



Universidade do Minho
Escola de Engenharia

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**Advanced technological approaches for 3-D
hierarchical living tissues**

Dissertação de Doutoramento

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

Trabalho efetuado sob a orientação do

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Doutora Alexandra Margarida Pinto Marques

STATEMENT OF INTEGRITY

I hereby declare having conducted my thesis with integrity. I confirm that I have not used plagiarism or any form of falsification of results in the process of the thesis elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

University of Minho, 26th March 2018

Full name: Raphaël Faustino Canadas

Signature:



To my parents

"One, remember to look up at the stars and not down at your feet. Two, never give up work. Work gives you meaning and purpose and life is empty without it. Three, if you are lucky enough to find love, remember it is there and don't throw it away." (2010)

— Stephen William Hawking (Oxford, 8 de janeiro de 1942 — Cambridge, 14 de março de 2018)



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HELIUM PRODUCTION IN AN ANISOTROPIC BIG-BANG COSMOLOGY

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THERE has recently been much interest in the helium content of the universe. A variety of observations, mainly on objects in our own Galaxy but some of nearby galaxies, have suggested that the helium abundance relative to hydrogen may be rather uniform with about one helium atom to every eleven hydrogen atoms. If such an abundance were universal, except for local irregularities mainly with a higher helium abundance, it would mean that about 27 per cent by mass of the universe is helium and it would seem plausible that the general helium abundance has been produced cosmologically, and that the local irregularities are due to helium production

but that the anisotropy rapidly disappeared in the expansion. It is the purpose of this article to point out that the existence of such an anisotropy, even if it only persisted for a few years, could have had a profound effect on the helium production.

The Early Stages of an Anisotropic Universe

It is well known that, in the early stages of the expansion of a homogeneous, isotropic hot big-bang universe in which radiation energy density dominates over material energy density, there is a unique relation between temperature and time. Hoyle and Bondi give the relation in

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ABSTRACT

In nature, isotropic and anisotropic hierarchical patterns characterize living tissues, in which cells assemble in multiple interacting three-dimensional (3-D) arrangements that associate both structure and function to compose complex organisms. Nowadays, *in vitro* generated biological models have been translated from two-dimensions (2-D) to 3-D, resulting in significantly improved biomimetic features of human native tissues. However, the area of cell culture systems has not evolved at the same rate as the state of the art of tissue engineering.

In the work described in this doctoral thesis, 3-D gradients, the control over porosity architecture, and the development of cell culture devices for 3-D sophisticated engineered tissues were targeted to achieve the development of advanced solutions either to fabricate hierarchical 3-D tissues matured *in vitro*, or to create cell culture systems adapted to the development of such biomimetic tissues. So, 3-D biocompatible structures were fabricated based on gradients of microparticles and applied *in vivo* for osteochondral defects regeneration. Furthermore, the porosity orientation of 3-D structures was controlled into anisotropic and isotropic architectures for the regeneration and mimicry of soft-hard interfaced tissues, such as the osteochondral unit, and to reproduce relevant patterned features of other tissues, such as the linearity of neocortex. Additionally, a bioreactor and a rotational platform were developed to enable a better spatial control over cell adhesion and to induce stem cell co-differentiation through gradient of growth factors in a single construct. This allows the creation of specific phenotypic profiles with controlled location within a single construct. Therefore, stem cell co-differentiations and co-cultures of different cell types in single constructs were used to study the expression of both osteogenic and chondrogenic phenotypes as well as the pre-vascularization of one part of the structure.

In a summary, the developed work has a direct impact in the cell culture of hierarchical 3-D tissues *in vitro* and in the development of interfaced tissue-like constructs, with potential interest for the drug development, as well as for regenerative medicine.

Keywords: Hierarchical structures, cell co-cultures, bioreactors, interfaces, gradients

RESUMO

Na natureza, os tecidos vivos são compostos por padrões hierárquicos e isotrópicos/anisotrópicos, nos quais as células se organizam em múltiplas disposições tri-dimensionais (3-D) que definem a sua função. Atualmente, a recriação de sistemas biológicos *in vitro* evoluiu de sistemas bidirecionais (2-D) para 3-D, o que resultou em melhorias significativas na reprodução das características nativas dos tecidos humanos. Contudo, a evolução dos sistemas de cultura celular não tem acontecido ao ritmo do estado da arte da engenharia de tecidos.

No trabalho de doutoramento aqui descrito, gradientes 3-D, o controlo sobre a arquitectura da porosidade e o desenvolvimento de sistemas de cultura para a engenharia de tecidos 3-D sofisticados, foram focados de forma a serem desenvolvidas soluções avançadas quer para a fabricação de tecidos 3-D hierárquicos *in vitro*, quer para se criarem sistemas de cultura celular adaptados ao desenvolvimento desses tecidos. Para isso, foram fabricadas estruturas 3-D biocompatíveis com base em gradientes de micropartículas, e aplicadas para a regeneração de defeitos osteocondrais. Para além disto, a orientação da porosidade em estruturas 3-D foi controlada para formar arquiteturas anisotrópicas ou isotrópicas capazes de mimetizar e regenerar tecidos em interfaces, transitando de “rígida-para-suave”, como a unidade osteocondral, assim como para reproduzir padrões de outros tecidos, como a linearidade do córtex cerebral. Adicionalmente, foram desenvolvidos um biorreator e uma plataforma rotacional para melhorar o controlo espacial da adesão celular, e a co-diferenciação de células estaminais numa única estrutura usando gradientes de fatores de crescimento, possibilitando a criação de estruturas únicas com fenótipos específicos em diferentes locais. Co-diferenciação de células estaminais ou co-culturas de diferentes tipos de células numa mesma estrutura foram então utilizadas para estudar a expressão de dois fenótipos, osteogénico e condrogénico, e, ainda, a indução do processo de prevascularização localizado numa região específica dessa estrutura.

Sumarizando, o trabalho desenvolvido tem impacto direto na cultura *in vitro* de células em estruturas hierárquicas 3-D, e no desenvolvimento de interfaces de tecidos, com potencial interesse para as áreas de desenvolvimento e rastreio de fármacos, bem como para a medicina regenerativa.

