promising results demonstrated that the bilayered scaffolds prepared in this study are good candidate for OCD tissue engineering applications.

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Section 4.

Chapter VIII

A Novel Silk Fibroin Hydrogel for Tissue Engineering and
Regenerative Medicine Applications

Chapter VIII

A Novel Silk Fibroin Hydrogel for Tissue Engineering and Regenerative Medicine Applications

Abstract

Up to now the general methods to prepare silk fibroin (SF) hydrogels are based on a SF conformation transition from amorphous to β -sheet in aqueous status. The main drawbacks of these methods are the long gelation time and harsh preparation conditions, which hinder the application of SF as an injectable system for cells' encapsulation and drug delivery. The present work provides a novel route for obtaining a SF hydrogel within a few minutes under physiological conditions, via peroxidase mediated cross-linking. The prepared hydrogels are of mainly amorphous conformation and transparent appearance. The gelation time of the SF hydrogels can be modulated within 5 minutes. The storage modulus ranges from around 200 Pa to around 5 kPa. Surprisingly, the enzymatically cross-linked SF hydrogels are ionic strength and pH stimuli responsive, *i.e.* it swells in solutions of low ionic strength or pH above 9, and shrink in solutions of high ionic strength or pH below 4. Additionally, the SF hydrogels are capable of incorporating cells and support their viability up to 7 days. The *in vivo* results showed that the SF hydrogels did not induce any acute inflammatory reaction, after 2 weeks subcutaneous implantation in mice model. These SF hydrogels underwent a β -sheet conformation transition after *in vitro* cell encapsulation for 10 days and *in vivo* implantation for 2 weeks. This study provides a facile approach to produce injectable SF hydrogels of dual stimuli response

This chapter is based on the following publications: (1) **Yan LP**, Silva-Correia J, Correia C, da Silva Morais A, Sousa RA, Oliveira AL, Oliveira JM, Reis RL. A Novel Silk Fibroin Hydrogel for Tissue Engineering and Regenerative Medicine Applications. 2014, Submitted.

(2) **Yan LP**, Oliveira AL, Oliveira JM, Pereira DR, Correia C, Sousa RA, Reis RL. Hydrogels Derived from Silk Fibroin: Methods and Uses Thereof. National Patent, Nr. 106041. Priority date: 06-12, 2011.

properties. The superior biocompatibility and fast gelation property allow the SF hydrogels to be used as an injectable material for filling tissue defects *in vivo* (such as defects in bone, cartilage, and so on), as a whole or combined with drugs or other bioactive factors. The enzymatically cross-linked SF hydrogels can also be used as drug delivery system, wound dressing, and biomedical optical device. These mechanically stable amorphous SF hydrogels open up new avenues for silk based biomaterials development, providing a useful model to elucidate the interactions between the protein conformation and hydrogel properties.

1. Introduction

Hydrogels are hydrophilic polymer networks which can absorb large amount of water and are insoluble due to its cross-linked structure [1]. Hydrogels can resemble the extracellular matrix (ECM) microenvironment, thus it have been extensively used in biomedical devices fabrication, drug delivery systems, tissue engineering and regenerative medicine [2]. Even though great advances have been achieved, development of novel hydrogel systems with improved physicochemical properties aiming for controlling cell fate and improving regeneration outcome is still a big challenge [3, 4].

Recently, hydrogels show great promise as model systems to understand the fundamental crosstalk between the microenvironments and the cells [3, 5]. Further dynamic manipulations of the hydrogels' physicochemical properties have been performed with light to achieve temporal and spatial regulation [4, 6]. The incorporation of growth factors or the micropatterning ECM proteins in the hydrogels brought new insights for future studies [7, 8]. Due to its clinical relevance the development of cytocompatible and injectable hydrogels is receiving specific attention [9, 10]. These systems can be easily applied through a minimal invasive injection procedure that will fill a defect site of any shape. They can also combine cells, drugs, growth factors, peptides, and genes with the precursor solutions before injection, and subsequently injected to form hydrogels in the site of application.

Numerous materials, including natural and synthetic polymers, have been studied for hydrogels' development [11, 12]. Naturally derived hydrogels attracts a special interest since they may provide important chemical cues to the cells due to its resemblance to the

natural extracellular matrix. The most studied natural hydrogels including hyaluronic acid, chitosan, alginate, fibrin, collagens, and gelatin [11]. Silk fibroin (SF) derived from *Bombyx mori* silkworm has been particularly studied as a biomaterial for tissue engineering [13, 14]. Its biodegradability and biocompatibility has been extensively validated *in vitro* and *in vivo*. SF is a versatile material and can be processed into different formats, such as membranes, sponges, fibers, non-woven/woven nets, hydrogels, etc. [15-20]. Normally, SF hydrogels are formed together with a structure transition from amorphous (random coil) into β -sheet (Silk II), which can happen by means of addition of solvents, decreasing the pH value or increasing the temperature/ion concentration in the aqueous silk solution [20-23]. The gelation time of SF hydrogels prepared by the above mentioned methods is typically very long, from hours to days. In order to shorten the gelation time, methods using external stimuli are explored, for example ultrasonication, vortex, and electrical stimuli [24-26]. In the case of ultrasonication treatment, the gelation time of SF hydrogels has decreased from 0.5 to 2 hours when encapsulated with cells [25]. Vortex induced SF hydrogels can be obtained within 2 hours after the stimulus [24].

Various approaches have been developed to prepare *in-situ* formed hydrogels, through chemical and physical methods [10]. Recently, several enzymatically mediated cross-linking approaches have shown promising potential to be used as injectable systems [27]. Comparing to other harsh cross-linking systems, the enzyme-mediated cross-linking system displays several advantages. The enzyme mediated gelation allows obtaining hydrogel systems at physiologic conditions, which is compatible for cell encapsulation and bioactive factors delivery [28]. Furthermore, it allows the cross-linking to occur *per se*, without any external stimulus. Moreover, the gelation time can go from a few seconds to some minutes, fulfilling many clinical application requirements [28]. In a pioneer work by Sofia *et al.* [29], it has been reported that the phenol group in tyrosine or tyramine can be conjugated to each other when catalyzed by peroxidase and hydrogen peroxide. Peroxidase mediated cross-linking of poly(aspartic acid) hydrogel was then achieved by functionalization of poly(aspartic acid) with phenol group-containing small molecules, such as tyrosine, tyramine and aminophenol. Following this work, other *in situ* forming hydrogels derived from different polymers were explored via enzyme mediated cross-linking [30-33]. The cell encapsulation behavior and *in vivo* biocompatibility test of these hydrogels were performed and promising outcomes have been achieved [28, 31, 34].

Even though great improvements have been made, ideal *in situ* injectable SF hydrogels were not achieved yet. To address this problem, the chemical composition of SF should be taken into account. Besides the large amount of glycine, alanine, serine in the backbone, SF also contains some reactive amino acid residues, such as tyrosine (Around 5 mol. %) [35]. The object of the present study is to develop an injectable fast formed SF hydrogel, capable for cell encapsulation and drug delivery applications, via peroxidase mediated cross-linking. In this way, the structural conformation and other physicochemical properties of the prepared SF hydrogels were characterized and the cytotoxicity and *in vivo* biocompatibility investigated.

2. Materials and Methods

2.1. Materials and reagents

Cocoons of *Bombyx mori* were provided by the Portuguese Association of Parents and Friends of Mentally Disabled Citizens (APPACDM, Castelo Branco, Portugal). Horseradish peroxidase (HRP, type VI, 260 U/mg) was purchased from Sigma-Aldrich (St. Louis, MO, USA). All the other reagents or materials were supplied by Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated.

2.2. Preparation of silk solution and hydrogels

SF was purified via removing the sericin from the cocoon in 0.02 M boiling sodium carbonate solution for 1 hour [36, 37]. The purified SF was dissolved in 9.3 M lithium bromide solution in an oven (BE500, Memmert, Schwabach, Germany) at 70°C for 60 minutes, followed by dialysis against distilled water for 48 hours in a benzoylated dialysis tubing (MWCO: 2 kDa). And then the SF solution was dialyzed in 0.2 M phosphate buffered saline (PBS, without calcium and magnesium ions) solution for 12 hours before concentration by 20 wt.% poly(ethylene glycol) solution. The final concentration of the SF was determined by drying the concentrated SF solution in the oven at 70°C overnight. The saline content in the SF was 1.73 ± 0.03 wt.% tested by thermal gravimetric analysis (TGA Q500, TA Instruments, DE, USA). The prepared SF solutions were stored in a room at temperature between 4-8°C before use. HRP solution (0.84 mg/mL) and

hydrogen peroxide solution (H_2O_2 , 0.36 wt.%) were prepared respectively in PBS solution. The SF solutions (pH 7.0-7.1) were diluted into 10%, 12% and 16 wt.% by PBS solution and used for the hydrogel preparation. SF hydrogels were prepared by mixing 1 mL SF solution with varied amount of HRP and H_2O_2 solutions in a 1.5 mL centrifuge tube (Eppendorf, Hamburg, Germany), and then the mixture were warmed in a water bath of 37°C . These formulations were achieved after some optimization. The gelation time was determined by inverting the vial, and observing no flow within 60 seconds was considered as the gel status. SF hydrogel discs were also prepared by the addition of 200 μL the mixture solutions in a polypropylene mold (8 mm in diameter, 5 mm in height), followed by placing the mold in the oven at 37°C . These discs hydrogels were used for the following test unless otherwise mentioned. The SF hydrogels prepared from 10, 12 and 16 wt.% SF solutions were denoted as Silk-10, Silk-12 and Silk-16, respectively. The SF hydrogels can also be prepared using SF solutions without dialysis in PBS solution.

2.3. Physicochemical characterization of the SF hydrogels

2.3.1. Structural characterization

SF solution of 16 wt.%, and HRP/SF and H_2O_2 /SF fixed at 0.26‰ and 1.1‰ (by wt.) respectively, were selected to prepare samples for the structural characterization. The optical absorbance of the SF before and after gelation was recorded by a microplate reader (Synergy HT, Bio-Tek, VT, USA). A mixture of the SF, HRP and H_2O_2 solutions (100 μL) was placed in a 96-well quartz plate (well diameter 7 mm) and read from 280-370 nm before and after gelation. Then, 50 μL of the same mixture was also placed into the quartz plate and read from 450-800 nm before and after gelation. The gelation of the mixture was performed by sealing the quartz plate with paraffin film (Parafilm, Pechiney Plastic Packaging Company, IL, USA), followed by placing the quartz plate in the oven at 37°C . SF solution, mixture of SF/HRP/ H_2O_2 , and formed SF hydrogels were further analyzed by attenuated total reflectance (ATR) model in a Fourier transform infrared spectroscopy (FTIR) equipped with a germanium crystal (IRPrestige-21, Shimadzu, Kyoto, Japan). Each specimen was scanned 48 times from $600\text{--}2000\text{ cm}^{-1}$ with a resolution of 4 cm^{-1} in wet state [38]. PBS solution was used as background in the FTIR.

2.3.2. Mechanical properties determination

The storage and loss moduli of the SF hydrogels were evaluated by using oscillatory model in a rheometer (MCR 300, Anton Paar, Graz, Austria), equipped with a cuvette accessory (CC10/Q1). The radiuses of the measuring bob and cup were 5.000 and 5.420 mm, respectively. The length of the gap was 14.985 mm, with a cone angle of 120°. For each measurement, 1 mL SF solution was mixed with varied amount HRP and H₂O₂, and then 1 mL of the mixture was transferred into the cup. The bob was immersed into the solution, followed by addition of one drop dodecane onto the surface of the solution. For the measurement of the modulus, the time sweep was first performed under constant strain (0.1%) and frequency (0.5 Hz) until the gel formed and reached a stable state, indicating by appearing a plateau in the storage and loss moduli curves. The storage and loss moduli were determined by averaging the values in the plateau. After the plateau of the storage modulus was reached, the frequency sweep (from 0.1-20 Hz) was conducted for 5 minutes with strain fixed at 0.1%. The strain sweep (0.1-100%) was following the frequency sweep and carried out for another 5 minutes under constant frequency at 1 Hz. All the data points were collected twice per minute for time sweep, frequency, and strain sweep. All measurements were conducted at 37°C.

2.3.3. Swelling ratio and degradation profile

The prepared discs Silk-10, Silk-12 and Silk-16 were used for the swelling and degradation study. Three formulations were used for each group hydrogels: 1/0.26‰/1.1‰, 1/0.52‰/1.1‰ and 1/0.26‰/1.45‰ (SF/HRP/H₂O₂, by wt.). The swelling ratios of the hydrogels were tested in both ultrapure water and PBS solution. Each piece of hydrogel was placed in a tube with 50 mL PBS solution or ultrapure water (0.55 uS/cm) prepared by a ultrapure water system (Genpure UV/UF, TKA GenPure, Niederelbert, Germany), subsequently the samples were placed in a thermostatic water bath (OLS200, Grant Instruments, Cambridgeshire, UK) at 37°C. The wet weight of the sample was measured at 1, 3, 6 and 12 hours. Before weighting, surface liquid in the hydrogels were absorbed by tissue. The ultrapure water was refreshed at the end of the first and the third hour. After 12 hours, the samples were dried in the oven at 70°C overnight. The swelling ratio at each time point was calculated as following equation 1:

$$\text{Swelling ratio (\%)} = \left(\frac{w_t - w_d}{w_d} \right) \times 100\% \quad (1)$$

In Equation 1, w_t referred to the wet weight of the sample tested in different time point, and w_d is the dry weight of the sample. It was assumed that the dry weight of each specimen was constant during the tested time period.

Protease XIV from *Streptomyces griseus* was used for the degradation study [25]. Each specimen was immersed in 5 ml PBS solution and placed in the oven at 37°C overnight. And then the wet weight of each specimen was recorded before the addition of 5 mL protease XIV solution. The protease XIV solution was prepared in PBS solution and yielded a concentration of 0.005 U/mL. The samples were placed in the thermostatic water bath at 37°C. The wet weight of each specimen was measured at 1, 2, 4, 6 and 12 hours. The weight loss ratio was defined as following equation 2:

$$\text{Weight loss ratio} = \left(\frac{w_i - w_t}{w_i} \right) \times 100\% \quad (2)$$

In Equation 2, w_i means the initial wet weight of the hydrogel, and w_t is the wet weight tested at each time point.

2.3.4. Ionic strength responsiveness

The Silk-16 hydrogels with formulation of 1/0.26%/1.45% (SF/HRP/H₂O₂, by wt.) were used for the stimulus response studies. For the ionic strength response test, the hydrogels were first alternately immersed in PBS solution and distilled water. Secondly, the hydrogels were also alternately placed in 0.154 M and 2 M sodium chloride solution. In the first part, each discs hydrogels was immersed in 5 mL PBS solution (pH 7.4) in a vial and kept in the oven at 37°C overnight, followed by measuring the diameter of the hydrogels and subsequently placing each hydrogels in 100 mL distilled water (pH 7.0-7.1, conductivity: 2.0 μS/cm) in a plastic bottle. And then the hydrogels were alternately immersed in distilled water and PBS solution every 12 hours. The samples were placed

in the thermostatic water bath at 37°C. Before every change between distilled water and PBS solution, the diameter of the hydrogel was measured. During each immersion procedure, the distilled water or PBS solution was refreshed at the third hour. For the second part, the prepared hydrogels were immersed in 5 mL 0.154 M sodium chloride solution (pH 7.4, adjusted by 1.0 M sodium hydroxide) in a vial and placed in the oven at 37°C overnight, and then the wet weight of each hydrogel was measured. Each hydrogel was then alternately immersed in 100 mL 2 M sodium chloride solution (pH 7.4, adjusted by 1 M sodium hydroxide) and 100 mL 0.154 M sodium chloride solution (pH 7.4) every one hour. The samples were placed in the thermostatic water bath at 37°C. Before every change of the solution, the wet weight of the hydrogel was recorded. And the wet weight variation ratio was calculated as following equation 3:

$$\text{Wet weight variation ratio (\%)} = \left(\frac{w_t - w_i}{w_i} \right) \times 100\% \quad (3)$$

In Equation 3, w_i means initial weight of the hydrogel after overnight immersion in 0.154 M sodium chloride solution, and w_t refers to the wet weight tested during the alternating immersion. The prepared discs hydrogels were also immersed in methanol for 3 hours, or in hydrochloric acid solution (pH 2.0) overnight to undergo β -sheet conversion, and then the opaque hydrogels were used as control for the ionic strength response test, as well as for swelling ratio and degradation tests.

2.3.5. pH responsiveness

Hydrogels of the same formulations used in the ionic strength response test were used for this test. Before the test, the specimens were immersed in 0.154 M sodium chloride solution (pH 7.4,) at 37°C overnight. This test included two parts. In the first part, the initial wet weights of the hydrogels were measured and then the discs were immersed in 100 mL sodium chloride solutions of different pH values (37°C): 2.5, 3.0, 4.0, 7.4, 9.0, 10.0 and 10.5. The ionic strength of these solutions was fixed at 0.154 M. After 2 hours, the wet weights of the hydrogels were recorded again after removing surface liquid. The basic solutions (pH above 7.0) were prepared by addition of disodium hydrogel

phosphate into the sodium chloride solution (0.137 M) and the pH values were adjusted by addition of sodium hydroxide solution. And the acid solutions were produced by supplementation of sodium dihydrogen phosphate into the sodium chloride solution (0.137 M) and the pH values were tuned by employing hydrochloric acid solution. The concentration of the phosphate buffered saline was fixed at 1 mM in all the solutions. Sodium chloride was added to modulate the final ionic strength to 0.154 M if necessary. In the second part, the overnight immersed hydrogels were alternately immersed in the above mentioned 100 mL basic (pH 10.5) and acid (pH 3.0) sodium chloride solutions, after measuring the initial wet weight. Before each change of the solutions, the wet weights of the samples were noted. The wet weight variation ratio was calculated as Equation 3. The prepared discs hydrogels were also immersed in methanol for 3 hours to undergo β -sheet conversion, and then used as control.