Palavras-chave: Estruturas hierárquicas, co-culturas celulares, biorreatores, interfaces, gradientes

TABLE OF CONTENTS

ABSTRACT	xi
RESUMO	xiii
LIST OF ABBREVIATIONS AND ACRONYMS.....	xxi
LIST OF FIGURES	xxv
LIST OF TABLES.....	xxxi
SHORT <i>CURRICULUM VITAE</i>	xxxiii
LIST OF PUBLICATIONS	xxxv
INTRODUCTION TO THE THESIS STRUCTURE	xlili
SECTION I: GENERAL INTRODUCTION.....	1
CHAPTER 1: GENERAL BACKGROUND.....	3
1. INTRODUCTION.....	5
1.1 HIERARCHICAL TISSUE ENGINEERING STRATEGIES.....	5
1.2 OSTEOCHONDRAL TISSUE ENGINEERING.....	7
1.2.1 Bone and Cartilage Phenotypes in Osteochondral unit	7
1.2.2 Engineering Osteochondral Composites.....	9
1.2.3 Biomaterials for Bone and Cartilage Regeneration	12
1.2.4 Stem Cells for Engineering Bone and Cartilage.....	15
1.3 NEURAL TISSUE ENGINEERING.....	16
1.3.1 Neocortex Phenotype	16
1.3.2 Engineering Neural <i>in vitro</i> Models	19
1.3.3 Biofabrication and Self-Assembling Techniques for 3-D Neural Models	20
1.4 DYNAMIC CELL CULTURE SYSTEMS	21
1.4.1 Mass Transport: Diffusion and Convection.....	22
1.4.2 Flow Conditions for Dynamic Cell Cultures	23
1.4.3 Flow Shear Stress and Related Stimuli	24
1.4.4 Bioreactor Designs.....	24
1.4.5 Microfluidic Designs.....	27
1.5 BIOREACTORS AND MICROFLUIDICS FOR OC <i>IN VITRO</i> TISSUE MODELLING.....	30
1.6 BIOREACTORS AND MICROFLUIDICS FOR NEURAL <i>IN VITRO</i> TISSUE MODELLING.....	33

1.7 GENERAL REMARKS.....	36
1.8 REFERENCES	38
CHAPTER 2: MARKET ANALYSIS.....	55
2. THREE-DIMENSIONAL STATIC AND DYNAMIC CELL CULTURE SYSTEMS: AN EMERGING MARKET FROM A SCIENTIFIC NEED	57
2.1 Abstract.....	57
2.2 Introduction.....	57
2.3 Significance and current limitations of 3-D cell culture	59
2.4 Market of the 3-D cell culture	62
2.5 Cell culture systems roadmap for startups	64
2.5.1 Pricing model	65
2.5.2 Financing culture systems.....	67
2.5.3 Distribution.....	67
2.5.4 Competition and market share	68
2.5.5 Marketing strategy	68
2.6 Adoption of 3-D cell culture devices.....	69
2.7 Patent landscape of 3-D cell culture systems.....	71
2.8 Regulatory demands	72
2.9 Competition and Cases of success.....	73
2.10 Survey analysis.....	80
2.11 Concluding remarks and future trends	81
2.12 Research Methodology.....	82
2.13 References	83
2.14 Acknowledgements	90
2.15 Supplementary Information.....	90
SECTION II: EXPERIMENTAL PART.....	95
CHAPTER 3: MATERIALS AND METHODS.....	97
3. MATERIALS AND METHODS.....	99
3.1 Abstract.....	99
3.2 Materials	99

3.2.1 Gellan Gum	99
3.2.2 Gelatin.....	100
3.2.3 Hydroxyapatite microparticles	101
3.3 Methods.....	103
3.3.1 Materials preparation/modification.....	103
3.3.2 Materials characterization	105
3.3.3 3-D structures preparation and characterization	107
CHAPTER 4: CONTROL OVER 3-D GRADIENTS.....	147
4. PROGRAMMED DENSITY-BASED GRADIENTS IN GELS WITH SPATIAL CONTROL OVER BIOACTIVE FACTORS FOR 3-D TISSUE INTERFACES	149
4.1 Abstract.....	149
4.2 Introduction.....	149
4.3 Results	150
4.4 Discussion.....	163
4.5 Material and methods.....	166
4.6 References	174
4.6 Acknowledgments.....	176
4.7 Supplementary Information.....	177
4.7.1 Supplementary Results	177
CHAPTER 5: CONTROL OVER 3-D POROSITY.....	183
5. TUNABLE ANISOTROPIC NETWORKS FOR 3-D ORIENTED NEURAL TISSUE MODELS	185
5.1 Abstract.....	185
5.2 Introduction.....	185
5.3 Results	188
5.3.1 Physicochemical control and characterization of hydrogel-based systems.....	188
5.3.2 Guiding porosity of 3-D oriented porous systems to obtain isotropic or anisotropic structures	192
5.3.3 Controlling diameter of linear porosity obtained on anisotropic structures for neurite guidance	194

5.3.4 Microfluidic custom design for tissue interfaces: heterotypic culture of HUVECS and primary neurons.....	196
5.4 Discussion.....	198
5.5 Materials and methods	201
5.6 References	206
5.7 Acknowledgments.....	209
5.8 Supplementary Information.....	210
5.8.1 Supplementary Materials and Methods.....	210
5.8.2 Supplementary Results	211
CHAPTER 6: BIOREACTOR FOR 3-D HIERARCHICAL CELL CULTURES.....	223
6. MULTI-CHAMBERS BIOREACTOR, METHODS AND USES	225
6.1 OBJECT OF INVENTION.....	225
6.2 DESCRIPTION	225
6.2.1 Background Art.....	225
6.2.2 General description of the invention	226
6.2.3 Brief description of the drawings, pictures and proof of concept.....	229
6.2.4 Description of preferred embodiments.....	232
6.3 CLAIMS.....	236
CHAPTER 7: DYNAMIC SYSTEM FOR AUTOMATED 3-D CELL CULTURE.....	239
7. DYNAMIC PLATFORM FOR A ROTATIONAL DUAL CHAMBER BIOREACTOR: METHODS AND USES THEREOF	241
7.1 OBJECT OF INVENTION.....	241
7.2 DESCRIPTION	241
7.2.2 Background Art.....	241
7.2.3 Summary	245
7.2.4 General description of the invention	247
7.2.5 Brief description of the drawings, pictures and proof of concept.....	249
7.2.6 Description of preferred embodiments.....	254
7.3 CLAIMS.....	256

CHAPTER 8: HIERARCHICAL CELL CULTURE UNDER CONTROLLED 3-D GRADIENT AND POROSITY..	259
8. SIMULTANEOUSLY CO-DIFFERENTIATED STEM CELLS APPLYING 3-D STRUCTURAL AND BIOCHEMICAL GRADIENTS MATCHING INTERFACED TISSUES.....	261
8.1 Abstract.....	261
8.2 Introduction.....	262
8.3 Results.....	265
8.4 Discussion.....	273
8.5 Materials and methods.....	275
8.6 References.....	280
8.7 Acknowledgments.....	283
8.8 Supplementary information.....	283
8.8.1 Supplementary methods.....	283
8.8.2 Supplementary results.....	284
SECTION III: FINAL REMARKS.....	289
CHAPTER 9: GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES.....	291
9. GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES.....	293
9.1 Conclusions.....	293
9.2 Future perspectives.....	297

LIST OF ABBREVIATIONS AND ACRONYMS

$^1\text{H-NMR}$ – Proton-Nuclear Magnetic Resonance

2D – Two-dimensional

3D – Three-dimensional

A

A/A – Antibiotic/antimycotic

ASCs – Adipose-derived Stem Cell(s)

ATR – Attenuated Total Reflectance

AB – Antibiotic

ALP – Alkaline Phosphatase

B

BM – Bone marrow

BMP – Bone morphogenic protein

BSA – Bovine serum albumin

C

CO_2 Carbon dioxide

CaP – Calcium phosphate

D

DAPI – 4',6-diamidino-2-phenylindole

DMEM – Dulbecco's modified eagle medium

DMA - Dynamic mechanical analysis

DNA - Deoxyribonucleic acid

dsDNA - Double-stranded DNA

E

ECM – Extracellular matrix

EDS - Energy-dispersive X-ray spectroscopy

E18 – Embryonic day 18

F

FBS – Fetal bovine serum

FDA - Food and Drug Administration

FGF – Fibroblast growth factor

FITC – Fluorescein isothiocyanate

FTIR – Fourier Transform Infrared Spectroscopy

G

G' – Storage modulus

G'' – Loss modulus

GFs – Growth factors

GAG – Glycosaminoglycan

GG – Gellan gum

GelMA – Methacrylated gelatin

H

h – hour

H&E – Hematoxylin and Eosin

H_2O_2 – Hydrogen peroxide

hASCs – Human adipose-derived stem cells

HA – Hydroxyapatite

HAp – Hydroxyapatite microparticles

HBSS – Hanks Balanced Salt Solution

HUVECs – Human umbilical vein endothelial cells

hAMECS – Humans adipose microvascular endothelial cells

I

iPS – Induced-Pluripotent Stem cells
IPFP – Infrapatellar fat pad (IPFP)

L

LAGG – Low acyl gellan gum

M

Min – minute
MEM – Minimum essential medium
Micro-CT – Micro-computed tomography
MMPs - Matrix metalloproteinases
MSC – Mesenchymal stem cell
MAGG – Methacrylated gellan gum
MT – Masson's trichrome

N

NSCs – Neural stem cells

O

OC – Osteochondral
OCD – Osteochondral Defect
OA – Osteoarthritis

P

PBS – Phosphate-buffered saline
PI – Propidium iodide
PMMA – Poly(methyl methacrylate)

R

RNA - Ribonucleic acid

RPM – Rotations per minute

RT – Room temperature

RM - Regenerative medicine

RUNX2 – Runt-related transcription factor 2

RGD – Tripeptide Arg-Gly-Asp

S

SD – Standard deviation

SEM – Scanning electron microscopy

SBF – Simulated body fluid

T

TE – Tissue engineering

TGF- β – Transforming growth factor β

TNBSA – 2,4,6-trinitrobenzenesulfonic acid

U

UV – Ultraviolet

LIST OF FIGURES

Figure 1.1 Major scientific challenges in RM. The main topics to have in consideration when designing a TE strategy are directly correlated to each other.....	6
Figure 1.2 Bone and cartilage micro- and nano-sized cylinder formation.....	8
Figure 1.3 Strategies based on materials engineering strategies to be coordinated with cellular strategies for OC TE.....	10
Figure 1.4 Strategies based cellular approached coordinated with scaffolds for OC TE.....	11
Figure 1.5 Cell-based strategies for bone and cartilage engineering.....	16
Figure 1.6 Layered neocortex schematic representation.....	17
Figure 1.7 Brain morphological shifts during embryonic development.....	18
Figure 1.8 Strategies mainly in two planes. 3-D layers better resemble the <i>in vivo</i> environment of cerebral cortex.....	20
Figure 1.9 Schematic representation of each bioreactor system for 3-D cell culture.....	26
Figure 1.10 Microfluidic devices designed over several substrates and for broad applications.....	29
Figure 1.11 Bioreactor systems adapted for high-throughput concept.....	32
Figure 1.12 SpinΩ Bioreactor-based Forebrain Organoid Culture System.....	34
Figure 1.13 A microfluidic platform for neurovascular unit including blood-brain barrier.....	35
Figure 2.1 Publications evolution related to 3-D cell culture from 1987 to 2017	60
Figure 2.2 World distribution of researchers per research topic in the field of RM.....	62
Figure 2.3 Market identification from the global one to the main niche.....	63
Figure 2.4 3-D cell culture systems market value chain considering drug testing as main application.....	70
Figure 2.5 Evolution of the number of patent publications worldwide for 3-D cell culture technologies, for the period of time comprised between 1985 and 2016.....	71
Figure 2.6 Dynamic dual-chamber bioreactor.....	79
Figure 3.1 Chemical structure of native and deacetylated GG.....	100
Figure 3.2 Gelatin as the result of collagen hydrolysis.....	101