2.4. Cell encapsulation and cytotoxicity

SF solution of 16 wt. % were sterilized by UV radiation for 15 minutes in a sterile cabinet and used for the cell encapsulation. ATDC-5 (European Collection of Cell Cultures, Salisbury, UK) was expanded in basal α -MEM medium, supplemented with 10% fetal bovine serum, and 1% Antibiotic-Antimycotic liquid (Life Technologies, Carlsbad, CA, USA). The cells were incubated in a CO₂ incubator under an atmosphere of 5% CO₂ at 37°C, with medium change every two days. As the cells reached around 90% confluence, they were detached from the culture flask by using TrypLE Express (1X) (Life Technologies, Carlsbad, CA, USA), and a diluted cell suspension (2×10^6 cells/mL) was prepared. The cell encapsulation procedure was performed under aseptic condition. Cell suspension containing 1 million cells was placed in a 1.5 mL centrifuge tube and subsequently centrifuged. A cell pellet was obtained after remove the supernatant. The SF solution (1 mL) was mixed with the HRP and H₂O₂ solutions and the mixture was warmed in the water bath (37°C) for 6 minutes. Two formulations were used: 1/0.26‰/1.1‰ and 1/0.26‰/1.45‰ (SF/HRP/H₂O₂, by wt.). A warmed mixture (1 mL) was mixed with the cell pellet and got a homogeneous cell suspension, and every 50 μ L of the cell suspension was transferred into one piece of cover slips with 13 mm diameter (Sarstedt, Newton, NC, USA) in a 24-well suspension cell culture plate. The plate was then placed into the CO₂ incubator for around 10 minutes to allow the gelation. After the

gel was formed, 1 mL basal α -MEM medium was supplemented into each well, and the medium was changed every two days. The Live/Dead of the incorporated cells was evaluated by Calcein AM and propidium iodide (Molecular Probes[®]; Life Technologies, Carlsbad, CA, USA) staining, after culturing for 1, 3, 7 and 10 days. For this assay, the hydrogels with cells were washed by PBS solution, and then immersed in 1 mL PBS solution supplemented with 1 μ g Calcein AM and 2 μ g propidium iodide for 10 minutes. The samples were observed in a transmitted and reflected light microscope (Axio Imager Z1m, Zeiss, Jena, Germany) after washing by PBS solution. Cell viability was also confirmed by a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay (MTS) (Promega, Fitchburg, WI, USA), at 1, 4, 7 and 10 days. For this assay, 500 μ L MTS working solution was added into each well, followed by incubated for 3 hours. The absorbance of 100 μ L supernatant from each well was read in a microplate reader (Synergy HT, Bio-Tek, VT, USA) at 490 nm. Hydrogels without cells were used as control.

The hydrogels encapsulated with cells were frozen and then lyophilized, after culturing for 6 and 10 days, respectively. The morphology of the hydrogels was observed by scanning electron microscopy (SEM). Before the SEM (Nova NanoSEM 200, FEI, Hillsboro, OR, USA) observation, the samples after coated with a layer of Au/Pd SC502-314B in an evaporator coater (E6700, Quorum Technologies, East Grinstead, UK).

2.5. *In vivo* implantation

The maintenance and use of animals were in accordance to the Ethics Committee of University of Minho. Silk-16 hydrogels with the same formulations for cell encapsulation were used for the *in vivo* implantation. The hydrogel discs (Diameter: 8 mm; Height: 3 mm) were prepared in a sterile condition using the sterilized silk solution. 4 Mice Hsd:ICR (CD-1) of 5 weeks old and average weight of 32 g (Charles River, Senneville, Quebec, Canada) were used for this study. Each mouse was anesthetized by intraperitoneal injection of 100 μ L of a mixture of Imalgene (Ketamina, 75 mg/Kg) and Domitor (Medetomidina 1 mg/Kg). If necessary, 50 μ L Antisedan (Atipamezol, 1 mg/Kg) was used to reverse the anesthesia. The hair in the implantation area of the mouse was removed by shaving, followed by disinfected by scrubbing with tincture of iodine. In each mouse, 4 skin incisions were made in the back near the midline below the ear, two in the right side

and another two in the left side. In the following, 4 pieces of hydrogel discs were implanted subcutaneously into respective pocket and the skin was sutured. For each formulation, 8 pieces of hydrogel discs were implanted. After 2 weeks post-surgery, the mice were euthanized by injection of overdose pentobarbital sodium, and the implants were retrieved. The explants were fixed by 10% formalin solution for 1 day at 4°C, followed by dehydration in grade ethanol solution (from 30% to 100%). And then the samples were embedded in paraffin, and slides were prepared by cutting the specimen into sections of 5 μm thick using a microtome (Spencer 820; American Optical Company, NY, USA). The sections were then stained with haematoxylin and eosin (H&E).

The SF hydrogels without fixing in formalin were analyzed by ATR-FTIR, following the same procedure mentioned in section 2.3.1. Before the analysis, the surface of the hydrogels was cleaned by removing the wrapping tissues and washing by PBS solution.

2.6. Statistical analysis

The data were presented by mean $\pm$ standard deviation (SD). The results were analyzed by one-way analysis of variance (ANOVA). The means of each group were compared by Tukey's test, and $p < 0.05$ was considered statistically significant.

3. Results

3.1. Structural characterization

SF hydrogel was successfully developed via HRP mediated cross-linking in physiological condition and presented transparent appearance, as presented in Figure 1a. The optical absorbance of the SF hydrogels was evaluated in both the visible light and the UV light ranges, respectively. When the wavelength increased from 450 to 800 nm, the optical density of the SF hydrogel, the SF solution, and the mixed solution of SF/HRP/H₂O₂ presented similar profiles, and all gradually decreased from around 0.02 to near 0 (Figure 1d, e). Regarding the UV absorbance, the formed SF hydrogel showed higher absorbance values in the range of 300-340 nm, compared with the one of the mixed solution of SF/HRP/H₂O₂ (Figure 1b). The ATR-FTIR spectra (Figure 1c) showed that one of the absorbance peaks of the hydrogels was at 1650 cm^{-1} , almost the same

position with the ones from the silk solution and the mixed solution of SF/HRP/H₂O₂, which were located at 1649 cm⁻¹. Additionally, all these three spectra presented absorbance peaks at 1538 cm⁻¹.

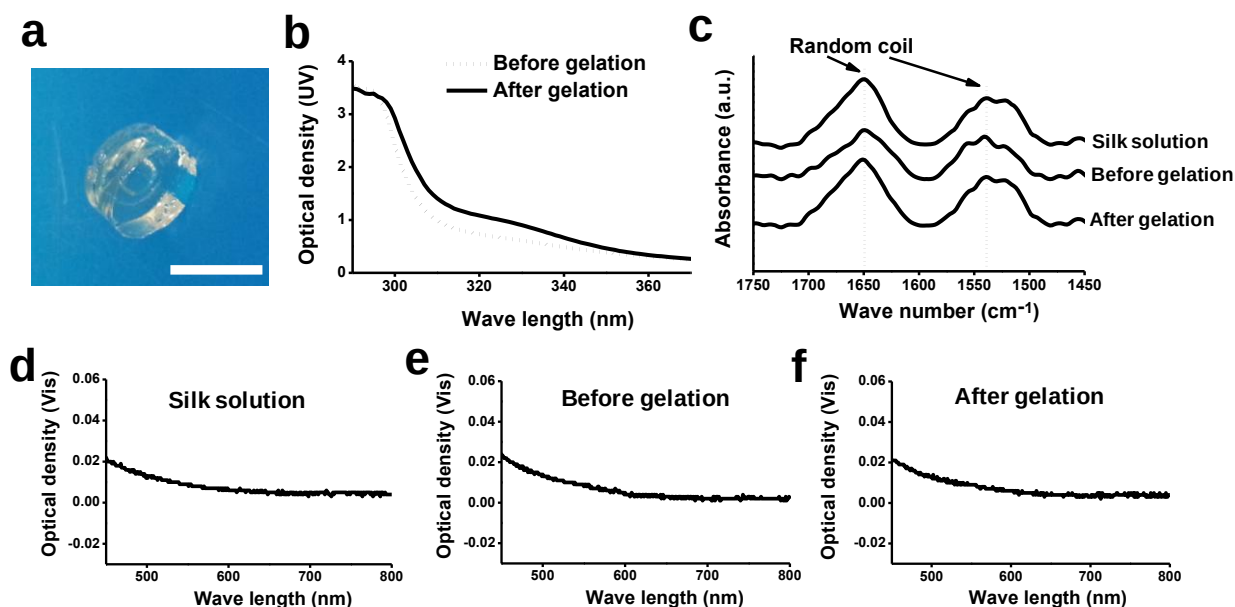
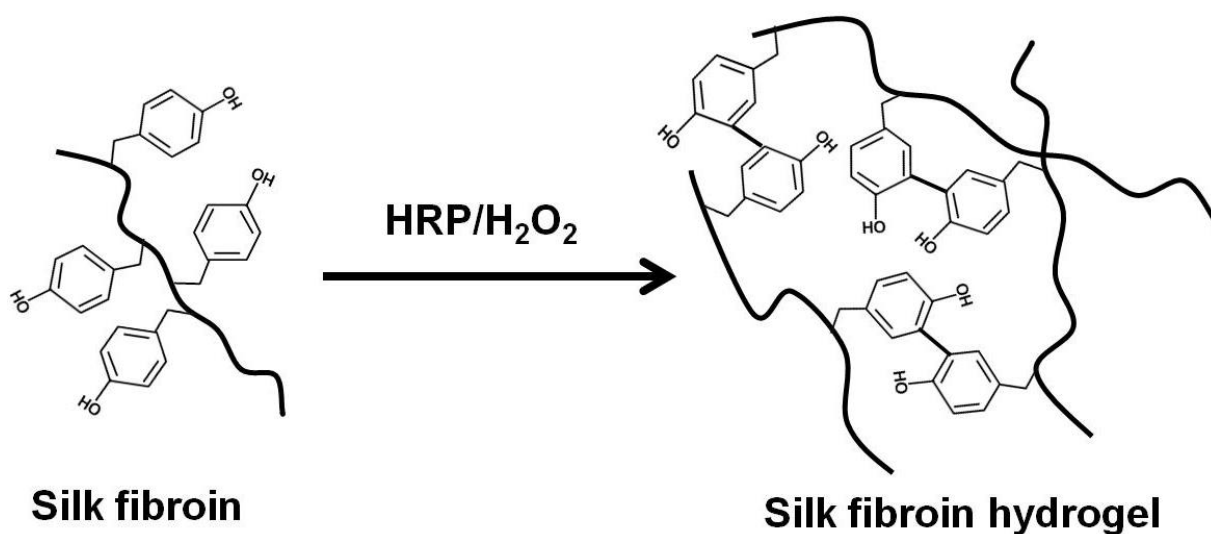


Figure 1. Structural analysis and optical absorbance profile of the SF hydrogels. (a) Macroscopic image of the formed hydrogels (Scale bar: 1 cm). (b) UV absorbance of the SF hydrogel before and after gelation. (c) ATR-FTIR spectra of the aqueous SF solution, the mixture of SF/HRP/H₂O₂ before gelation, and the formed SF hydrogel. (d-f) Visible light absorbance (Vis) of the aqueous SF solution, mixture of SF/HRP/H₂O₂ before gelation, and the formed SF hydrogel, respectively.



Scheme 1. Illustration of the cross-linking of SF hydrogls via peroxidase mediation.

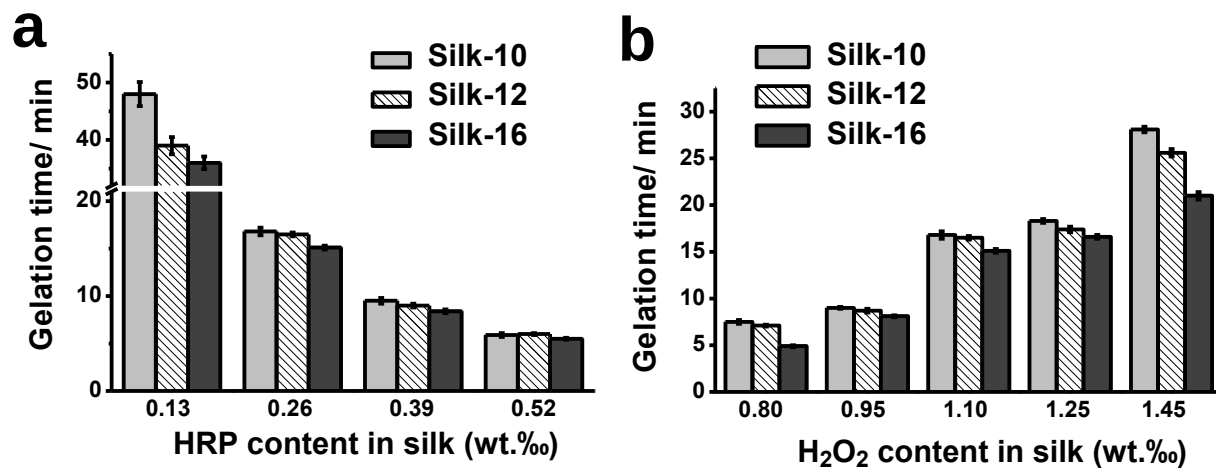


Figure 2. Influence of (a) HRP and (b) H₂O₂ contents on the gelation time of the SF hydrogels. (a) H₂O₂/SF was fixed at 1.10‰ (by wt.), (b) HRP/SF was fixed at 0.26‰ (by wt.).

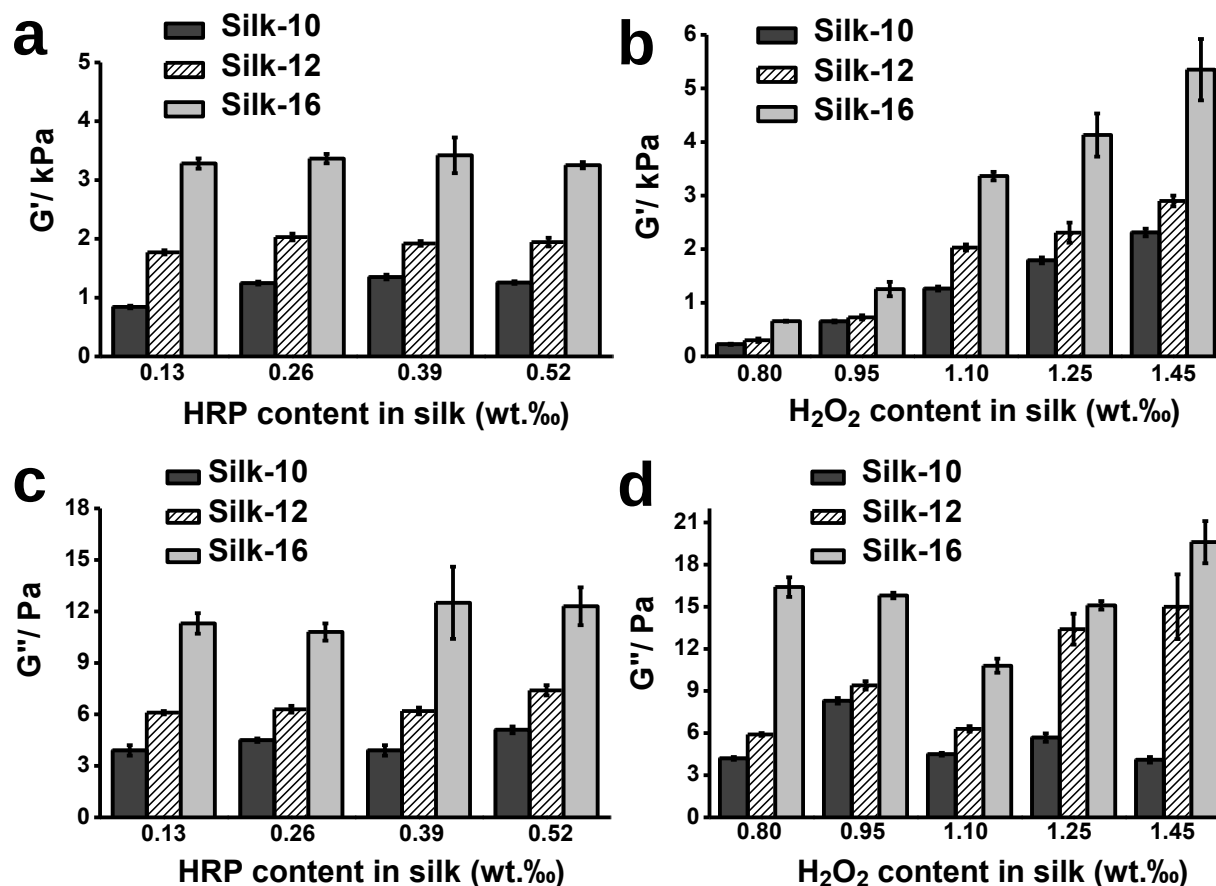


Figure 3. Influence of (a, c) HRP and (b, d) H₂O₂ contents on the mechanical properties of the SF hydrogels tested in a rheometer in oscillatory model. (a, c) H₂O₂/SF was fixed at 1.10‰ (by wt.); (b, d) HRP/SF was fixed at 0.26‰ (by wt.); (a, b) storage modulus; (c, d) loss modulus.

3.2. Gelation time and mechanical properties

The influence of HRP and H_2O_2 contents in the gelation time of the SF hydrogels are presented in Figure 2. It was found that the gelation time of the Silk-10, Silk-12 and Silk-16 all decreased significantly as increasing the HRP content (Figure 2a). Silk-16 demonstrated the shortest gelation time (around 5.0 minutes) when HRP was 0.52‰ (Figure 2a). An opposite trend was observed when increasing the H_2O_2 content. In this case, the gelation time increased greatly for all the three groups of hydrogels (Figure 2b). Silk-16 presented the lowest gelation time among the three groups of hydrogels over all the H_2O_2 concentrations used. In the case of 0.80‰ H_2O_2 , the gelation time of Silk-16 was within 5 minutes (4.9 ± 0.1 minutes).