Figure 3.3 Spherical CaP particles obtained using different methods.....	102
Figure 3.4 Gradient structures fabrication made of GG-HAp.....	108
Figure 3.5 The fabrication steps of anisotropic structures is represented by pictures....	109
Figure 3.6 Fabrication and control of oriented porous structures (anisotropic).....	110
Figure 3.7 The fabrication steps of anisotropic gradient structures is represented.....	111
Figure 3.8 Fabrication of isotropic and anisotropic porous gradient structures.....	111
Figure 3.9 Perspective view of the dual-chamber (with open view for the inside).....	120
Figure 3.10 Dimensions of the dual-chambers.....	120
Figure 3.11 Dynamic rotational platform.....	121
Figure 3.12 Microsoft-compatible software to control the rotational platform dynamics.....	122
Figure 3.13 Android-compatible application to control the rotational platform dynamics.....	122
Figure 3.14 Schematic of a microfluidic system adapted to create interfaces and perfused co-cultures.....	123
Figure 3.15 Characteristics, culture phenotypes and cell-surface markers of MSCs isolated from tissues within the knee joint.....	124
Figure 3.16 Reduction of resazurin to resorufin in the presence of metabolic active/viable cells.....	130
Figure 4.1 Controlled fluid dynamics for hierarchical hydrogels formation.....	151
Figure 4.2 Gradients patterned by HAp controlled dispersion in 3-D structures programmed by gels viscosity/temperature.....	153
Figure 4.3 Evidence of convection-driven HAp dispersion in the experimental and simulation models.....	155
Figure 4.4 Control of the 3-D gradient by the volume of the gels and HAp concentration.....	156
Figure 4.5 Mediation of the transition from bioactive to non-bioactive, and mechanical properties character by the HAp gradient within the 3-D structures.....	158
Figure 4.6 Mediation of stem cells response by the HAp gradient within the 3-D structures.....	160
Figure 4.7 <i>In vivo</i> behavior of gradient 3-D structures implanted subcutaneously and in an OCD.....	162

Figure S4.1 Gradient of HAp variations in GG hydrogels enabled by HAp concentration and injected volumes.....	177
Figure S4.2 Phasic composition and structural characterization of 3-D graded structures.....	178
Figure S4.3 Bioactive to non-bioactive gradient transition mediated by microparticles.....	179
Figure S4.4 Water uptake and weight loss.....	179
Figure S4.5 Dynamic mechanical analysis was performed to characterize the whole structure and each layer.....	180
Figure S4.6 Initial temperature gradient and screening of Grashof numbers.....	180
Figure S4.7 Subcutaneous assay performed in mice to study inflammatory response and to assess the maintenance of a HAp gradient during the <i>in vivo</i> implantation.....	181
Figure S4.8 X-rays cross-sectional and macroscopic views of explanted rabbit knees....	181
Figure 5.1 Fabrication and manipulation of the properties of 3-D oriented porous systems for <i>in vitro</i> modeling	187
Figure 5.2 Physicochemical characterization of GelMA-MAGG based hydrogels.....	189
Figure 5.3 3-D porous structures characterization and cell response assessment in GelMA-MAGG formulations.....	191
Figure 5.4 Control over porosity architecture as isotropic and anisotropic porous geometry.....	193
Figure 5.5 Anisotropic porosity guiding neurite outgrowth from mice primary neurons.....	195
Figure 5.6 Organ-on-a-chip microfluidic system adapted to create interfaces and perfused co-cultures	197
Figure S5.1 Methacrylation of gelatin and GG and hydrogel fabrication.....	210
Figure S5.2 Characterization gelatin and GG methacrylation.....	211
Figure S5.3 Physicochemical characterization of GelMA and MAGG.....	212
Figure S5.4 Biological assessment for cytotoxicity and sterilization efficiency.....	212
Figure S5.5 Biological assessment for cytotoxicity and sterilization efficiency.....	213
Figure S5.6 Neurites extension assessment and neural viability.....	213
Figure S5.7 Guided crosslinking set-up and oriented porosity reconstruction.....	214
Figure S5.8 Guided crosslinking set-up and oriented porosity reconstruction.....	215

Figure S5.9 SH-S5Y5 neurite guidance assessment.....	215
Figure S5.10 Primary neuron neurite guidance and columns formation assessment.....	216
Figure S5.11 Porosity guiding neurite extension.....	217
Figure S5.12 Neurite quantitative characterization.....	218
Figure S5.13 Post-synaptic protein expression.....	219
Figure S5.14 Microfluidic device assembling and gradient formation quantification.....	220
Figure S5.15 Interface microfluidic approach on OC differentiation.....	221
Figure 6.1 Dual-chambers.....	228
Figure 6.2 Cross section of the dual chamber, with a detail of the screw adapted to fit top hole of the dual chamber bioreactor, motor, and bottom view detail.....	229
Figure 6.3 Schematic representation of the versatile dynamics of the dual-chambers...	229
Figure 6.4 Represents examples of multilayered tissues and barriers in human body that could be mitigated by the present subject-matter.....	230
Figure 6.4 Proliferative NSCs region interfaced with differentiated neurons.....	230
Figure 7.1 Perspective view of the dual chamber (with open view for the inside).....	250
Figure 7.2 Perspective picture or the dual-chambers.....	250
Figure 7.3 Lateral view of the holder (2nd generation) for the dynamic platform.....	251
Figure 7.4 Perspective view of the holder (2nd generation) for the dynamic platform.....	251
Figure 7.5 Perspective view (from bottom) of the top plate to be used for allowing mechanical compression.....	252
Figure 7.6 Dynamic platform of the dynamic platform (2nd generation version 1) that performs the rotating movements (horizontal and vertical).....	252
Figure 7.7 2nd and 3rd generation of the dynamic platform assembled with the dual-chambers on top.....	253
Figure 7.8 Metabolic activity of cells cultured in a dual-chamber under horizontal rotation vs static.....	253
Figure 7.9 Represents the metabolic activity obtained using the dual-chamber bioreactor of the present disclosure compared to a static culture condition.....	254
Figure 8.1 Schematic representation of 3-D gradient and porosity guided structures fabrication.....	264
Figure 8.2 Structural characterization of 3-D gradient structures.....	266
Figure 8.3 Physicochemical characterization of gradient structures.....	267

Figure 8.4 Cell orientation assessment in 3-D gradient structures.....	269
Figure 8.5 Structural and biochemical gradients for OC differentiation in isotropic and anisotropic 3-D structures and endothelization.....	271
Figure S8.1 Scheme of the strategy for 3-D OC <i>in vitro</i> tissue development.....	283
Figure S8.3 Gelatin modification by methacrylation.....	284
Figure S8.4 X-ray microtomographic analysis of lyophilized GelMA-GG structures.....	284
Figure S8.5 Bioactivity assay on GelMA-GG 3:1 scaffolds.....	285
Figure S8.6 Multipotency of ASCs isolated from Hoffa's body.....	285
Figure S8.7 Flow cytometry analysis of fat pad-ASCs over 21 days.....	286
Figure S8.8 Scaffolds sterilized by O₂ plasma.....	286
Figure S8.9 Adhesion, viability and proliferation of fat pad-ASCs in GelMA-GG formulations	287
Figure S8.10. Metabolic activity of fat pad-ASCs cultured in GelMA-GG formulations.....	288

LIST OF TABLES

Table 1.1 OC constructs design strategies for TE.....	9
Table 1.2 Bioreactor designs compared in dynamics, application, advantages and issues.....	25
Table 2.1 Market niches influenced by 3-D cell culture.....	64
Table 2.2 The main market players for 3-D disposable cell culture systems.....	74
Table 2.3 Most significant patented bioreactors for TE applications.....	76
Table S2.1 Results obtained from the survey about 3-D cell culture systems.....	89
Table 3.1 Nominal ion concentrations of SBF in comparison with those in human blood plasma.....	119
Table 3.2. Characterization and phenotypic properties of MSCs in the IPFP tissue.....	124
Table 3.3 Antibodies used to perform immunocytochemistry	132

SHORT *CURRICULUM VITAE*

Raphaël Faustino Canadas was born in 1988 in Paris, France, having been raised in Portugal since he was 2 years old. Nowadays, he works as a researcher at 3B's Research Group (Biomaterials, Biodegradables and Biomimetics), University of Minho, Portugal, under the supervision of Doctor Joaquim Miguel Oliveira and Doctor Alexandra Pinto Marques.

Raphaël F. Canadas concluded his Degree in Cellular and Molecular Biology in 2009 at Faculty of Science and Technology from Nova University of Lisbon. In 2011, his Master was accomplished in Bioengineering and Nanosystems at Instituto Superior Técnico from University of Lisbon. From March 2012 to March 2014 he was enrolled in OsteoCart and NOVOMAR Projects as a researcher at 3B's. From April 2014 he is enrolled on his Graduation under the Doctoral Program Tissue Engineering, Regenerative Medicine and Stem Cells from University of Minho. His PhD thesis aims at studying and developing "Advanced technological approaches for 3-D hierarchical living tissues", in cooperation between 3B's and BAMM Lab – Canary Center at Stanford for Cancer Early Detection from Stanford University, under the Direction of Prof. Rui Reis of Prof. Utkan Demirci, respectively. Over the last 4 years Raphaël has been awarded with grants from the Portuguese Foundation for Science and Technology and from the Luso-American Development Foundation on the scope of his PhD project. In 2014 the researcher was selected by the COHITEC Entrepreneurship Program for the translation of a patented bioreactor system to the market. In 2015, his PhD project was highlighted by the TV program 'Future Today' from the Portuguese TV Channel SIC. He was also invited by the TERM&SC Doctoral Program from University of Minho to give a lecture for the course "Bioreactors I", and later to collaborate on the "Hands-on Training Practical session" from the 3rd International Meeting "The Meniscus 2016", giving a lecture about micro-computed tomography analysis. In 2016 Raphaël was part of the Organizing Team of the 11th European Young Chemists' Network Delegates Assembly. He has been awarded an Honorable Mention by the D. Manuel II Foundation from the Portuguese Royal Family, which distinguishes the excellence on Biomedical Research undertaken by a scientist under 40 years. More recently, the researcher has been awarded a second Honorable Mention for the selection of the project BIOreACT for the final phase of the TERMIS-EU 2016 Business Plan Competition. In 2017, Raphaël was invited by the University of Porto, Faculty of Pharmacy, to give a lecture on Entrepreneurship, Innovation and Business Creation entitled "Creating technology for regenerative medicine: a multidisciplinary and multicultural approach". He has also been acting as a referee of the peer reviewed Journal of Tissue Engineering and Regenerative Medicine. Additionally, he

collaborated with his colleagues in varied research works, having been co-author of several research articles, as well as supporting junior researchers and colleagues in tasks as, for example, conducting micro-computed tomography trainings and analysis.

Raphaël F. Canadas has been integrating stem cells and biomaterials knowledge into tissue engineering and creating devices as a way to define innovative strategies to improve the functionality and reliability of biological tissues. Osteochondral and cerebral cortex tissue engineered constructs were targeted to be applied *in vivo* and *in vitro* as 3-D platforms to understand cellular crosstalk. As a result of the research carried out, he is inventor of patents (2), author and co-author of publications in international scientific meetings (28), book chapters (6) and papers (total 10; 1st author of 1 published and 3 submitted papers) in international journals.

LIST OF PUBLICATIONS

Author and co-author in the following scientific publications (*directly correlated with the present PhD work)

PATENTS (2)

***Canadas RF**, Oliveira JM, Marques AP, Reis RL, Multi-chamber bioreactor, methods and uses, PCT/IB2015/057210, 2016.

***Canadas RF**, Oliveira JM, Marques AP, Reis RL, Rotational dual chamber bioreactor: methods and uses thereof, PCT/IB2014/059740, 2014.

ARTICLES IN SCIENTIFIC JOURNALS WITH PEER-REVIEW (10)

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***Raphaël F. Canadas**, Alexandra P. Marques, Joaquim M. Oliveira, Rui L. Reis, Utkan Demirci. Simultaneously co-differentiated stem cells applying 3-D structural and biochemical gradients matching interfaced tissues. (Submitted)

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***RF Canadas**, T Ren, A Tocchio, E Karaca, M Miansari, AP Marques, JM Oliveira, RL Reis, U Demirci, Anisotropic methacrylated network for the development of an *in vitro* three-dimensional neural model, TERMIS-AP17, Nantong, China, 2017

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***R. F. Canadas**, D. R. Pereira, J. Silva-Correia, C. Viegas, A. da Silva Morais, R. A. Sousa, A. P. Marques, R. L. Reis and J. M. Oliveira, *In vivo* performance of gellan gum-based structures: a comparison study between hydrogels and spongy-like scaffolds for osteochondral tissue regeneration, V Annual Meeting of the ICVS/3B's Associate Laboratory, Braga, 2015