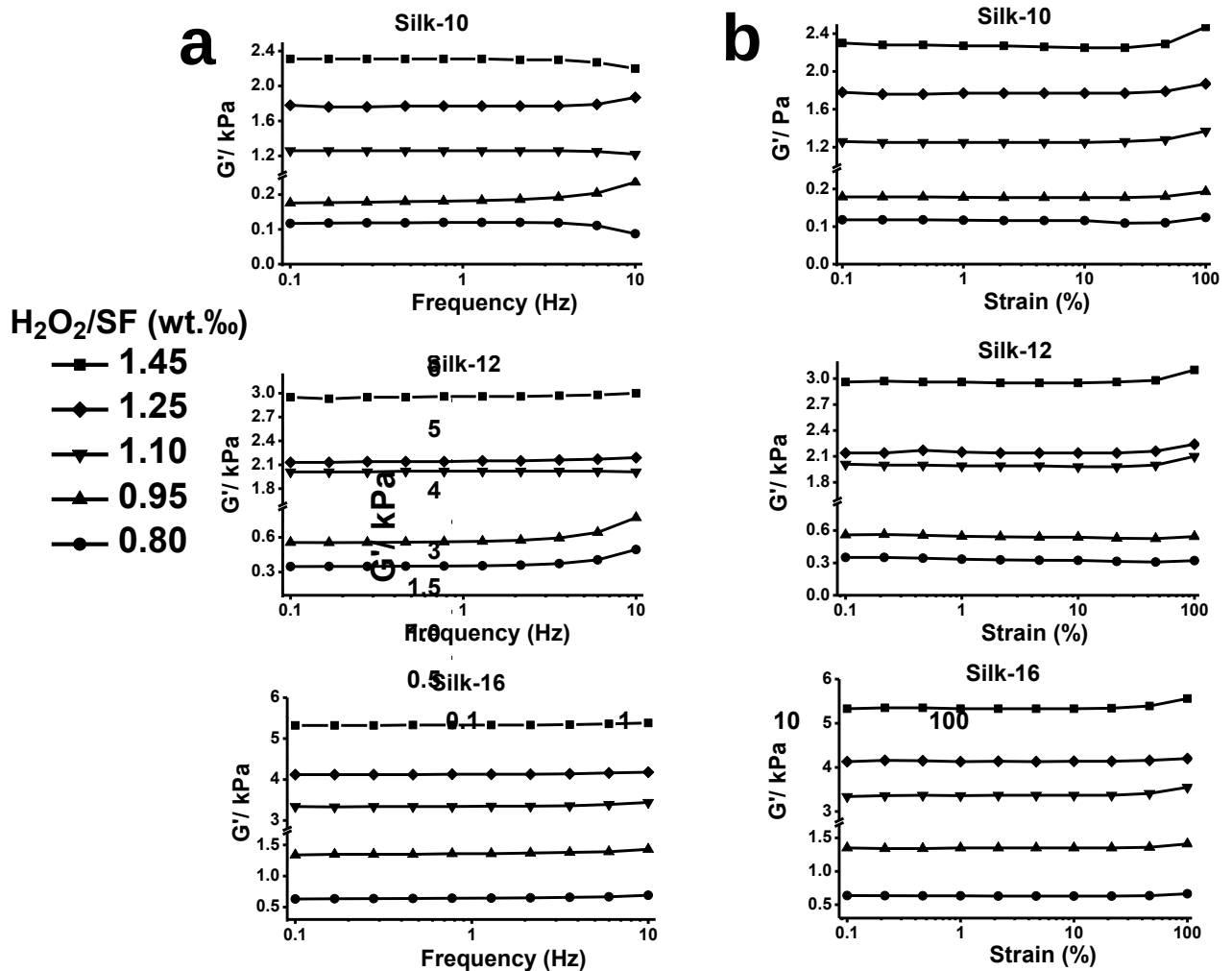


Figure 4. The frequency and strain sweeps of the SF hydrogels. (a) Frequency sweep; (b) strain sweep. HRP/SF was fixed at 0.26‰ (by wt.).

The mechanical properties of these hydrogels were tested in a rheometer. As showed in Figure 3a, the storage moduli (G') increased significantly when enhancing the SF concentration under the same HRP content, but it was only affected slightly by varying the HRP content in each group hydrogel. In contrast to the effect of HRP, increasing the H_2O_2 content greatly improved the G' of all the three groups of hydrogels (Figure 3b). The highest G' was observed in Silk-16 in all the tested H_2O_2 content. The G' of the SF hydrogels can be modulated in a wide range, from several hundred Pa to around 5 kPa. Additionally, the G' of hydrogels in each group was improved for increasing H_2O_2 content (Figure 3b). The loss moduli (G'') of the SF hydrogels were in a range of 3 to 21 Pa according to the HRP/ H_2O_2 proportions.

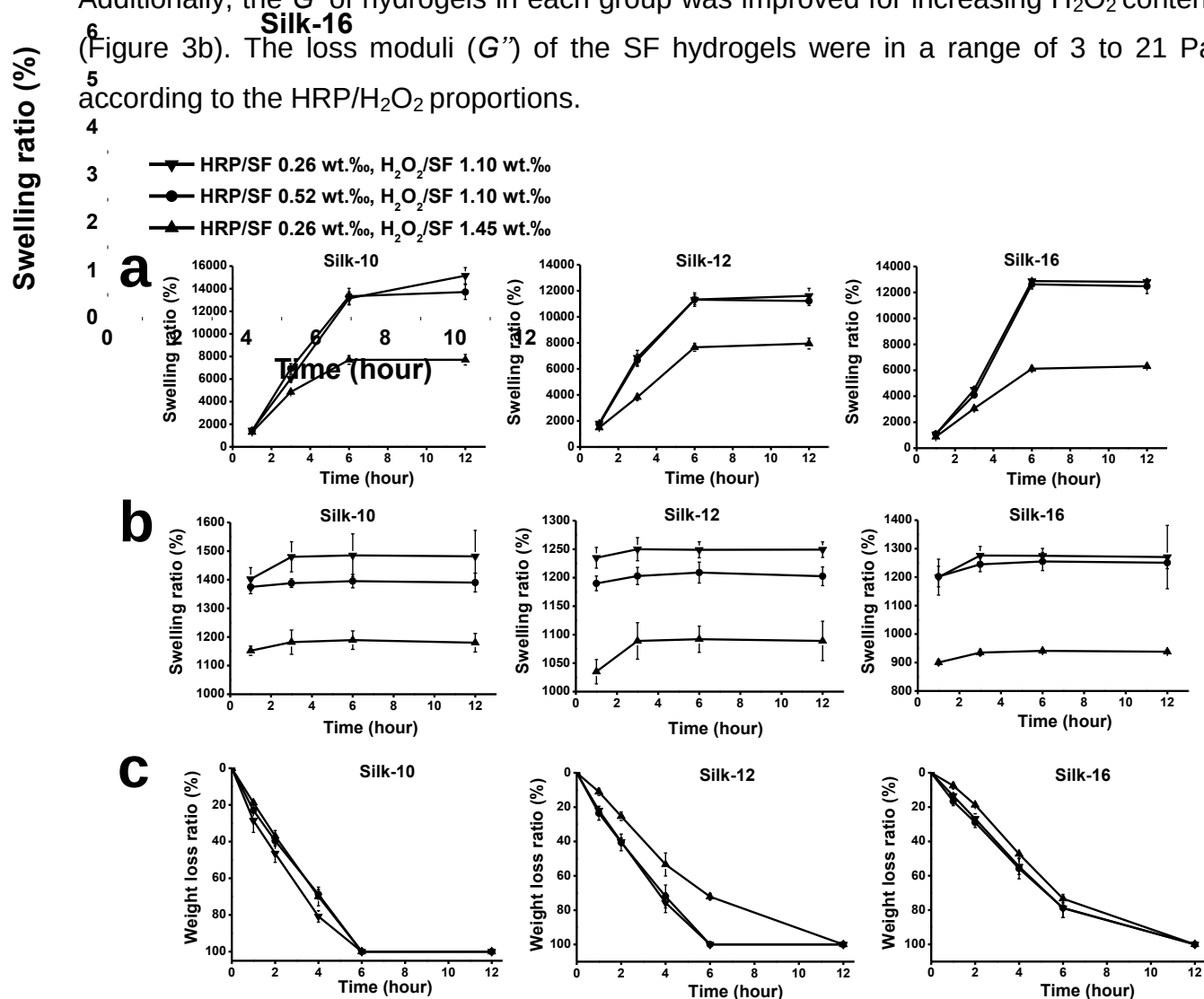


Figure 5. Swelling ratio and enzymatic degradation profiles of the SF hydrogels. (a) Ultrapure water, (b) PBS solution, and (c) protease XIV solution (0.005 U/mL).

Table 1. Comparison of SF hydrogels

Ref	Method	Shortest gelation time	Modulus (kPa)	Main conformation	SF concentration
[22]	Storing SF solution at 4°C	~3 days	—	β-sheet	2 wt. %
[22]	Addition of glycerol in SF solution	~2 days	—	β-sheet	2 wt. %
[21]	Addition of citric acid in SF solution and storing at 50°C	Overnight	—	β-sheet	2 wt. %
[20]	Increasing SF concentration or temperature, decreasing pH, addition of ions, or polyethylene glycol	<1 day	~200-6000 (C)	β-sheet	4-20 wt. %
[23]	Freezing the SF solution with organic solvents at -20°C	>6 hours	~3-50 (C)	β-sheet	6 wt. %
[25] ^a	Sonication treatment on SF solution	>0.5 hour	369-1712 (C)	β-sheet	4-12 wt. %
[24]	Vortex treatment on SF solution	~35 minutes	0.1-70 (S)	β-sheet	1.3-5.2 wt. %
[26]	Electrical (direct current) treatment on SF solution	~3 minutes	~1 (S)	Amorphous	8.4 wt. %
[50]	Addition of ethylene glycol diglycidyl ether in SF solution at 50°C	Within 2 hours	0.01-100 (S)	β-sheet	4.2 wt. %
[47]	Addition of sodium dodecyl sulfate in SF solution	~15 minutes	—	β-sheet	4 % (wt/vol)
[48]	Addition of methylcellulose in SF solution at 50°C	~40 hours	—	β-sheet	2 wt. %
[49]	Freezing the SF solution, and then immersion them in ethanol	Overnight	—	β-sheet	5 wt. %

Ref: reference; (C): compressive modulus; (S): storage modulus tested in rheometer.

^aAqueous SF solution was mixed with cells (final concentration: 0.5 million/mL). The mixture would gel in 0.5-2 hours. The compression modulus was tested without cells.

Silk-16 presented the steadiest G' profile in the tested frequency range compared with the ones of Silk-10 and Silk-12 (Figure 4a). The G' of Silk-10 were maintained until 3 Hz, and Silk-12 group showed similar G' until 10 Hz, when H_2O_2 /SF was between 1.1 and 1.45 %. Regarding the strain sweep (Figure 4b), all the three groups showed stable G'

until 50% strain was applied. Above 50% strain, all the silk compositions presented only a slight difference in G' .

3.3. Swelling behavior and degradation profile

Figure 5a and b showed the swelling behavior of the SF hydrogels in ultrapure water and PBS solution. All the SF hydrogels reached equilibrium after 6 hours immersion in ultrapure water (Figure 5a). SF hydrogels presented a high swelling ratio, ranging from 6,000 to 15,000. When immersed in PBS solution, the SF hydrogels presented a different swelling behavior, after reaching the equilibrium only after 3 hours (Figure 5b). The swelling ratios of the SF hydrogels in this case ranged from 900-1500, about 10 times less than in case of ultrapure water. The control samples (SF hydrogels undergone post-acid or post-methanol treatment) presented similar swelling ratios in ultrapure water and in PBS solution. In each group, the SF hydrogels with higher H_2O_2 /SF ratio presented lower swelling ratios, either in ultrapure water or in PBS solution (Figure 5a, b).

The enzymatic degradation profiles of these hydrogels were showed in Figure 5c. It was found that these hydrogels degraded completely within 12 hours even in low enzyme concentration (0.005 U/mL). All the formulations of Silk-10 and two formulations of Silk-12 with less H_2O_2 content degraded completely within 6 hours. The three formulations of Silk-16 and the formulation of Silk-12 of higher H_2O_2 content degraded around 70-80% in 6 hours. The control samples (SF hydrogels undergone post-acid or post-methanol treatment) did not show any notable degradation during the time tested.

3.4. Stimuli-responsiveness

Interestingly, the diameter of the SF hydrogel was maintained during immersion in PBS solution, and then increased around 100% when replaced by distilled water, finally recovering its original size during subsequent immersion in PBS solution (Figure 6a). The changes in the diameter were reversible as demonstrated in Figure 6b. In order to isolate the effect of the presence of different ions, another system was used by alternative immersing SF hydrogels in 0.154 and 2.0 M sodium chloride solutions, both of pH 7.4. The results showed that the wet weight of the SF hydrogels decreased around 14% when

immersed in 2.0 M sodium chloride solution for 1 hour, and then went back to its original value after 1 hour of immersion in 0.154 M sodium chloride solution (Figure 6c), demonstrating reversibility also in this system.

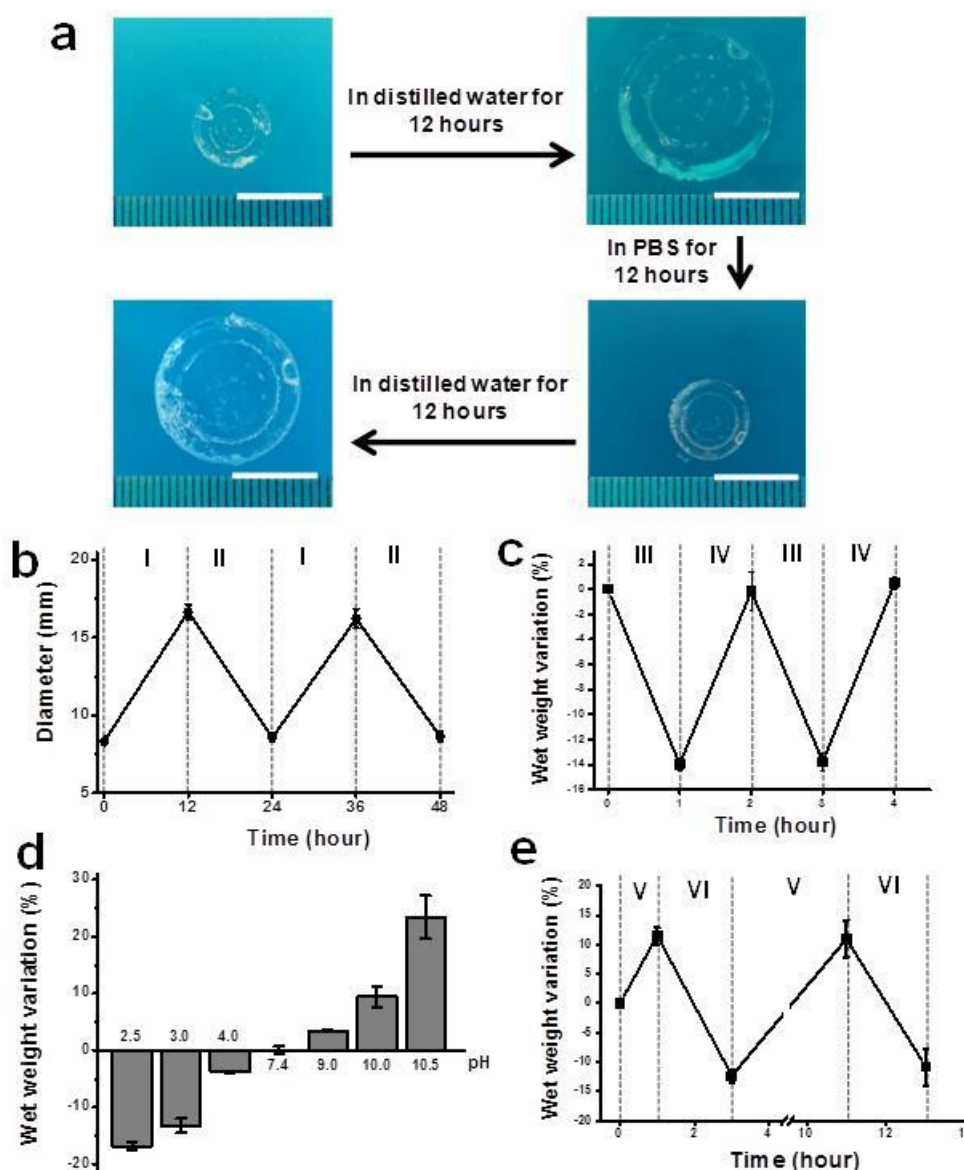


Figure 6. Ionic strength and pH stimuli response of SF hydrogels. (a) The prepared hydrogel discs were alternatively immersed in distilled water and PBS solution, and each immersion lasted for 12 hours (Scale bar: 1 cm). (b) Changes in the diameter of the hydrogel during the alternative immersion in (I) distilled water and (II) PBS solution; (c) Wet weight variation of the hydrogel during the alternative immersion in (III) 1.0 M and (IV) 0.154 M sodium chloride solutions (both of pH 7.4). (d) Wet weight variation of the hydrogels after immersion in solutions of different pH values for 2 hours, respectively. (e) Wet weight variation of the hydrogels during the alternative immersion in acid (pH 3.0, V) and basic (pH 10.5, VI) sodium chloride solutions.

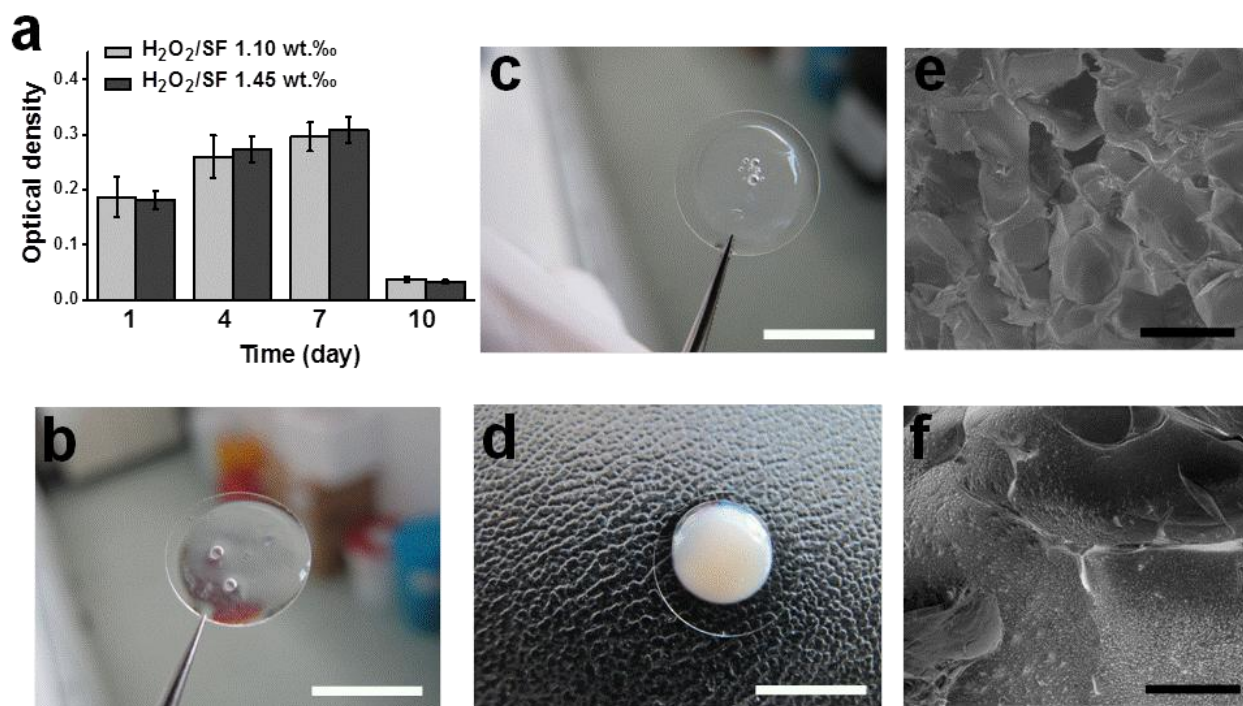


Figure 7. Cell encapsulation in the SF hydrogels. (a) The cell viability after encapsulation analyzed by MTS assay. SF solution: 16 wt.%; HRP/SF: 0.26‰ (by wt.). (b-d) Macroscopic images of the SF hydrogels incorporated with cells and cultured for 1, 6 and 10 days, respectively (Scale bar: 1 cm). (e-f) SEM images of the lyophilized SF hydrogels incorporated with cells and cultured for 6 and 10 days, respectively (Scale bar: 200 μ m). In (b-f), H₂O₂/SF was fixed at 1.1‰ (by wt.).

The influence of the pH on the SF hydrogels behavior was also evaluated, using solutions of the same ionic strength. In Figure 6d, it was found that these hydrogels swelled in basic conditions and shrank in acid conditions after immersion for 2 hours. The wet weight increased from ~3.5% to ~25%, when increasing the pH from 9.0 to 10.5. As the pH decreased from 4.0 to 2.5, the wet weight of the SF hydrogels decreased ranging from ~4% to ~18%. There were no obvious changes in the wet weight when the pH was between 5.0 and 8.0. Furthermore, the wet weight of the SF hydrogels can be reversed for a few cycles when alternate immersion in solution of pH 10.5 and pH 3.0 (Figure 6e). The recovering of the wet weight was more prolonged in basic condition than in acid condition.

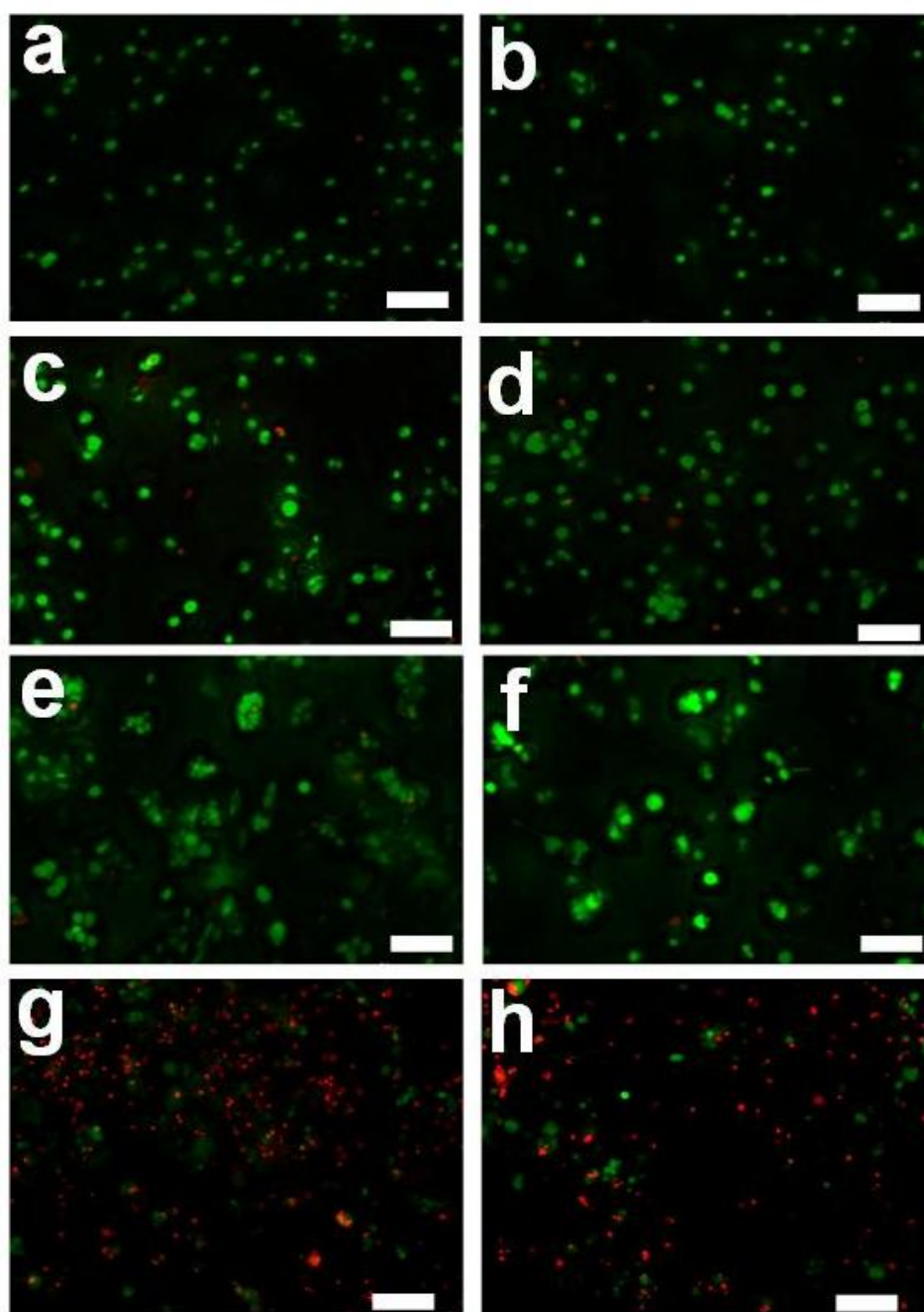


Figure 8. Live/dead staining of the ATDC-5 cells encapsulated in the SF hydrogels for 10 days. SF solution: 16 wt.%; HRP/SF: 0.26‰ (by wt.). (a, b) Day 1; (c, d) day 3; (e, f) day 7; (g, h) day 10. (a, c, e, and g) H_2O_2 /SF was fixed at 1.1‰ (by wt.). (b, d, f, and h) H_2O_2 /SF was fixed at 1.45 ‰ (by wt.). Scale bar: 100 μm .

3.5. Cell encapsulation and *in vivo* biocompatibility

The quantitative MTS evaluation demonstrated that the metabolic activity of the cells improved from day 1 to day 4, and was maintained until day 7 (Figure 7a). But the cell viability sharply decreased at day 10. There were no significant differences in cell viability

between these two formulations. As showed in Figure 7b, the SF hydrogels were still stable after incorporation with the cells, and maintained the transparent appearance. Similar observation was obtained at day 6 (Figure 7c). However, the SF hydrogels became opaque at day 10 (Figure 7d). Moreover, the hydrogels changed from elastic to stiff. The SEM images showed that the SF hydrogels displayed porous structure at day 6 (Figure 7e). A more dense morphology was observed in the hydrogels at day 10 (Figure 7f). From the Live/Dead staining, it was found that most of the incorporated cells were alive in the two formulations up to 7 days. The cells formed some clusters at day 3, and the size of the clusters became larger at day 7 (Figure 8c-f). However at day 10, the dead cells number increased for both formulations (Figure 8g, h).

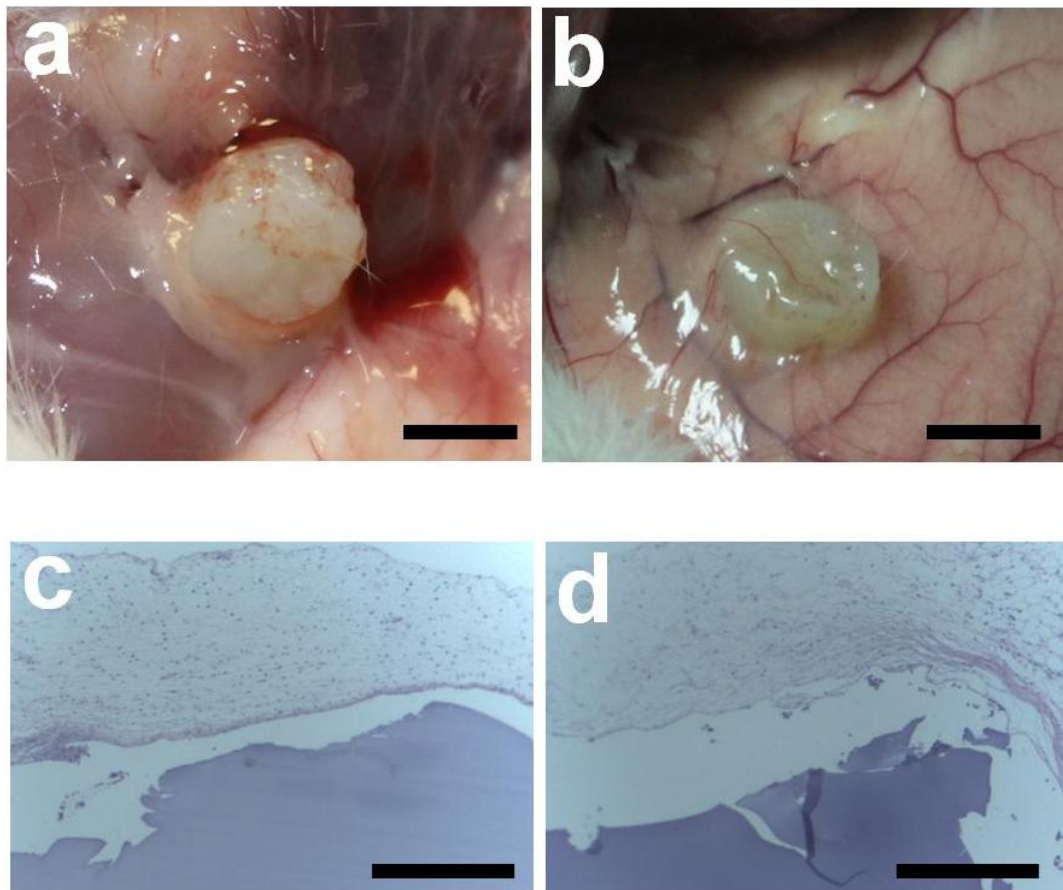


Figure 9. Subcutaneous implantation of the SF hydrogels in mice for 2 weeks. SF solution: 16 wt. %; HRP/SF: 0.26‰ (by wt.). (a, b) Macroscopic images of the explants (Scale bar: 5 mm). (c, d) H&E staining of the explants (Scale bar: 400 μ m). (a, c) H_2O_2 /SF was fixed at 1.1‰ (by wt.); (b, d) H_2O_2 /SF was fixed at 1.45‰ (by wt.).

The *in vivo* biocompatibility of these hydrogels was studied by subcutaneously implantation in mice for two weeks. The *in vivo* results showed that the hydrogels maintained their shape during the implantation time period (Figure 9a, b). There were no infections detected for all the implants. Only limited amount macrophages were observed in the connective tissue wrapping the hydrogels (Figure 9 c, d). Additionally, no invasions of cells or vessels in the hydrogels were observed. After two weeks implantation, the properties of the hydrogels evolved from elastic and transparent to stiff and opaque. The structural conformation of the explants was studied by ATR-FTIR in the amide I and amide II region (Figure 10). The ATR-FTIR spectra showed that both hydrogels presented strong peaks located at 1650 cm^{-1} , 1627 cm^{-1} , 1539 cm^{-1} and 1522 cm^{-1} , respectively.

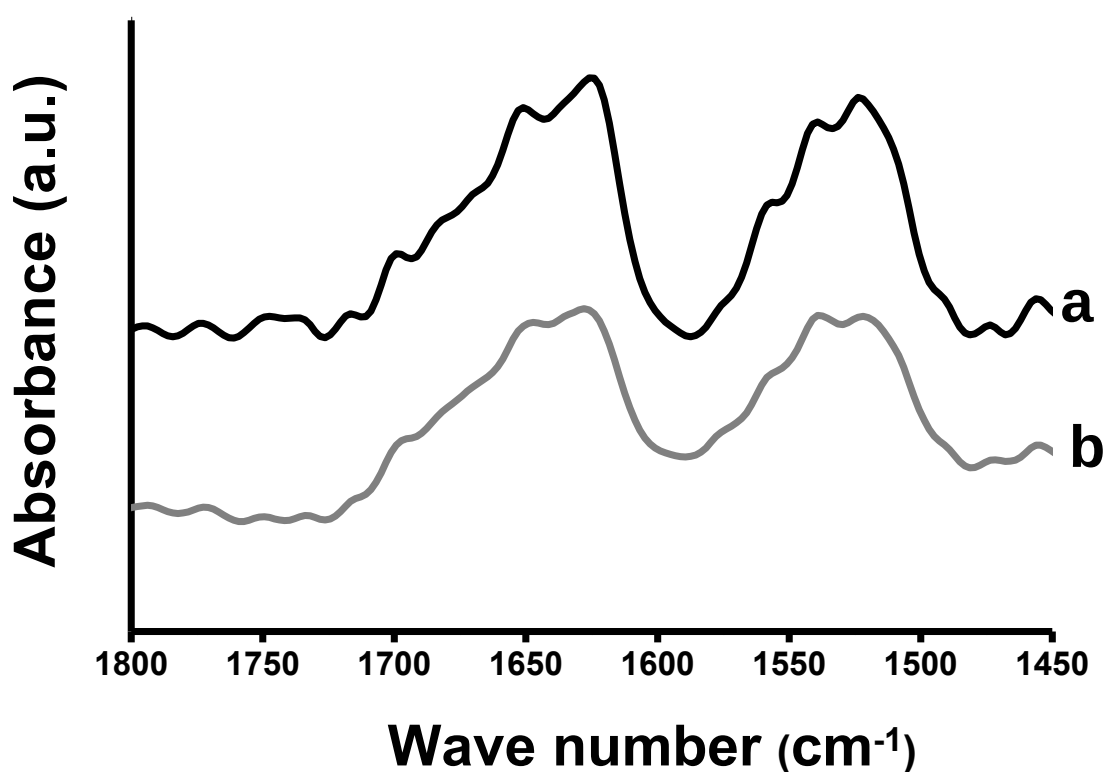


Figure 10. ATR-FTIR spectra of the SF hydrogels after subcutaneous implantation in mice for 2 weeks. SF solution: 16 wt.%; HRP/SF: 0.26‰ (by wt.). (a) H_2O_2 /SF was 1.1‰ (by wt.); (b) H_2O_2 /SF was 1.45‰ (by wt.).

4. Discussion

Hydrogels derived from biomacromolecules (such as peptide, protein and DNA) are attracting increasing attention in the latest years. Besides their good biocompatibility, these hydrogels also present other fascinating aspects, such as stimulus responsiveness,

self-assembly capability, or metamaterial characteristics [39-41]. In case of protein based hydrogels, to have a defined conformation is of particular interest, as their properties mainly depended on their structural conformation [42].

Despite the numerous strategies to develop SF hydrogels proposed so far, mechanical stability has only be achieved with the presence of dominant β -sheet conformation, leading to a characteristic opaque appearance [23, 43]. In this study, transparent SF hydrogels were developed, maintaining the amorphous conformation from the SF aqueous solution. An increasing in UV absorbance (Figure 1b) was observed due to the formation of dityrosine groups by the cross-linking of tyrosine groups in the SF solution. Malencik *et al.* [44] reported that in basic condition, the UV absorbance spectra of dityrosine group was around 300-360 nm, with a maximum fluorescence sensitivity under excitation and emission wavelengths at 320 and 400 nm, respectively. Our results were consistent with this previous observation. SF it is known to present an excellent *in vivo* biocompatibility when contacting with soft tissues [19]. The presently developed transparent SF hydrogels have potential for example as a temporary cornea substitute or as a material for *in vivo* optical detection devices.

FTIR is a useful tool to evaluate the subtle changes of SF conformation. It has been accepted that the FTIR peaks located at 1624 cm^{-1} and 1528 cm^{-1} were assigned to the β -sheet conformation [45]. The FTIR peaks between 1640 and 1650 cm^{-1} and 1538 cm^{-1} were assigned to the amorphous conformation in SF [22, 26, 45, 46]. Thus, both the prepared SF hydrogels and SF solution presented dominantly amorphous conformation (Figure 1c). Table 1 shows that the almost all the previous developed SF hydrogels were of β -sheet conformation, except the electrically induced SF hydrogel. However, the electrically induced hydrogels were not suitable for cell encapsulation, since the direct current used and their unstable mechanical properties [26]. In current study, the peroxidase mediated sol-gel transition was a very mild procedure, which is critical for cells encapsulation and/or drugs delivery. Since the SF hydrogels developed herein maintained the optical properties of the SF solution, they can be used as a three dimensional (3D) platform to monitor the SF molecular responses against various stimuli, which is impossible to be performed in liquid status.

The gelation time is crucial for injectable hydrogels. Many efforts have been devoted to produce SF hydrogels in a short time. SF hydrogels can be formed by changing the

amorphous conformation of SF into β -sheet, via physical stimuli (such as sonication [25], vortex [24], temperature modulation [20, 22], electrical stimulus [26]) and chemical treatment (lower the pH [21], incorporation of chemical compounds [47, 48], immersion in organic solvents [23, 49]). Table 1 summarized the preparation methods and properties of various SF hydrogels. Motta *et al.* developed several pioneering methods for SF hydrogel preparation by storing a SF solution at 4°C for 3 days [22], or addition of glycerol into the SF solution for 2 days [22], or addition of citric acid into SF solution and stored at 50°C overnight [21]. Kim *et al.* comprehensively studied the influence of temperature, ion contents and poly(ethylene oxide) content on the gelation time of SF solutions, and found that decreasing the pH at high temperature can induce faster gelation (less than one day) compared with other treatments [20]. Organic solvents and diepoxide have been introduced to accelerate SF hydrogel formation in a few hours [23, 49, 50]. Wang *et al.* found that sonication treatment on SF solutions for a few seconds can also induce hydrogel formation after 0.5-2 hours incubation [25]. Another physical stimulus-vortex was also employed. The post-vortex assembly time of the SF molecular structure can be modulated up to 35 minutes when increasing the vortex time [24]. The SF hydrogels can be formed in 3 minutes by electrical stimuli, but these hydrogels were in a metastable status and lack of consistent mechanical property [26]. Recently surfactant sodium dodecyl sulfate was used to trigger the SF gel formation and the shortest gelation time was between 15 and 18 minutes [47]. All the above mentioned methods for preparing SF hydrogels presented a relatively long gelation time and/or harsh preparation conditions. The development of a fast forming SF hydrogel able to sustain cell viability during the process remains a big challenge for silk based biomaterials.

In the present study, SF hydrogels were successfully generated in a physiological condition with a gelation time in a few minutes. During the peroxidase mediated cross-linking procedure (Scheme 1), the H_2O_2 decomposed to water after oxidizing the HRP. Subsequently, the oxidized HRP was reduced by tyrosine groups which were oxidized and became the phenol radical species for the cross-linking reaction. The recovered HRP would enter the next catalysis cycle [51]. Therefore, the cross-linking density depends on the initial H_2O_2 and the amount of tyrosine groups in the SF solution. Excess of H_2O_2 can inactivate the function of HRP, thus the higher H_2O_2 content induced the longer the gelation time (Figure 2b). Increase in the HRP content can accelerate the speed of

tyrosine radical species formation which subsequently led to the decrease of gelation time, when SF concentration and HRP content were fixed (Figure 2a). The 16 wt.% SF solution presented the highest amount of tyrosine groups as compared to the 10 wt.% and 12 wt.% SF solutions, which led to the fastest gelation speed among the three groups tested formulations (Figure 2). Our data were consistent with previous studies on peroxidase mediated cross-linked hydrogels [30-32]. In those studies, it was found that the gelation time of the hydrogels decreased with increasing HRP content and polymer concentration, and increased with the increasing of H_2O_2 content. The shortest gelation time is critical for the development of injectable hydrogels for cell loading in a tissue engineering approach or as a drug delivery system (DDS). This study showed that SF hydrogels can be formed within 5 minutes, and the gelation time can be modulated more precisely compared with previous systems (Table 1). More importantly they were prepared under physiological condition which does not compromise the cell viability during gel formation.