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R. F. Canadas, J. M. B. T. Cavalheiro; J. D. T. Guerreiro, M. C. M. D. de Almeida, E. Pollet, C. Lobato da Silva, M. M. R. da Fonseca, F. C. Ferreira “Polyhydroxyalkanoates Fibrous Scaffolds for Support of

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***Canadas R. F.**, Pereira D. R., Silva-Correia J., Marques A. P., Oliveira J. M., and Reis R. L., Novel bilayered Gellan gum/Gellan gum hydroxyapatite scaffolds for osteochondral tissue engineering applications, TERM STEM 2012, 2012

INTRODUCTION TO THE THESIS STRUCTURE

The present thesis is divided into 3 Sections subdivided into 9 Chapters. These sections were organized according to the defined aims, the nature of the experiments performed and the derived conclusions. The sub-division in chapters is based in a series of related papers, book chapters, and patents published or submitted in international journals, which are identified in the front page of each chapter. Therefore, each thesis chapter corresponds to a published or submitted manuscript with minor format changes for ensuring a homogeneous style and consistent structure combining the various thesis chapters.

Section I contains a 1st Chapter with an overview of the state-of-the-art, and a 2nd Chapter with a market analysis. The **1st Chapter** consists of a comprehensive and detailed literature review about current approaches to stratify and pattern engineered tissues *in vitro*. A special focus is given to osteochondral and neocortex, because the first is a gradient tissue addressed in this PhD work, and the second is characterized by an anisotropic phenotype, which is usually affected by relevant disorders as Alzheimer's. Furthermore, the current systems for 3-D cell culture are reviewed, mainly those able to perform high-throughput or automated approaches, such as bioreactors and microfluidics.

The **2nd Chapter** of the thesis includes a detailed analysis of the cell culture systems market, focusing the ones related with 3-D approaches. The main problem and associated need were screened and surveyed. Moreover, some industrial players in Portugal were directly contacted. International market reports were reviewed, and a survey, as well as scientific reviews, reporting the opinion of industrial and academic leaders were analysed. As a result, market size, roadmap, competition and cases of success are discussed in this chapter.

The **Section II** refers to the experimental part (Chapter 3, 4, 5, 6 and 8). This section starts with the **3rd Chapter**, which presents a detailed description of the materials used, the processing technologies and the techniques applied or developed for the scaffolds preparation and characterization. The development of cell culture systems is summarized. The biological tests, including the used cellular sources and performed assays to characterize the cell seeded/cultured constructs are described. The *in vivo* tests are described as well.

The **4th Chapter** describes the control over gradient by programming the temperature of two mixing gels. The application of convection as a driving mechanism to distribute hydroxyapatite microparticles in patterns is presented. The performance of such gradient structures either *in vitro* and *in vivo* for osteochondral tissue engineering is analysed.

The **5th Chapter** describes a fabrication method to obtain anisotropic structures with control over the size of the linear pores. Anisotropic structures are cultured with primary neurons to achieve oriented neurite outgrowth and compared against constructs with isotropic architecture. The synaptic junctions are characterized to observe their anisotropic organization. A microfluidic device designed is tested as a platform for endothelial-neural interfaces.

The **6th Chapter** refers the design and fabrication of a dual-chamber bioreactor to control gradients of growth factors and cell phenotypes in 3-D cell culture environments. The perfusable chamber conceived to fit 6-well culture plates is fully described. As a proof of concept, a 3-D hydrogel encapsulating neural stem cells submitted to proliferation and differentiation conditions is assessed for phenotypic control in different regions in a single cell culture.

The **7th Chapter** presents the development of a dynamic platform to which the previously referred dual-chambers can be attached. This platform created to introduce dynamic rotations during 3-D cell cultures, is described in detail. The dynamics performed by the platform are highlighted as a proof of concept. avoiding cell sedimentation and increasing metabolic activity of cells cultured in 3-D dynamic conditions.

The **8th Chapter** reports the combination of the previous chapters, where anisotropic and isotropic structures are fabricated with incorporation of a hydroxyapatite microparticles gradient. The dual-chamber bioreactor is then used to achieve osteogenic and chondrogenic simultaneous differentiations in a single 3-D construct. Furthermore, the spatial control over pre-vasculature is similarly shown.

Finally, **Section III** addresses the final remarks. In this section, the **9th Chapter** of this thesis, the final one, summarizes the overall conclusions obtained from the performed research work and discusses the future perspectives and lines of research to be followed.

SECTION I

GENERAL INTRODUCTION

CHAPTER 1

GENERAL BACKGROUND

This Chapter was based on the following publications:

R. F. Canadas, R. P. Pirraco, J. M. Oliveira, R. L. Reis, A. P. Marques. Chapter 25: Stem Cells for Osteochondral Regeneration, in *Osteochondral Tissue Engineering: Challenges, Current Strategies, and Technological Advances*, ed. J. M. Oliveira, S. Pina, R. L. Reis, J. San-Román, (in press). Springer

R. F. Canadas, A. P. Marques, R. L. Reis and J. M. Oliveira. Chapter 26: Bioreactors and Microfluidics for Osteochondral Interface Maturation, in *Osteochondral Tissue Engineering: Challenges, Current Strategies, and Technological Advances*, J. M. Oliveira, S. Pina, R. L. Reis, J. San-Román, (in press). Springer

R. F. Canadas, S. Pina, A. P. Marques, J. M. Oliveira, R. L. Reis. Chapter: *Cartilage and Bone Regeneration – How close are we to bedside?*, in *Translating Regenerative Medicine to the Clinic*, ed. Jeffrey Laurence, Pedro Baptista, Anthony Atala, 2016, Elsevier. DOI: 10.1016/b978-0-12-800548-4.00007-3

R. F. Canadas, A. P. Marques, R. L. Reis and J. M. Oliveira. Chapter X: *Osteochondral Tissue Engineering and Regenerative Strategies*, in *Progress on Biomaterials and Regenerative Strategies for the Treatment of Knee Joint Disabilities*, ed. J. M. Oliveira and R. L. Reis, (2015), Springer. DOI10.1007/978-3-319-44785-8_11