Previously developed SF hydrogels of β -sheet conformation have presented robust mechanical properties [20]. On the contrary, electrical stimulated hydrogels prepared from fresh SF solution were reported to present amorphous conformation, however they were not mechanically stable [26]. In the present study it was possible to produce an amorphous and stable SF hydrogel with mechanical properties can be modulating in a broad range. This was achieved by controlling the cross-linking density, *i.e.*, the amounts of the available tyrosine groups and H_2O_2 [32]. By the raising of SF concentration (from 10 to 16 wt.%,) in all the HRP concentrations tested (Figure 3a), or increasing the H_2O_2 content (Figure 3b) it was possible to increase G' . The low G'' indicated that the prepared SF hydrogels were of high elasticity, which was further confirmed by the frequency and strain sweep after gelation (Figure 4). In practical terms, these hydrogels can easily recover their original shape even when they undergo cycling compression with 100% strain, while a SF hydrogel of β -sheet conformation (SF hydrogels of the same formulation but after post-acid treatment) are stiff and brittle. In a previous study on sonicated SF hydrogels, it was shown that the compressive moduli of the hydrogels were between 369-1712 kPa [25], while in the present study the compressive moduli of the Silk-16 were less than 25 kPa. These results proved that the SF hydrogels herein developed are of remarkable elasticity. This property is associated to the SF amorphous domain, which is dominant in the present hydrogels. SF molecular chains with

amorphous conformation have higher mobility than those in crystalline state, thus the amorphous hydrogels displayed higher elasticity as compared with those of β -sheet structure.

The present SF hydrogels present a high swelling ratio which is mainly related with the hydrophilic domain in the SF molecular chains, including the C- and N-termini, the repetitive short internal hydrophilic blocks in the heavy chain, and the counterbalanced amphiphilic domains in the L-chains [52]. Moreover, the swelling ratio also partially depended on the cross-linking density. Higher H_2O_2 can induce a higher cross-linking density, which in turn can lead to a reduced mesh size and lower swelling ratio. The differences in the swelling ratio of these hydrogels between ultrapure water and in PBS solution were from the varied osmotic pressures of these hydrogels in ultrapure water and in PBS solution.

These SF hydrogels degraded much faster in the protease XIV solution as compared to the hydrogels which undergone post-acid or post-methanol treatment (Figure 5c). The fast degradation of these hydrogels was attributed to its higher amorphous content. In the amorphous status, the SF molecular chains were highly hydrated, thus the enzyme had a good access to the molecular chains, leading to a fast degradation. The minor differences of degradation ratio between the formulations result from the differences in cross-linking density. Higher cross-linking density would delay the degradation procedure. In previous studies using sonication SF hydrogels (derived from a 12 wt.% SF solution and exhibiting β -sheet conformation) degraded around 10% by mass after incubation in 5 U protease XIV solutions for 24 hours [25]. SF films of high Silk-I content showed around 40% weight loss after 24 hours incubation in 9.2 U protease XIV solutions [53]. In current study SF hydrogels degraded 100% after 12 hours of incubation in 0.025 U protease XIV solutions. These results demonstrate that the protein conformation plays the main role during enzymatic degradation. In previous studies, SF based biomaterials of β -sheet conformation have degraded relatively slow both *in vitro* and *in vivo* [21, 37]. Some other studies have been conducted to modulate the degradation profile via reducing the β -sheet content in SF [45]. The present study opens a new door to prepare a mechanically stable SF biomaterial with a degradation profile which is tailorable by altering its conformation. This possibility is quite interesting for example when considering a short-term drug delivery system.

The results from the swelling ratio test revealed that the SF hydrogels developed in this study were ionic strength responsive. There were only a few studies about the responsive properties of SF hydrogels. In a study by Leisk *et al.* [26], it was presented a SF hydrogel formed under electric field that could returned to solution state through a reverse electric process or by modulating the temperature. The responsive property of the SF hydrogels, either in previous study or in the currently presented study, was related to its predominant amorphous domain. Due to this property, the hydrogels developed in current study have potential to be used as sensors for ionic detection, which is an important application because hydrogel arrays for sensing ionic strength has been initially proposed by Orthner *et al.* [54].

Besides the ionic strength response, these SF hydrogels have further presented a pH response. This characteristic was partially related with the isoelectric point (pI: 3.8) of SF. As a protein, SF is composed of amphoteric molecules which contain acid and basic groups. When pH is lower than 3.8, the amorphous hydrogels become protonated resulting in the decrease of mobile counterions and electrostatic repulsion inside the hydrogels, leading to the gel shrinking [52]. In basic conditions, the deprotonation of the amorphous hydrogels occurred, and both the mobile counterions and electrostatic repulsion between the SF molecular chains increased, thus the gel swelled. The pH stimuli response property of the SF hydrogels is mainly attributed to the amorphous conformation of the SF molecules. Under the varied external stimuli, the amorphous SF molecular chains can be easily hydrated, protonated, and deprotonated, inducing the different swelling behaviors of the hydrogels. Neither ionic strength response nor pH response were observed in the controls. Previous studies on SF hydrogels did not report external stimuli responsiveness, when SF molecular chains undergo self-assembly to form a crystalline structure, which increases the hydrophobic interaction and mechanical properties of the hydrogels [46]. The amorphous SF hydrogels produced in this study provide a novel 3D model to study the SF molecular behavior or interaction against various stimuli, such as the presence of metal ions, organic solvents, antigens, antibodies, among others.

The developed SF hydrogels were able to be used for cell encapsulation. In the previously developed sonicated SF hydrogels it was possible to achieve cell encapsulation [25]. Wang *et al.* performed the first study on cell encapsulation in sonicated SF hydrogels that formed after 30 minutes. Compared with this previous work,

the present SF hydrogels are advantageous since they present a shorter gelation time within 10 to 15 minutes after cell encapsulation. These results showed that the SF hydrogels developed under the present methodology were non-cytotoxic, and can support the cell viability up to 7 days. However, the SF hydrogels showed their irreversible tendency for β -sheet transition after some days. The spontaneously conformation evolution has improved the hydrogels stiffness, leading to the cells death. This observation was in line with a study by Kim *et al.* [20], where the amorphous silk solution would spontaneously form a gel (β -sheet) after weeks or months, due to the self-assembly of SF molecules. This unique property of SF hydrogels may bring new insights to study cell destruction in a hydrogel model only by an alteration of its physicochemical properties, *e.g.* it may be used as a model for studying cancer cells destruction.

SF hydrogels induced by acid treatment have been previously used for the healing of critical size cancellous defects [21]. However, those SF hydrogels were formed after overnight immersion in acid solution, and they needed to be neutralized before the contact with cells or implantation. In the present study, the SF hydrogels were generated in a couple of minutes under physiological condition, and then implanted directly. The HRP or H_2O_2 did not show any negative effect during the implantation. It was noticed that the implanted SF hydrogels also underwent a conformation transition from amorphous to β -sheet (Figure 10). This result is in good agreement with the *in vitro* study (Figure 7d). The SF hydrogels developed herein can be used as injectable system in clinics (for instance filling irregular tissue defects), since their short gelation time, superior *in vitro* and *in vivo* compatibility. The good integration between host tissue and injected materials are crucial for successful tissue regeneration. Moreira Teixeira *et al.* [28] showed that tyrosine groups in cartilage and in the hydrogels can be covalently bonded. Therefore, the SF hydrogels would probably present good affinity to host tissue when injecting *in vivo*, such as for defects in cartilage or skin. The *in vitro* and *in vivo* intrinsic conformation transition from amorphous to β -sheet brings new application potentials for these SF hydrogels, such as studying cancer cell self-destruction *in vitro* and *in vivo*. Overall, these SF hydrogels presented numerous advantages to be used as implants for tissue regeneration/replacement or in drug delivery applications.

5. Conclusions

In the present study, a new class of SF hydrogels was developed by peroxidase mediated cross-linking. These hydrogels present completely distinct properties compared to the SF hydrogels of β -sheet conformation presented in the literature. They are of amorphous conformation, transparent appearance, and outstanding elasticity. The gelation time and mechanical properties can be tuned from 1 hour to within 5 minutes and from several hundred Pa to around 5 kPa, respectively. Notably, these hydrogels displayed ionic strength and pH stimuli response properties. More importantly, they are non-cytotoxic and biocompatible *in vitro* and *in vivo*. These versatile and extremely easy to use SF hydrogels not only bring new insights for the fundamental study of hydrogel-based biomaterials, but also provides new candidate for drug delivery, medical devices, tissue regeneration and regenerative medicine applications.

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Chapter IX

**Core-Shell Silk Fibroin Hydrogels: Modulating the Release
of Bioactive Molecules through Controlled Spatial
Conformation**

Chapter IX

Core-Shell Silk Fibroin Hydrogels: Modulating the Release of Bioactive Molecules through Controlled Spatial Conformation

Abstract

Hydrogels with spatially controlled physicochemical properties are very appealing, since they can better fulfill the complex requirements from tissue engineering or drug delivery systems. This study aimed to provide a simple approach towards preparing core-shell silk fibroin (SF) hydrogel composed of dominant β -sheet conformation in the shell layer and mainly amorphous conformation in the core layer. The core-shell hydrogels were prepared by means of soaking the developed peroxidase mediated cross-linked amorphous SF hydrogel in methanol. The thickness of the outer shell layer was measured in wet state. The morphology of the shell and core layer of the SF hydrogel was observed by scanning electron microscopy (SEM). The conformation of different domains in the hydrogels was analyzed by Fourier transform infrared spectroscopy in wet state. The biostability and hydration degree were screened by immersion the hydrogels in protease XIV solution and phosphate buffered saline solution, respectively. The mechanical properties of the hydrogels were determined under compression testing in wet state. Albumin was incorporated in the developed core shell hydrogels and its *in vitro* release profile was determined. The SEM analysis revealed that the thickness of the shell layer in the hydrogel can be tuned from about 200 to around 900 μm . The shell layer displayed a compact morphology and dominant β -sheet conformation, while the core layer was porous and maintained the amorphous conformation. The shell layer presented a higher stability during the enzymatic degradation and also a lower hydration degree,

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compared to the core layer. The compressive modulus of the core-shell hydrogels had increased from around 25 kPa to about 1.1 MPa with increasing immersion time in methanol. The core-shell SF hydrogels demonstrated slower and more controllable release profiles as compared with the non-treated hydrogel. The developed core-shell SF hydrogels can be very useful and tunable systems for biomedical applications, such as for drug delivery system or tissue substitutes, and as models for protein structure investigation. This study also provides a new strategy to generate hydrogels with sophisticated and hierarchical structure via modulation of the protein conformations.

1. Introduction

Biocompatible and biodegradable hydrogels have been attracting great deal of interest in biomedical applications, due to the possibility of tuning its physicochemical properties and similarity to the extracellular matrix (ECM) [1]. These hydrogels have been used in the fabrication of medical devices, drug delivery, food science, tissue engineering and regenerative medicine (TERM) [1]. Its physicochemical properties, such as the degradation profile and mechanical and chemical properties are crucial for cell encapsulation and greatly dictate the growth, differentiation, or *de novo* tissue forming ability [2, 3]. Typically, hydrogels present homogeneous characteristics and lack sufficient control on their properties [4]. However, organs or tissues are heterogeneous or highly hierarchical organized structures, showing different mechanical properties and permeability. When hydrogels are applied for tissue regeneration, its physicochemical properties should be tunable over time to fulfill the dynamic requirements at different stages [4]. Because of the increased understanding on the crosstalk between tissues and biomaterials, the development of hydrogels with spatiotemporally controlled properties constitutes great demand [5]. Several interesting studies have been performed to address this great challenge [4, 6]. Photodegradable hydrogels whose properties can be regulated on-demand with light after cell encapsulation have been prepared [4, 5]. The immobilization of the growth factors in distinct regions in the hydrogels has been also achieved [6]. Furthermore, hydrogels with spatically controlled micropatterned ECM were produced for manipulating cell invasion [7]. The success of those studies was based on the use of photochemical approaches.

Besides, the biological system has already provided useful hints on spatially tuning the tissue properties via changing the arrangement of its molecules [8]. The heterogeneous properties of the tissues mainly result from the different orientation adopted by the biomacromolecules in the different regions of its ECM. Taking the cartilage as an example, it is mainly composed of collagen type II, in its four zones [9]. The compressive modulus of the deep zone is much higher than the superficial zone, partially owing to the different orientations of collagen fibrils in these two areas [9]. The resulting anisotropy of these natural tissues serves as an inspiration for spatially modulation of the hydrogel mechanical properties by playing with their structural conformation or molecular orientation [10, 11]. For instances, multi-membrane hydrogel was developed by interrupted neutralization of a polyelectrolyte alcohol gel in sodium hydroxide solution [12]. The layered hydrogels have been proposed as a micro-bioreactor system for evaluation of cell behavior or the interaction between materials and cells [13]. These are also advantageous systems for the regeneration of the stratified structure of injured tissues, such as cartilage, by endowing defined properties in each layer [14]. Furthermore, different drugs can be incorporated in the layered hydrogels systems to develop multiple or spatially control drug release systems [15].

Due to its non-toxic nature and outstanding compatibility with cells and tissues, protein-based hydrogels have received particular interest in TERM field [16]. The silk fibroin (SF) hydrogels can be obtained from aqueous solutions by means of decreasing the pH value [17], increasing the temperature [18] or applying external stimulus such as vortex and ultrasonication [19, 20]. The addition of organic solvents/saline [18], or surfactants [21] can also lead to the formation of SF hydrogels. All SF hydrogels reported by the previously aforementioned methods presented a β -sheet conformation. Electrical stimulus was also able to generate SF adhesive hydrogels with dominant amorphous conformation, but presented no mechanical stability [22]. Recently, it has been reported a new class of SF hydrogels using peroxidase to mediate cross-linking [23, 24]. Gelation of the SF hydrogels can be induced within few minutes and presenting mainly amorphous conformation. To our knowledge, SF based hydrogels with spatially controlled conformations have not ever been reported.

In this study, it is described an approach to prepare novel core-shell SF hydrogel prepared by immersion the amorphous SF hydrogels in methanol. The thickness of the shell layer was recorded by a micrometer. The morphology of the developed hydrogels

was evaluated by scanning electron microscope (SEM). The structure of the hydrogels was studied by Fourier transform infrared spectroscopy (FTIR). The biostability and hydration degree were evaluated by immersion the hydrogels in protease XIV solution and phosphate buffered saline solution, respectively. Compressive test was performed to study the mechanical properties of the hydrogels. The drug delivery ability was investigated by incorporation albumin in the core-shell SF hydrogels and conducting *in vitro* release up to one week.

2. Materials and Methods

2.1. Materials and reagents

Cocoons of *Bombyx mori* were purchased from the Portuguese Association of Parents and Friends of Mentally Disabled Citizens (APPACDM, Castelo Branco, Portugal). Horseradish peroxidase (HRP, type VI, 260 U/mg solid) was provided from Sigma-Aldrich. All the other materials or reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless mentioned otherwise.

2.2. Preparation of the SF solution

The SF was purified by degumming in 0.02 mol/L sodium carbonate boiling solution for 1 hour, in order to remove the sericin [25, 26]. The dried SF was dissolved in 9.3 mol/L lithium bromide solution at 70°C for 1 hour. In the following, the SF solution was transferred into a benzoylated dialysis tubing (Molecular weight cut off: 2 kDa) and dialyzed in distilled water for 48 hours. Then, the SF solution was dialyzed against 0.2 M phosphate buffered saline (PBS, without calcium and magnesium ions) solution for 12 hours, followed by concentration in 20 wt.% poly(ethylene glycol) solution. The final concentration of the SF aqueous solution was tested by drying the SF solution in an oven at 70°C overnight. The SF solution was stored in a room with temperature ranged from 4 to 8°C before use. The saline ratio in the SF was 1.73±0.03 wt.% analyzed by thermal gravimetric analysis (TGA Q500, TA Instruments, DE, USA). The pH of the SF solution was around 7.1 tested by a pH meter.

2.3. Preparation of the core-shell SF hydrogels

The SF solution was diluted into 16 wt.% by the addition of PBS solution. The SF hydrogels were obtained via HRP mediated gelation [23, 24]. Briefly, 1 mL SF solution was mixed with 100 μ L of HRP solution (0.84 mg/mL) and 65 μ L hydrogen peroxide solution (0.36 wt.%), followed by transferring 200 μ L the mixture into a polypropylene mold (diameter: 8 mm) and placing the molds into the 37°C oven until the gel formed. The SF hydrogel discs were removed from the moulds and used for the preparation of the layered SF hydrogels.

The core-shell SF hydrogels were prepared by immersion the prepared gel discs in methanol for 1, 3, 5 and 10 minutes. At the end of each time point, the hydrogel discs were removed from the methanol and washed in PBS solution for three times to eliminate the organic solvent. After the methanol treatment these hydrogel discs formed a core-shell structure, with a stiff outer shell and a soft core.

2.4. Characterization of the core-shell SF hydrogels

2.4.1. Determination of the thickness of the shell layer of the core-shell SF hydrogel

The prepared core-shell SF hydrogel discs were longitudinally cut, and the soft core layers were separated from the stiff shell layers. The thickness of the wall in the shell layers was measured by a micrometer. Three areas in one disc were measured and the values were averaged. For each group of discs, at least 4 specimens were tested.

The specimens were also analyzed by SEM (Nova NanoSEM 200; FEI, Hillsboro, OR, USA). The core-shell and non-treated hydrogels were longitudinally cut, followed by frozen in -20°C for at least 3 hours. And then the samples were lyophilized and observed by SEM. Before SEM evaluation, the samples were coated with Au/Pd SC502-314B in an evaporator coater (E6700; Quorum Technologies, East Grinstead, UK).

2.4.2. Structure characterization of the core-shell hydrogels

The conformation of the different domains in the core-shell SF hydrogels was characterized by FTIR in attenuated total reflectance (ATR) model (IRPrestige-21;

Shimadzu, Kyoto, Japan). The samples after methanol treatment were washed and immediately tested by ATR-FTIR. The tested domains were the external surface of the shell layer, the inner surface of the shell layer, the interface area between the shell and the core layers, and the core layer. The samples were analyzed by contacting the germanium crystal in the FTIR. Each specimen was scanned 48 times from 500-4000 cm^{-1} with a resolution of 4 cm^{-1} in wet state. Silk solution and hydrogels without methanol treatment were used as controls. PBS solution was scanned as background. Three specimens were analyzed in each group.

2.4.3. Enzymatic degradation of the core-shell SF hydrogels

The shell layer of the core-shell SF hydrogel was degraded in protease XIV solution. The non-treated SF hydrogel and the core layer of the core-shell SF hydrogels which immersed in methanol for 10 minutes were also tested. Around 50 mg hydrogel (wet weight after removing surface liquid by filter paper) was immersed in 5 mL protease XIV solution and kept in a thermostatic water bath at 37°C. The enzyme solutions of 0.2 U/mL and 0.005 U/mL were used for the shell layer and the core layer hydrogels, respectively. The samples were degraded for 1, 2, 4, 6 and 12 hours, and the weight loss ratio was calculated as following the equation 1:

$$\text{Weight loss ratio (\%)} = \left(\frac{w_i - w_t}{w_i} \right) \times 100\% \quad (1)$$

In Equation 1, w_i means the initial wet weight of the hydrogel, and w_t is the wet weight tested at each time point. Four specimens were used for each group hydrogel.

2.4.4. Hydration degree of the core-shell SF hydrogels

The hydration degree of the shell layer and the core layer of the hydrogels were evaluated. The samples were immersed in PBS solution for 1 hour, and then the wet weights were recorded after removing the surface liquid by filter paper. In the following,

the samples were dried at 70°C in an oven overnight. The dry weight of each sample was measured. The hydration degree was defined as following the equation 2:

$$\text{Hydration degree (\%)} = \left(\frac{w_i - w_d}{w_d} \right) \times 100\% \quad (2)$$

In Equation 2, w_i refers to the initial wet weight of the sample, and w_d is the dry weight of the sample. Four specimens were screened in each group.

2.4.5. Compression testing of the core-shell SF hydrogels

The compressive modulus of the core-shell SF hydrogels was tested in a universal testing machine (Instron 4505, Instron, Norwood, MA, USA). The specimens were tested in an unconfined compression model, after removing the surface liquid by filter paper. The compressive rate was set at 2 mm/minute until reaching 50% strain. The modulus was determined from the slope of the initial linear domain in the compressive curve. At least six specimens were examined for each group.

2.5. Release profile of the core-shell SF hydrogels

The albumin-fluorescein isothiocyanate conjugate (Albumin-FITC) was used as a model drug to study the release profile from the core-shell SF hydrogels. The hydrogel discs were first hydrated in PBS solution for 1 hour after prepared, followed by immersion in 100 µg/mL Albumin-FITC solution overnight and at room temperature (1.5 mL/disc). Afterwards, the hydrogel discs were removed from the Albumin-FITC solution and rinsed in PBS solution. The SF hydrogels discs were used to prepare the core-shell hydrogels by immersion in methanol for 3, 5 and 10 minutes. The Albumin-FITC release profiles of the non-treated and core-shell hydrogels were evaluated by immersion of each specimen in PBS solution. For the non-treated specimens and specimens treated by methanol for 3 minutes, 4 mL PBS solution was used for each disc. Due to the low amount of albumin incorporation in the specimens with methanol treatment for 5 and 10 minutes, 2 mL PBS

solution was used for each disc in these two groups. The release of Albumin-FITC was tested at 2, 4, 6, 24, 48, 72, 120 and 168 hours. At the end of each time point, the supernatant from each specimen was removed and equal volume of fresh PBS solution was added. For the quantification of the released Albumin-FITC, the fluorescence intensity of 100 μ L supernatant of the removed PBS solution was read by a microplate reader (Synergy HT, Bio-Tek, VT, USA), with the excitation wavelength at 485/20 nm and the emission wavelength at 528/20 nm. The samples without Albumin-FITC incorporation were used as controls. Five specimens were used for each group. For the determination of the total Albumin-FITC in the hydrogels, the discs were immersed in 4 mL PBS solution and the supernatants were analysed periodically.

2.6. Statistical analysis

The data were presented by mean $\pm$ standard deviation (SD). The results were analyzed by one-way analysis of variance (ANOVA). The mean values for each group were compared by Tukey's test and $p < 0.05$ was considered statistically significant.

3. Results

Figure 1 shows the macroscopic appearance of the core-shell SF hydrogels. It was found that while the non-treated hydrogel was transparent, the methanol treated hydrogel became opaque (Figures 1a, b). An obvious core-shell structure was formed in the methanol treated hydrogels (Figures 1c). The two layers integrated well, without an obvious interface. The shell layer was opaque and became thicker when increasing the immersion time from 0 to 10 minutes. The core layer was still transparent after methanol treatment for 10 minutes. The thickness of the shell layer increased from less than 200 μ m to around 850 μ m in wet state, as enhancing the immersion time in methanol from 1 to 10 minutes (Figure 1d).

The morphology of the core-shell SF hydrogels was observed by SEM. A compact structure was observed in the shell layer of the methanol treated SF hydrogels (Figures 2a-d). The SEM images revealed that the wall thickness of the dried core-shell hydrogels increased from around 100 μ m to about 500 μ m when immersion in methanol for 1 to 10

minutes (Figure 2a-d). The non-treated SF hydrogel displayed no core-shell structure (Figure 2m). The core layer of the core-shell hydrogels demonstrated loose and porous structures (Figure 2e-h), similar to the control (Figure 2n). The shell surface of the core-shell hydrogels was smooth (Figure i-l), while the one of the non-treated hydrogel was porous and rough (Figure 2o).

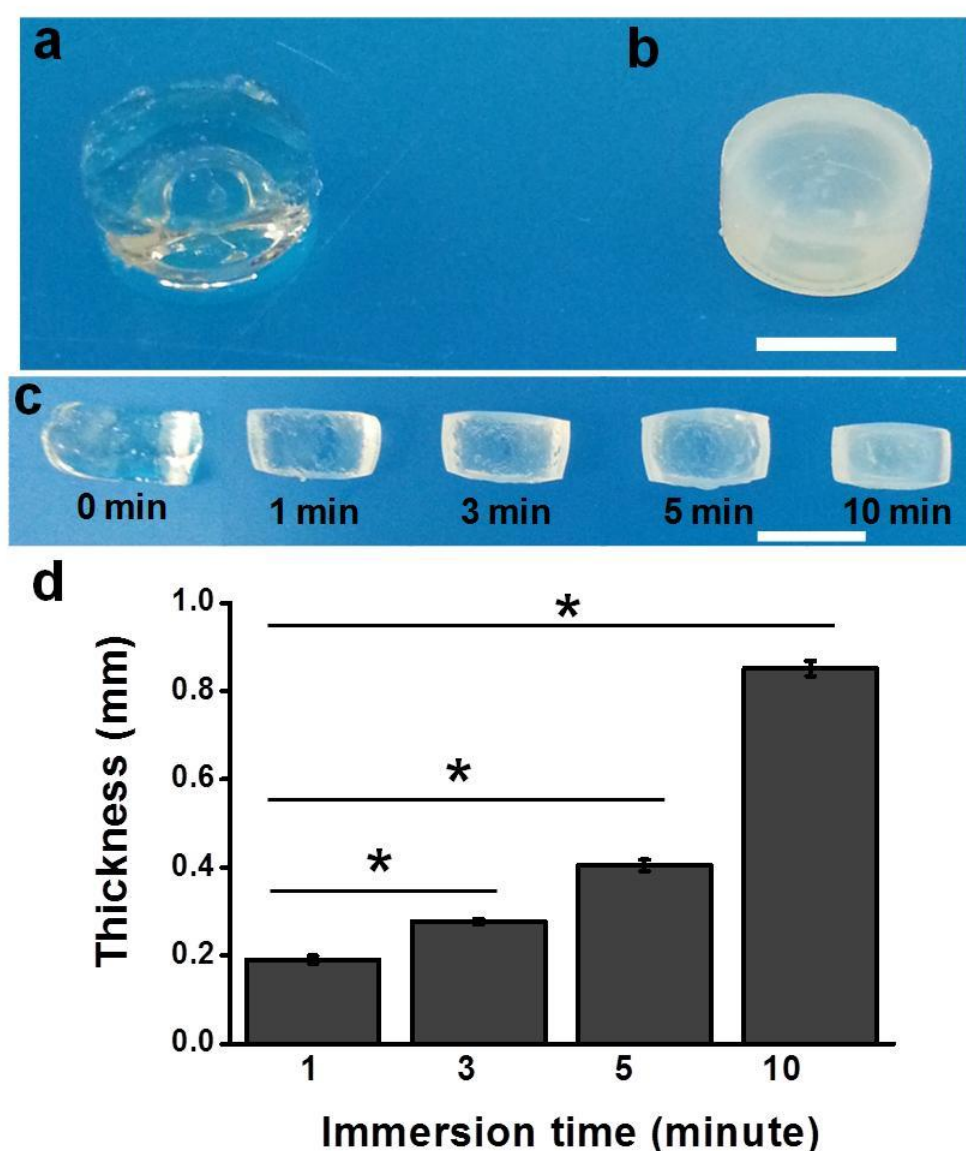


Figure 1. SF hydrogels with core-shell structure. (a) SF hydrogel without methanol treatment; (b) SF hydrogel after immersion in methanol for 10 minutes; (c) from left to right: longitudinal sections of the SF hydrogels after immersion in methanol for 0, 1, 3, 5 and 10 minutes, respectively; (d) thickness of the shell layer of the core-shell SF hydrogels after immersion in methanol for 1, 3, 5 and 10 minutes, respectively. * indicated statistically significant ($p < 0.05$). Scale bar: 5 mm.

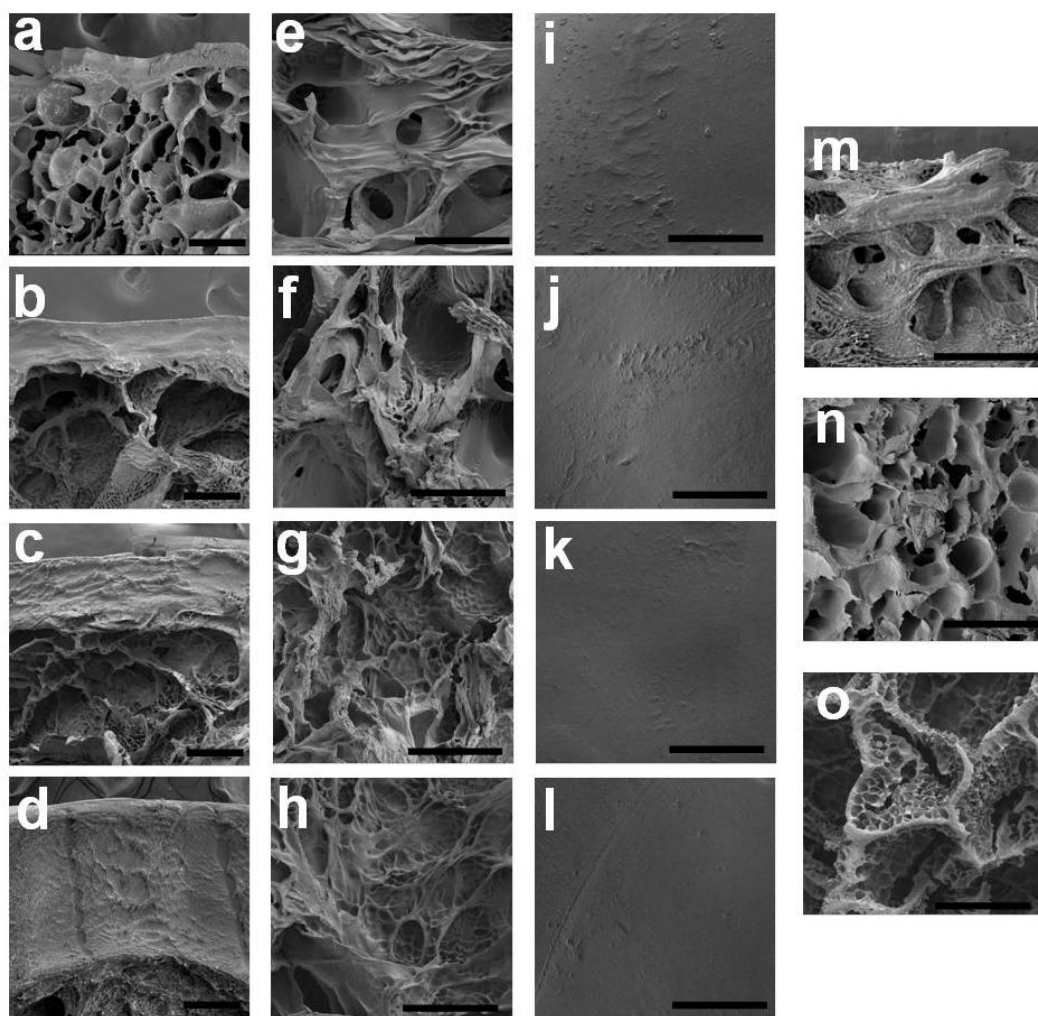


Figure 2. The SEM images of the core-shell SF hydrogels. (a-d) The shell layer after immersion in methanol for 1, 3, 5 and 10 minutes, respectively. (e-h) The core layer after immersion in methanol for 1, 3, 5 and 10 minutes, respectively. (i-l) The shell surface of the core-shell hydrogels after immersion in methanol for 1, 3, 5 and 10 minutes, respectively. (m-o) The outer region, inner region, and the external surface of the SF hydrogels without methanol treatment, respectively. Scale bar: 200 μm .

The structural conformation of the core-shell hydrogels was confirmed by ATR-FTIR analysis, as presented in Figure 3. It was found that a main peak located at around 1648 cm^{-1} appeared in the spectra of the aqueous silk solution, the non-treated SF hydrogel, and the core layer of the treated hydrogel (Figure 3a). It was depicted a small shoulder located at 1627 cm^{-1} when immersion the hydrogel for 10 minutes, however not dominant. In the interface region, two strong peaks appeared at 1648 cm^{-1} at 1627 cm^{-1} (Figure 3b). The intensity of the peak located at 1627 cm^{-1} gradually increased as increasing the immersion time in methanol (Figure 3b). When immersed more than 3

minutes, the intensity of this peak was slightly higher than the one of the peak located at 1648 cm^{-1} . The inner side of the shell layer in the core-shell hydrogels showed a dominant absorbance peak at 1627 cm^{-1} and an obvious shoulder peak at 1648 cm^{-1} (Figure 3c). The external surface of the shell layer in the core-shell hydrogels all showed a main peak around 1627 cm^{-1} and a very small shoulder at 1648 cm^{-1} (Figure 3d). These peaks were sharper than the ones of the inner side of the shell layer. When immersing in methanol for 10 minutes, a peak shift from 1627 cm^{-1} to 1621 cm^{-1} was observed.

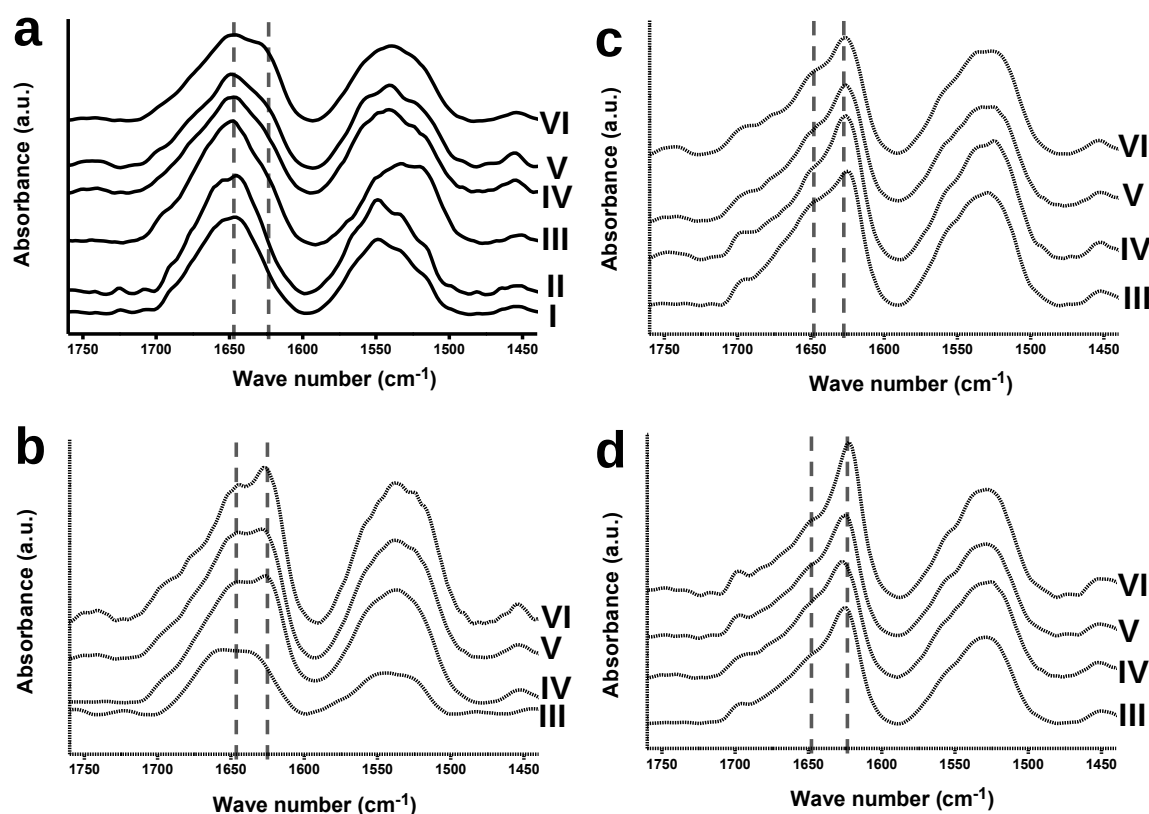


Figure 3. ATR-FTIR spectra of the core-shell SF hydrogels. (a) The core layer, (b) the interface region, (c) the inner side of the shell layer, and (d) the external side of the shell layer of the core-shell SF hydrogels. (a) I and II are corresponding to SF solution and SF hydrogels without methanol treatment, respectively. (a-d) III, IV, V and VI are corresponding to the core-shell hydrogels after immersion in methanol for 1, 3, 5 and 10 minutes, respectively.

It has been reported that the peak located between 1640 cm^{-1} and 1650 cm^{-1} indicates the amorphous conformation of SF, while the peak located at 1627 cm^{-1} is assigned to β -sheet conformation [22]. Figure 3a clearly shows that the dominant conformation in the aqueous silk solution, the non-treated SF hydrogel, and the core layer hydrogel was

amorphous. After immersion in methanol for 10 minutes, the core hydrogel also presented small amount of β -sheet conformation. In the interface region, both the amorphous and β -sheet conformations were dominant (Figure 3b). The inner side of the shell layer hydrogels was of dominant β -sheet conformation and certain amount of amorphous content (Figure 3c). The external side of the shell layer of the core-shell hydrogels all showed superior β -sheet conformation and very little amount of amorphous content in this side (Figure 3d).