1. INTRODUCTION

Organized networks are common in nature showing specific tissue micro-architecture, where cells can be found isotropically or anisotropically distributed. All the cells in the human body are surrounded by topographical and biochemical signals. The physical structures comprise nanopores, nanofibers, and nanocrystals. Some examples of such structures in physiological settings are pores in capillaries shape in the brain cortex, and gradients of nanocrystals in the form of hydroxyapatite (HA) in the bone microstructure. Layered patterns are also very prevalent in both tissues. For instance, maintenance of cell alignment is essential for muscle, cardiovascular/blood vessel, corneal, brain cortex, and peripheral nerve tissue engineering (TE) in which controlling tissue microarchitecture and biological function are tightly connected. Among these functional patterns, interfaces play a vital role in nature, where interfaced fluidic systems are a common phenomenon and a building base for living complex organisms, which consist of several distinct yet interfaced tissues. Gradients of three-dimensional (3-D) tissue interfaces organized at micro-scale are important to mimic in numerous fields such as drug development, micro-fabrication, and TE. Currently, methods based on materials, micro-scale engineering, and microfluidic devices have been applied to fabricate such 3-D gradient microenvironments. However, since native tissues present characteristic stiffness and cell arrangements, when addressing an *in vitro* tissue model, it is important to grant control over mechanical properties while achieving iso- or anisotropic polymeric networks, especially in 3-D. Furthermore, a gradient tissue needs a robust spatial control not only over architecture but also of the biochemical environment, especially when heterogeneous cell differentiation has to occur simultaneously in a single construct. Hence, a challenging need still exists for 3-D gradient structures with controlled porous orientation in spatially controlled biochemical signals, to better mimic specific hierarchical tissues. In this thesis general introduction, we revised the existing TE approaches for osteochondral (OC) and cerebral cortex surrogate and model tissues development. In addition, the dynamic 3-D cell culture is addressed, focusing on bioreactors and microfluidic systems, and the future directions and challenges to be faced are discussed. Basic principles, advantages and challenges of each technology are described.

1.1 HIERARCHICAL TISSUE ENGINEERING STRATEGIES

Cell culture is being pushed by the development of RM and even more since the creation of the branch of TE 30 years ago. The term “Tissue Engineering” as it is nowadays used was introduced in medicine

in 1987¹. The definition that was agreed on was: “TE is the application of the principles and methods of engineering and life sciences toward the fundamental understanding of structure-function relationships in normal and pathologic mammalian tissue and the development of biological substitutes to restore, maintain, or improve function”¹.

Together with the evolution of TE and cell culture systems, 3-D *in vitro* models have been raised for the last decade for two main reasons: first, envisioning decreasing animal experimentation and its related ethical concerns; second, because these can be more representative of the human condition². The use of animal models in drug discovery studies presents issues with feasibility and ethical concerns. To address these limitations, *in vitro* tissue models have been developed to provide a means for systematic, repetitive, and quantitative investigation of drugs. The evolution of TE and nanotechnologies fields over the last decades allowed moving from 2-D to 3-D and high-throughput techniques³.

The impact of TE in the future of the health care and pharmaceutical industries is likely to be comparable with that of antibiotics (ABs), vaccines and most recently, monoclonal antibodies. However, many challenges have been delaying this impact and are yet to be solved, which are summarized and interrelated in figure 1.1. These challenges include finding the best cell source, the most appropriate biomaterials, and to identify reliable ways of expanding and growing the cells in a 3-D environment without losing their phenotype⁴.

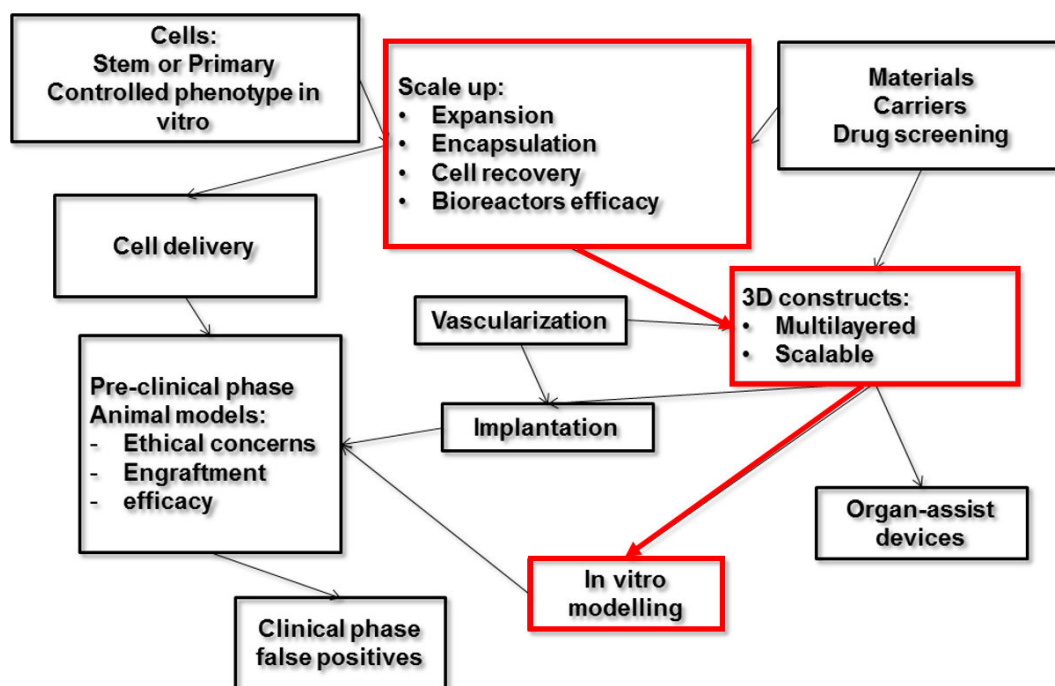


Figure 1.1 | Major scientific challenges in RM. The main topics to have in consideration when designing a TE strategy are directly correlated to each other. The main challenging tasks

are the ones chosen to build the represented schematic. Red lines are highlighting the main needs addressed during the present PhD project.