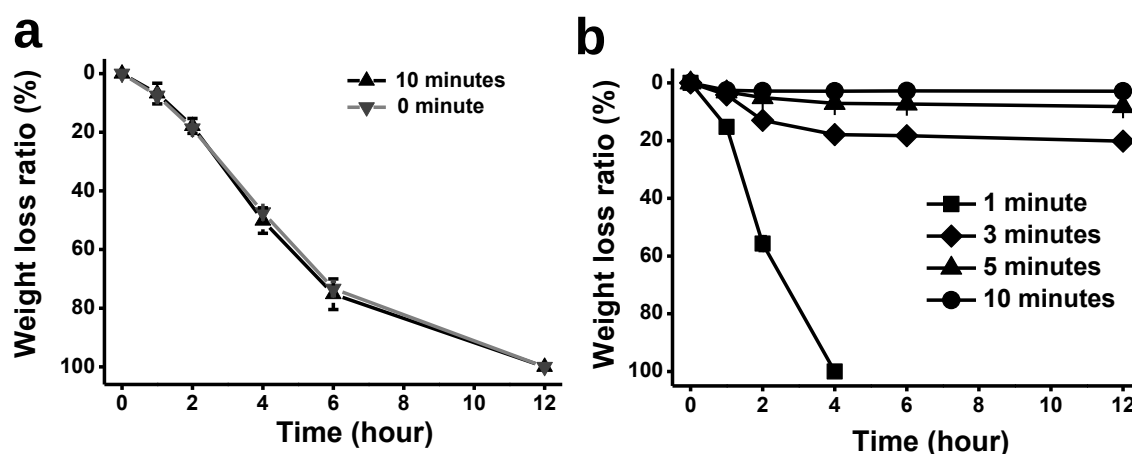


Figure 4. The enzymatic degradation of (a) the core layer and (b) the shell layer of the core-shell SF hydrogels. (a) 0 minute and 10 minutes indicate hydrogels without methanol treatment and hydrogels treated by methanol for 10 minutes, respectively. (b) 1 minute, 3 minutes, 5 minutes and 10 minutes indicated hydrogels after immersion in methanol for 1, 3, 5 and 10 minutes, respectively.

The biostability of the core-shell SF hydrogels was studied by *in vitro* enzymatic degradation. Figure 4a shows the enzymatic degradation profile of the non-treated hydrogel and the hydrogel in the core layer of the methanol treated samples. For each time point, these two groups of hydrogels presented a similar weight loss ratio. Both hydrogels degraded completely within 12 hours in a low concentration of protease XIV solution (Figure 4a). A mass loss of around 50% was observed for these two groups of hydrogels. The concentration of protease solution used for the degradation of the shell layer was 20 times the one used for the degradation of the core layer. After immersed in methanol for 3, 5 and 10 minutes, the shell layers of the core-shell hydrogels showed around 20%, 10% and 5% weight loss within 12 hours, respectively (Figure 4b). While the shell layer of specimens immersed in methanol for 1 minute was completely degraded within 4 hours. There was no obvious mass loss after 2 hours for the shell layer

of the core-shell hydrogels immersed in methanol for 10 minutes. The weight loss was quite slow after 4 hours for the shell layers of the core-shell hydrogels immersed in methanol for 3 and 5 minutes.

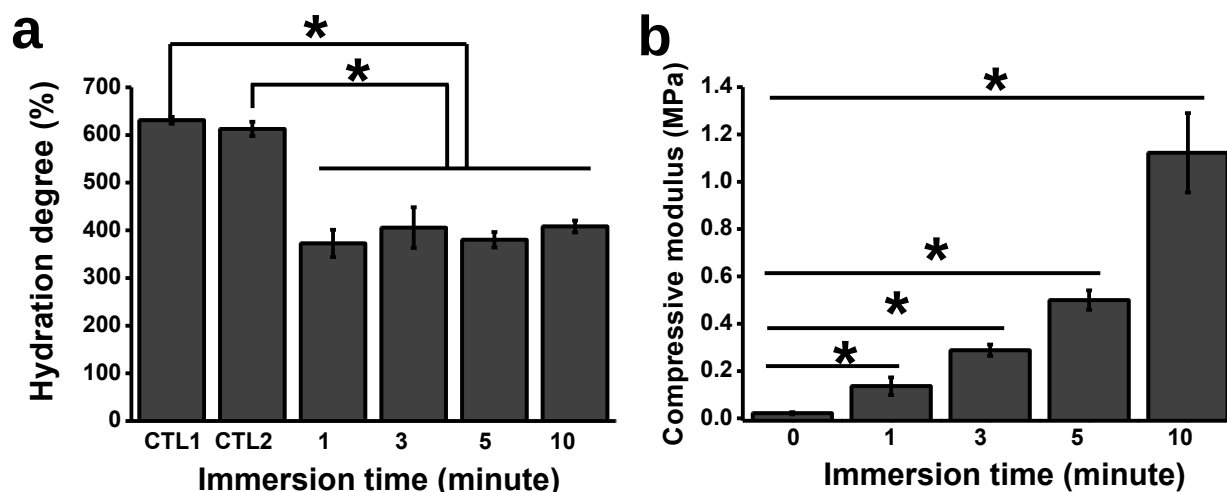


Figure 5. (a) Hydration degree and (b) compressive modulus of the core-shell SF hydrogels after immersion in methanol for different time periods. (a) CTL1 and CTL2 correspond to the SF hydrogels without methanol treatment and the core layer of the SF hydrogels after methanol treatment for 10 minutes, respectively. * indicated statistically significant ($p < 0.05$).

The core layer of the core-shell hydrogels presented similar hydration degree to the one of the non-treated hydrogels (Figure 5a). There were no significant differences in the hydration degree for the shell layers of methanol treated samples. However, the hydration degree of the shell layer was much less than the one of the core layer in the core-shell hydrogels (Figure 5a). The compressive modulus of the core-shell hydrogels increased dramatically when extending the immersion time in methanol (Figure 5b). The non-treated SF hydrogels showed a modulus around 22 kPa. After 10 minutes of immersion in methanol, the compressive modulus of the core-shell hydrogels increased more than 50 times as compared to the one for non-treated samples.

The core-shell SF hydrogels were evaluated as a drug delivery system, using albumin as a model drug, as presented in Figure 6. The non-treated SF hydrogel discs were able to incorporate 30.72 ± 1.09 μg albumin per disc. There were 23.09 ± 1.56 , 17.96 ± 1.14 , and 13.86 ± 0.87 μg albumin encapsulated in the core-shell SF hydrogels discs after immersion in methanol for 3, 5 and 10 minutes, respectively. As shown in Figure 6a and

b, the non-treated hydrogels present a lower intensity of fluorescence signal as compared to the core-shell hydrogels, after 24 hours release. The cumulative drug release results showed that more than 60% incorporated albumin was released in the non-treated hydrogels within 24 hours period (Figure 6c). After 72 hours, this group released nearly the total amount of the incorporated albumin. However, the core-shell hydrogels presented much slower release profiles compared to the one of the non-treated group (Figure 6c). The cumulative release ratio decreased as increasing the immersion time in methanol. In the first 24 hours, the core-shell hydrogels showed around 30%, 18% and 12% release of incorporated albumin for the hydrogels treated by methanol for 3, 5 and 10 minutes, respectively (Figure 6c). After 72 hours, the core-shell SF hydrogels only released around 22% to 47% of the incorporated albumin. One week later, it was observed that around 30%, 60% and 70% of incorporated albumin still remained in the core-shell hydrogels treated by methanol for 3, 5 and 10 minutes, respectively.

4. Discussion

In order to better fulfill the complex demand for tissue regeneration, the development of hydrogels with spatially and temporally tunable properties is an emerging trend in TERM applications [4-7]. The core-shell or multi-layered hydrogels can provide a favorable system for the tuning and achieving a spatial controlled degradation, mechanical properties, and permeability in order to control the cell behaviors or drug release profiles [12-15].

Different strategies for core-shell hydrogels development have been reported [12, 27-30]. Ladet *et al.* [12] have previously developed multi-membrane polysaccharide hydrogels by a multi-step interrupted neutralization of the alcohol gel in sodium hydroxide solution. In that procedure, the water replaced the alcohol of the initial alcohol gel and subsequently induced the formation of a physical gel containing only water. Thermo-responsive polymers have been explored for core-shell hydrogel preparation. Gao *et al.* [27] prepared poly-N-isopropylacrylamide core-shell nanoparticles via seed and feed precipitation polymerization. These particles were of tunable de-swelling properties. Another thermo-sensitive core-shell hydrogels were produced by Iizawa *et al.* [28], with poly(N-alkylacrylamide) as shell layer and poly(acrylic acid) in the core. The release of

methyl orange from the core-shell hydrogel presented on-off profile in response to stepwise temperature changes. Natural polymer or synthetic peptides were also used for core-shell structure materials manufacture. Alginate core-shell microcapsules were developed by Ma *et al.* [29] with islets encapsulation in the core, via a two-fluid co-axial electro-jetting method. These capsules were able to secrete insulin and control the mice blood glucose to normoglycemic level when implantation in diabetic mice. Koutsopoulos *et al.* [30] generated two-layered self-assembling peptide hydrogels for long-term delivery of human antibodies.

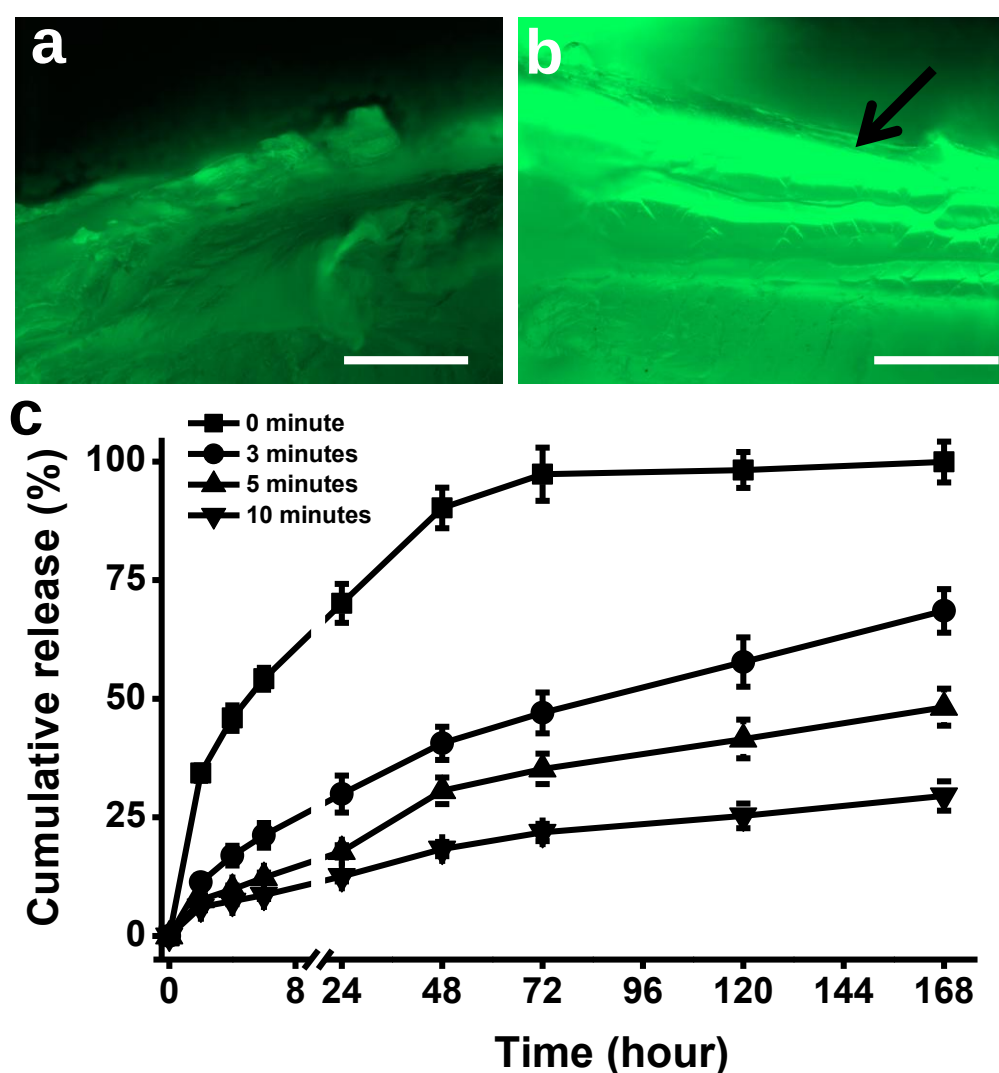


Figure 6. Albumin-FITC release profile of the core-shell SF hydrogels. (a, b) Fluorescence images of the non-treated and the core-shell SF hydrogels (treated by methanol for 3 minutes) after releasing albumin for 24 hours, respectively. Arrow indicated the shell layer of the core-shell SF hydrogels (Scale bar: 300 μm). (c) Albumin-FITC release profile from the SF hydrogels without methanol treatment (0 minute) and the core-shell SF hydrogels after methanol treatment for 3, 5 and 10 minutes, respectively.

The above mentioned core-shell systems were formed by the electrostatic force, polymerization of monomers, ionic cross-linking of biopolymers, or self-assembly. The current study provides a new strategy to produce core-shell structural hydrogels, namely by controlling the conformations of proteins. This strategy can be performed very easily and finished in a short time, with high efficiency and reproducibility. In our previous study, by using the very mild peroxidase mediated cross-linking procedure it was possible to produce SF hydrogels with mainly amorphous conformation [24]. The conformation of the amorphous SF hydrogels would change to β -sheet after encapsulation of cells for 7 days or subcutaneous implantation in mice for 2 weeks [24]. Methanol and other organic solvents were able to induce fast β -sheet formation in SF [18, 31, 32]. By simply controlling of the immersion time of these amorphous SF hydrogels in alcohol solvents (such as methanol or ethanol), it was possible to form a core-shell structure with distinct properties in each regions (Figure 1). The thickness of the shell layer can be easily controlled by the immersion time. The ATR-FTIR analysis clearly showed the conformation transition and distribution in the core-shell SF hydrogels (Figure 3). The shell layer of β -sheet conformation was formed immediately when immersion the SF hydrogel in methanol. As the diffusion of methanol into the inner region, the thickness of the shell layer increased. Due to the protection of the compact shell layer, the core region maintained mainly amorphous conformation. The interface was a region where the hydrogel just met the methanol and the SF molecules was at an intermediate status between the amorphous and β -sheet.

Up to now most of the developed SF based biomaterials have been mainly of crystallized structure, such as β -sheet or Silk-I structure [33, 34]. Some exceptions were found, for instances electrically generated SF hydrogels developed by Leisk *et al.* [22] were of dominant amorphous structure. However, these hydrogels are mechanically unstable without chemical cross-linking [22]. Silk films developed by controlled water annealing were able to present different amounts of β -sheet content and silk-I ratio, but without a spatial controlled conformation [34-36]. This study provides a facile method to control the properties of the SF hydrogels spatially. The core layer and the shell layer hydrogel present distinct properties.

The biostability and hydration degree of the core layer and the shell layer hydrogels were obviously different. The core layer hydrogels and the non-treated hydrogels degraded easily in low concentration protease solution (Figure 4a), which was related with their

amorphous conformation (Figure 3a) and consistent with our previous observation. Previously, amorphous SF hydrogels, derived from different concentrations of SF solution, all completely degraded in 12 hours in the protease XIV solution (0.005 U/mL) [24]. The shell layer presented much better biostability than the core layer (Figure 4b). The good stability of the shell layer was assigned to its β -sheet conformation (Figure 3c and d). When immersing the SF hydrogels in methanol for 1 minute, the β -sheet transition ratio in the shell layer was too low. Thus this group degraded faster compared with other groups. The longer immersion time in methanol led to the improved stability (Figure 4b), which came from the higher β -sheet transition ratio in the shell layer. The shell layer and core layer presented different wetting properties (Figure 5a) due to the conformation distinction. Koutsopoulos *et al.* [30] found that double-layered hydrogels released the human antibodies slower than the single layer hydrogel, owing to the differences of the density and chemical properties of the two layer hydrogels. When these core-shell SF hydrogels are loaded with drugs, the shell layer of high stability is useful for protection the drug activity *in vivo*, such as for oral delivery of insulin. Besides the protection role, the hydrophobic and compact shell layer can act as a barrier to control the hydrophilic drug diffusion from the core layer to the external environment. These properties endow the core-shell SF hydrogels with great potential for using as drug delivery systems.

The enormous improvement of the mechanical properties was induced by the increased volume of the crystallized hydrogel in the core-shell hydrogels. The amorphous SF hydrogels of different formulations presented storage modulus ranged from around 200 Pa to 5 kPa [24]. The core-shell hydrogels provided even wider tailored window in the mechanical properties. These hydrogels can fulfill several mechanical requirements for different tissue regeneration application, such as for bone, cartilage, and meniscus. Nguyen *et al.* [14] prepared multi-layered poly(ethylene glycol) based hydrogels mimicking the native cartilage. The compressive modulus of these layers ranged from ~200 kPa up to ~1 MPa. The core-shell SF hydrogels presented broader modulus range than the one in the previous study [14]. Besides, the core-shell SF hydrogels with more than 5 minutes methanol treatment presented comparable modulus to human cartilage, since the average compressive modulus of the human cartilage is around 1 MPa [37]. Thus these SF core-shell hydrogels display great potentials as tissue substitutes.

Controlled release of the model drug was achieved in the core-shell SF hydrogels with spatially controlled conformation (Figure 6). Besides albumin, it is also possible to control

the release profiles of different drugs in the core-shell SF hydrogels, by fine tuning of the conformation changes. The compact structure and the thickness of the shell layer played the key role for the controlled release of albumin in the core-shell hydrogels. In amorphous hydrogels, the molecules were highly hydrated and swelled, inducing large mesh size in these hydrogels. Thus the albumin released fast and easily from the amorphous SF hydrogels. In the core-shell hydrogels, the SF molecular chains became hydrophobic and shrank in the shell layer, forming a compact and stiff structure (Figure 2a-d and Figure 5b). By this way, the core-shell hydrogels displayed slower and more controllable release profiles compared with the amorphous hydrogels. The core-shell hydrogels with thicker shell layer would lead to the slower release profile. The obtained results were consistent with other multi-layer hydrogels or core-shell hydrogels reported in previous studies [15, 30]. Choi *et al.* [15, 38] incorporated paclitaxel in multi-layered phospholipid polymer hydrogels, and found that the paclitaxel release depended on the location and concentration of the drug containing polymer layer. Different from the multi-components systems and the time-consuming preparation procedures in previous studies [15, 30, 38], this study only used one component and a very facile procedure to prepare the core-shell structure. This proof-of-concept study opens the application possibility of the core-shell SF hydrogels for controlled release of growth factors or other bioactive macromolecules.

In addition to the possibility to be used in drug delivery systems or as tissue substitutes, the core-shell hydrogels can find interesting applications in other areas. The amorphous SF hydrogels have showed superior biocompatibility in previous cell encapsulation and *in vivo* implantation studies [24]. SF hydrogels of β -sheet conformation are also biocompatible [17]. Thus, the cross-section of the core-shell SF hydrogels can act as a biocompatible platform for short-term cell culture, and for studying the influence of different mechanical properties, conformations, and effect of selective nutrient or bioactive agents diffusion on the cell behaviors. By injection cells into the core layer, this core-shell hydrogels may also be used as a hypoxia bioreactor for investigation the cells performance. The amorphous SF hydrogels was pH and ionic strength stimuli response [24]. Therefore, the core-shell hydrogels can be processed into sensors when coated in the biomedical devices.

5. Conclusions

In this study, core-shell SF hydrogels with spatially controlled physicochemical properties were developed by spatial manipulation of the SF conformation. These hydrogels are composed by stiff shell layer with main β -sheet conformation and an elastic soft core layer of dominant amorphous conformation. The distribution of these two layers can be easily tailored by varying the immersion time in alcohol solution. The shell layer demonstrated higher biostability and lower hydration properties as compared to the core layer, making this core-shell hydrogels suitable as a potential drug delivery system (e.g., oral delivery system or subcutaneous implantation system). The mechanical properties of these core-shell hydrogels can be tuned in a broad range, showing high potential for various tissue substitute applications, such as for bone and cartilage. Moreover, the core-shell hydrogels were able to provide a sustained system for drug delivery. Overall, the core-shell SF hydrogel with spatially tailored structure produced in this study opens a new window for the application of SF based biomaterials in tissue engineering and regenerative medicine, as well as in drug delivery system. This study also brings new insights in development of biomaterials with sophisticated structure by tuning the silk fibroin conformation.

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Section 5.

Chapter X

General Conclusions and Final Remarks

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1. General Conclusions

Biomaterials have been considered as one of the key elements in tissue engineering and regenerative medicine. Silk fibroin (SF) is an appealing biomaterial which can be processed into different forms and whose properties can be tuned by controlling its molecular conformation.

As porous scaffolds or hydrogels, SF can act as a three-dimensional substrate for cells to adhere and grow. These structures also play an important role as mechanical support in the defect site when implanted. SF based scaffolds have been prepared and applied for tissue engineering, such as for bone and cartilage regeneration. The mechanical properties of SF scaffolds were inferior to those found in human bone or cartilage. Thus, the improvement of the mechanical properties and integration of the SF scaffolds is critical for the successful tissue regeneration. The goal of this thesis was to develop silk fibroin (SF) based scaffolds and enzymatically cross-linked hydrogels with advanced properties for better fulfill the requirements of tissue engineering and regenerative medicine.

Up to now, only a few studies have been performed to improve the mechanical properties of SF scaffolds, for instance by fiber or particle reinforcement. In this thesis, a simple strategy was used for improving the strength of SF scaffolds without deleterious affecting its total porosity and pore size distribution. Highly concentrated aqueous SF solution and salt-leaching were combined and employed to produce macro/microporous scaffolds. In order to endow the developed SF scaffolds with osteoconductive properties aiming at bone regeneration, nano calcium phosphate particles were introduced into the SF scaffolds (Silk-NanoCaP) via an *in-situ* synthesis approach. The *in-situ* synthesis approach led to the homogeneous dispersion of the calcium phosphate (CaP) particles in the silk matrix. Based on the initial works on SF and Silk-NanoCaP scaffolds in this thesis, bilayered Silk/Silk-NanoCaP scaffolds were also generated for osteochondral tissue engineering. These bilayered scaffolds were capable to induce fast subchondral

bone formation. Besides the development of the SF based scaffolds, this thesis also focus on hydrogel preparation. In the literature, several SF hydrogels have been developed. However they were not suitable to be used as injectable systems due to their long gelation time or harsh preparation conditions. Herein, a novel gelation method was proposed to prepare injectable SF hydrogels, namely peroxidase mediated cross-linking in the presence of different concentrations of oxygen peroxide. Furthermore, SF hydrogels with spatially controlled properties were produced by tuning the SF molecular conformation. In the following Sections are presented the general conclusions of the experimental work, from Chapters III to IX.

1.1. Macro/microporous SF scaffolds with potential for cartilage and meniscus tissue engineering applications

SF based scaffolds have been prepared and applied for tissue engineering, such as for cartilage, meniscus, and bone regeneration. However, the mechanical properties of SF scaffolds were inferior to those found in human bone or cartilage. Thus, the improvement of the mechanical properties of the SF scaffolds is critical for the successful tissue regeneration. Chapter III described the feasibility of preparation of SF scaffolds with superior mechanical properties derived from high concentration aqueous SF solution. The novelty of this work consisted on the fact that it was possible to prepare salt-leached SF scaffolds with more than 10 wt.% SF aqueous solution. In this study, an initial physicochemical characterization is presented on SF scaffolds derived from high concentration aqueous SF solution and prepared by combining salt leaching and freeze-drying methodologies. The results indicated that the developed scaffolds presented β -sheet conformation. The morphological study revealed that the scaffolds possessed both macro- and micro-porous structures, and the morphology varied depending on the initial concentration. The micro-CT analysis further demonstrated the prepared scaffolds possessed high porosity and interconnectivity, which seemed to decrease with increasing SF concentration. An opposite trend was exhibited in terms of the trabecular thickness of the scaffolds. The compressive test and DMA analysis showed that the mechanical properties of the SF scaffolds increased dramatically with the increasing of SF concentration. The viscosity properties of the SF scaffolds were stable for the testing frequencies. The water-uptake data demonstrated that the scaffolds presented a high swelling capability that increased with increasing porosity. It should be highlighted that the prepared scaffolds were able to keep their original structure and morphology, as well

as their original mechanical properties, after 30 days of immersion. Therefore, the developed SF scaffolds were good candidates to be used in tissue engineering scaffolding, namely for cartilage and meniscus regeneration. This study also opens a new window to prepare load-bearing multifunctional SF based scaffolds for other specific tissue engineering applications, such as bone and osteochondral tissue.

1.2. Bioactive macro/microporous Silk-NanoCaP scaffolds with potential for bone regeneration

SF based scaffolds have been developed and applied for bone tissue engineering. However, these scaffolds were not osteoconductive and their mechanical properties were inferior to those found in human bone. Therefore, the enhancement of the mechanical properties of the SF scaffolds and improvement of the integration between SF scaffolds and host bone are crucial in bone tissue engineering. Chapter IV reported the production of macro/microporous Silk-NanoCaP scaffolds, through the *in-situ* synthesis of nano-sized CaP in a high concentration aqueous SF solution (16 wt.%) followed by scaffolding using a salt-leaching/lyophilization approach. This study presented a good example of how to bridge the nano-sized bioactive particles with a three-dimensional porous scaffold by using combined facile approaches, namely *in-situ* synthesis and salt-leaching/lyophilization. The CaP particles consisted of poorly crystalline HA and the SF presented β -sheet conformation. The synergetic effect of the *in-situ* synthesis method and the highly concentrated SF aqueous solution allowed to uniformly distributing the CaP particles in the scaffolds, at both microscopic and macroscopic scales. The combination of salt-leaching/lyophilization approaches allowed the formation of highly interconnected macro-pores, homogeneous porosity distribution, and high interconnectivity in the Silk-NanoCaP scaffolds. The Silk-NanoCaP scaffolds with the theoretical CaP content of 16 wt.% present the highest wet status storage modulus. The porosity and hydration degree of the Silk-NanoCaP scaffolds can be controlled by the amount of CaP particles incorporated. The developed SF and Silk-NanoCaP scaffolds were non-cytotoxic. The Silk-NanoCaP scaffolds developed present promising mechanical properties, suitable architecture and stability, superior bioactivity and no cytotoxicity, which make them suitable for possible application in bone tissue engineering scaffolding.

1.3. *In vitro* and *in vivo* characterization of the SF and Silk-NanoCaP scaffolds

Chapter V examined the *in vitro* biological performance of the developed SF and Silk-NanoCaP scaffolds. The incorporation of CaP in the SF matrix further improved the stability of the scaffolds during enzymatic degradation. Both SF and Silk-NanoCaP scaffolds were non-cytotoxic, and promoted the attachment, viability, proliferation, and migration of the human adipose tissue derived stromal cells (hASCs). The microporous structure favored the adhesion of the hASCs and the macroporous structure promoted the proliferation and migration of the cells. The culture of hASCs upgraded the biomechanical properties of these SF based scaffolds.

Chapter VI demonstrated the *in vivo* bone regeneration ability of the SF and Silk-NanoCaP scaffolds. In this study, the novel salt-leached Silk-NanoCaP scaffold presented a rapid bioactive response *in vitro*, evidenced by the formation of apatite crystals on its surface after one day of immersion in a simulated body fluid (SBF) solution. During long-term degradation, both the SF and Silk-NanoCaP scaffolds had an adequate biostability in terms of hydration degree along with a slow weight loss. After 3 weeks implantation in rat bone defects, both scaffold types supported new bone in-growth and no acute inflammatory response was observed. The Silk-NanoCaP scaffolds were shown to be osteoconductive since they supported new bone formation on their surface. Furthermore, this group of scaffolds induced significantly higher amount of new bone formation as compared to the observed for SF scaffolds. Silk-NanoCaP scaffolds are good candidates for bone tissue engineering.

1.4. Bilayered Silk/Silk-NanoCaP scaffolds for osteochondral tissue engineering

Chapter VII proposed a novel bilayered SF based scaffold for osteochondral defect (OCD) regeneration. This scaffold fully integrates a SF layer and a Silk-NanoCaP layer. The *in situ* synthesis route allowed controlling the size of CaP particles in the bone-like layer. These scaffolds presented superior mechanical properties and suitable stability due to the β -sheet conformation in the SF and the high concentration of SF aqueous solution for scaffold preparation. Spatially controllable porosity and CaP distribution/confinement were also obtained with these bilayered scaffolds. Apatite formation was induced after immersion in SBF solution clearly restricted to the Silk-

NanoCaP layer. This layer promoted higher ALP activity when seeded with rabbit bone marrow mesenchymal stromal cells (RBMSCs) and cultured in osteogenic condition, as compared to the SF layer. The scaffolds supported cells' attachment, viability, and proliferation when cultured with RBMSCs *in vitro*. Furthermore, these scaffolds allowed tissue ingrowth and induced very weak foreign body reaction when subcutaneously implanted in rabbit for 4 weeks. When implanted in the knee critical OCD in rabbit for 4 weeks, the bilayered scaffolds were able to integrate well with the host tissues and induced no acute inflammation. These scaffolds matched the mechanical environment of the OCD and maintained their stability. Moreover, the bilayered scaffolds supported the cartilage regeneration in the top silk layer. A large amount of subchondral bone ingrowths was achieved exclusively in the Silk-NanoCaP layer. These promising results demonstrated that the bilayered scaffolds prepared in this study are good candidates for OCD tissue engineering applications, where the properties at the interface between both tissues should be replicated.

1.5. Peroxidase mediated cross-linked SF hydrogels for tissue engineering and regenerative medicine applications

In chapter VIII it is demonstrated that injectable SF hydrogels can be prepared by peroxidase mediated cross-linking under physiological condition. When compared with SF hydrogels prepared elsewhere, these hydrogels are able to present a shorter gelation time and less aggressive preparation conditions. The SF hydrogels presented completely distinct properties compared to the SF hydrogels of β -sheet conformation in previous studies. They were of amorphous conformation, transparent appearance, and outstanding elasticity. The gelation time and mechanical properties can be tuned from 1 hour to within 5 minutes and from around 200 Pa to around 5 kPa, respectively. Notably, these hydrogels displayed ionic strength and pH stimuli response properties. Additionally, these hydrogels were non-cytotoxic and biocompatible *in vivo*. These versatile SF hydrogels not only bring new insights in the fundamental study of SF based biomaterials, but also constitute a new candidate for several biomedical applications, such as drug delivery, medical devices, tissue regeneration and regenerative medicine.

1.6. Core-shell SF hydrogels with spatially controlled conformations

Chapter IX presented core-shell SF hydrogels with spatially controlled physicochemical properties that were developed by spatial manipulation of SF conformations. These hydrogels contained a stiff shell layer with main β -sheet conformation and an elastic soft core layer of dominant amorphous conformation. The distribution of these two layers can be easily tailored by varying the immersion time in alcohol solvents. The shell layer demonstrated higher biostability and lower hydration properties as compared to the core layer, making this core-shell hydrogels suitable as a potential drug delivery system (e.g., oral delivery system or subcutaneous implantation system). The mechanical properties of these core-shell hydrogels can be tuned in a broad range, showing high potentials of these hydrogels for various tissue substitute applications, such as for bone and cartilage. Moreover, the core-shell hydrogels were able to provide a sustained system for drug delivery, approving these hydrogels can be a superior controlled release system. Overall, the core-shell SF hydrogel with spatially tailored structure produced in this study opens a new window for the application of SF based biomaterials in tissue engineering and regenerative medicine, as well as in drug delivery systems. This study also brings new insights in development of biomaterials with sophisticated structure for biomedical applications, specific for protein-based biomaterials.

2. Final Remarks

The herein developed SF scaffolds and hydrogels can find numerous applications for tissue engineering and regenerative medicine, or drug delivery systems. These scaffolds and hydrogels can also be tuned or functionalized to better fulfill the application requirements.

Regarding the SF scaffolds, they have potential for cartilage and meniscus repair/regeneration. The pore size of current developed SF scaffolds is around several hundred micrometers which is good for bone tissue engineering. For cartilage and meniscus regeneration, the porosity and pore size of the scaffolds must be further screened by *in vitro* and *in vivo* experiments. In the future, the *in vitro* biological evaluation should be performed using primary cells (such as chondrocytes or meniscus cells) or stem cells (e.g., bone marrow stromal cells, adipose-tissue derived stromal

cells). Specific bioreactor can be combined to increase the cell seeding efficiency and facilitate cell proliferation. Hydrostatic pressure bioreactor may be applied to enhance the chondrogenic differentiation of the cells in the scaffolds. More specific chondrogenic evaluation studies need to be performed, such as the glycosaminoglycan content, collagen II content, and quantitative chondrogenic gene expressions (e.g., Sox 9 and collagen II). *In vivo* studies using cellular or acellular strategy need to be pursued. The biomechanical properties and degradation of the implants should be evaluated *in vivo*, aiming to select the best scaffold candidate for cartilage or meniscus regeneration. Biological factors, such as growth factors or hormones, can be encapsulated into the scaffolds to promote regeneration outcome.

In case of the Silk-NanoCaP scaffolds, they should be fully evaluated for bone regeneration. *In vitro*, comprehensive characterization should be carried out to study the interaction between scaffolds and cells. Bone marrow stromal cells or other source stem cells can be used for osteogenesis differentiation. The osteogenic gene expression is worthy to screen, such as the ALP, BMP-2, osteocalcin, osteopontin genes. The vascularization in the scaffolds is important for bone tissue engineering. Thus co-culture of endothelial cells and osteoblasts (or osteogenic stem cells) is worthy to advance. Since short-term bone defect regeneration had been done, following the long-term *in vivo* experiment should be performed. Large bone defect model and big animal models could be used. During the *in vivo* study, the degradation of the scaffolds and the biomechanical properties of the explants must be monitored. Drugs favoring bone regeneration can be incorporated in the scaffolds, such as biphosphonates (alendronate and zoledronate). Growth factors could be used to promote *in vivo* new bone or vessel formation, these including BMP-2 and vascular endothelial growth factor.

Concerning the bilayered Silk/Silk-NanoCaP scaffolds, further *in vitro* and *in vivo* biological evaluation should be performed. *In vitro*, specific double chamber bioreactors could be used for seeding and culturing different cells in the chondral and subchondral layers, respectively. For example, the chondral layer and the subchondral layer can be seeded with chondrocytes and osteoblasts, respectively. Other possibilities include the seeding of bone marrow stromal cells into both layers and subsequently performing chondrogenesis and osteogenesis, respectively. Besides the evaluation of bony tissue and chondral tissue formation in specific layer, the engineered osteochondral interface should also be carefully analyzed. This includes the integrated strength, and the

formation of calcified cartilage tissue, specific gene expression (such as collagen X, MMP-13, Ihh, PTHrP genes). *In vivo*, long-term implantation time should be performed. Cellular strategy can also be investigated. For instance, the *in vitro* engineered osteochondral tissue can be implanted. Growth factors (e.g., BMP-2 or TGF- β 1) may be incorporated into the chondral layer to promote neocartilage formation. During the OCD implantation, external mechanical stimulus on the implanted site may be employed, such as continuous positive motions. Other stimulus, like shock wave, microwave, and magnetic therapy may be used.

The peroxidase mediated cross-linked SF hydrogels show great promise in a wide range of applications. They can be used as injectable materials for filling tissue defects, such as for bone and cartilage. Since tyrosine group is common in host tissues, these SF hydrogels could covalently bind to the host tissue during the enzymatically cross-linking, thus enhancing material/tissue affinity. Since these hydrogels would become crystalline, they may be suitable for long-term implantation in cartilage and bone. Furthermore, these hydrogels can act as short-term tissue substitute, such as for skin wound dressing or cornea substitute. Drugs or growth factors can be encapsulated in these hydrogels as short-term release system to improve the tissue regeneration. When implanting medical detectors *in vivo* to monitor or detect the organs or tissues (such as brain), these hydrogels could be promising encapsulation materials due to their superior *in vivo* tissue biocompatibility. Moreover, the hydrogels can be incorporated with cancer cells and then studying the cell destruction behavior during the formation of β -sheet structure. These hydrogels can also be used as *in vitro* models of diseases or as coating for medical devices. In addition to the biomedical application, since they are stimuli-responsive, it can be processed as actuators by changing the pH or ionic strength. Additionally, they have potential as sensors for detecting ionic strength and pH value.

The core-shell hydrogels can be used as tissue substitutes, such as for bone, cartilage, and meniscus. Different tissue substitute requires varied mechanical properties. The core-shell hydrogels demonstrate tunable properties in broad ranges. They can fulfill these requirements easily by changing the immersion time in organic solvents. Additionally, they are promising drug delivery systems and it can be potentially applied as oral delivery or subcutaneous implantation delivery systems. For example, they can be encapsulated with insulin and release through oral delivery for diabetic therapy. They can also be incorporated with drugs or growth factors during the implantation as tissue

substitute to enhance tissue regeneration outcome. This core-shell structure may also act as a micro-bioreactor to study cells' behavior or as models for disease or tumors.

There are many interesting ideas that can be explored having this thesis as a basis. Some of them are currently ongoing by other colleagues.