Among the main challenges for 3-D constructs technological evolution, the mimicry of hierarchical tissues, such as gradient interfaces or spatially organized phenotypes, are yet to be effectively addressed. Currently, the state of the art of hierarchical tissues biofabrication is mainly categorized by techniques combining heterogeneous layers of different materials, which is limited in achieving continuous interconnected but heterogeneous interfaced tissues phenotypes. Furthermore, the developed constructs are mainly characterized by random architectures, which is not efficient to obtain functional tissue patterns. In addition to these challenges, the commercially available culture systems limit the stem cell differentiation to one lineage when cultured in a 3-D environment. So, advanced technological approaches for hierarchical tissue models are urgent. In the present PhD project, we focused on developing strategies for these referred needs. Taking in consideration that specific tissues functions can be a results from specific architectures, as for example OC and neocortex, which are organized in gradients and anisotropic patterns, we focused our efforts on developing strategies to achieve these features. OC unit was chosen as a target tissue for gradient phenotypes. Additionally, brain cortex was selected to address engineering solutions for anisotropic tissues. To address such challenging technologic advances, cell culture systems have to follow the evolution of engineered scaffolds. Specialized bioreactor and microfluidic systems were created during this PhD project and are addressed in the thesis.

Considering the importance of both tissues as *in vitro* models for drug testing of relevant disorders, the state of the art on OC and brain cortex TE will be the focus of this introductory background. A special attention will also be addressed to *in vitro* cell culture techniques and devices with direct impact on 3-D *in vitro* models development, namely bioreactors and microfluidics, applied to OC engineering and neural *in vitro* modulation.

1.2 OSTEOCHONDRAL TISSUE ENGINEERING

1.2.1 Bone and Cartilage Phenotypes in Osteochondral unit

Bone and cartilage have a 3-D architecture with several levels of organization comprising micro- and nano-structures (figure 1.2)⁵. Cancellous bone is a porous structure, whereas cortical bone is composed of osteons that consists of a concentric series of layers (lamellae) of mineralized collagen type I matrix.

Articular cartilage presents a stratified architecture, which consists of three zones where in the superficial zone collagen type II fibers are oriented tangentially to the articular surface, in the transitional zone have no predominant orientation and become aligned perpendicularly to the calcified cartilage, anchored in subchondral bone in the deep zone. Articular cartilage has poor intrinsic ability for healing due to its isolation from vessels and nerve supply. On the other hand, bone is a vascular and innervated tissue. While bone is mineralized, normal cartilage tissue is not mineralized and is a highly hydrated tissue.

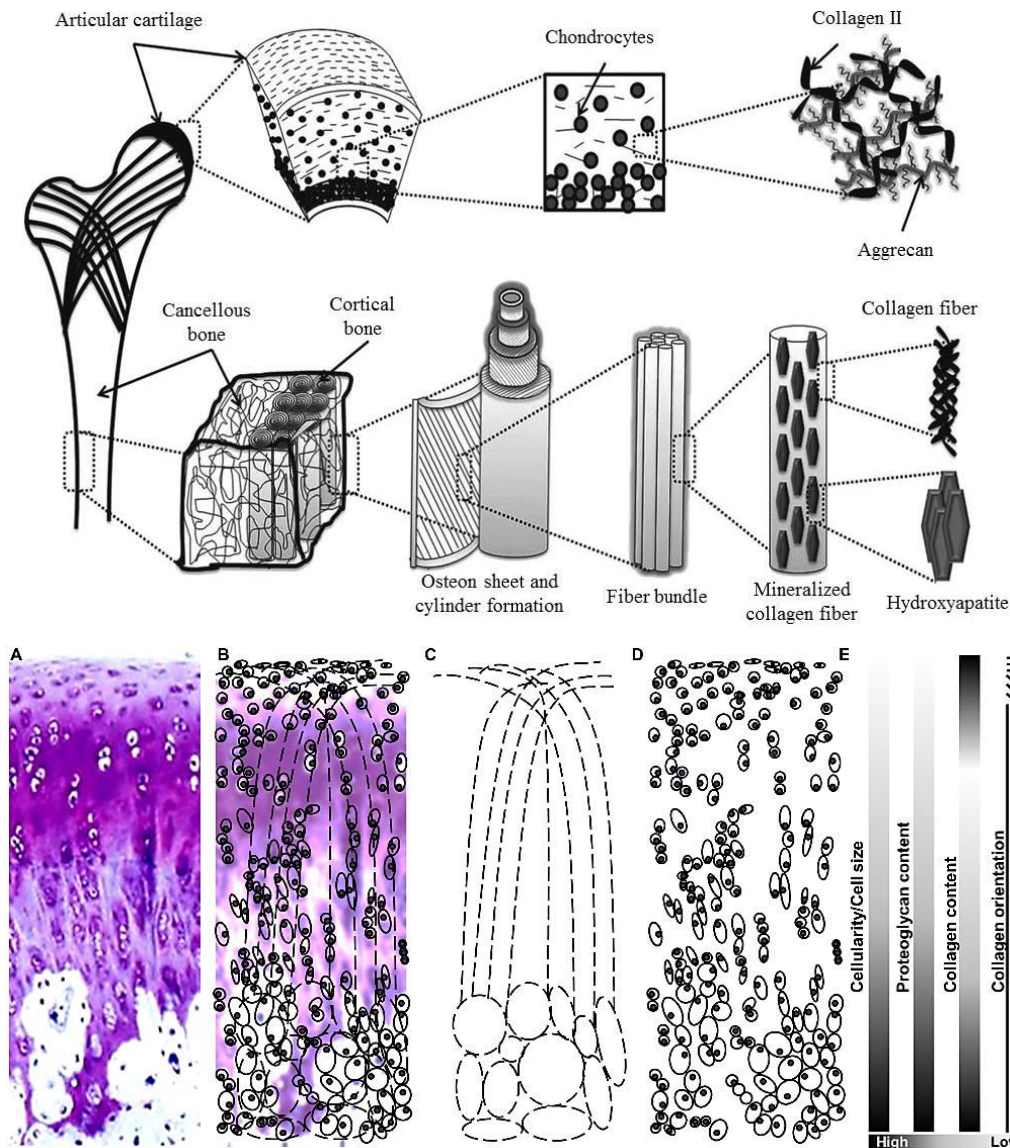


Figure 1.2 | Bone and cartilage micro- and nano-sized cylinder formation. First scheme *reprinted from Ref. ⁶, with permission*. Subchondral bone and articular cartilage interface make the OC tissue which is characterized by specific collagen orientation and several content gradients. (A). Histological H&E section of healthy human OC tissue. (B) Representation of the organization of collagen

and cells. (C) Scheme of collagen orientation. (D) Scheme of cellular distribution. (E) Representation of gradients and collagen orientation.

Taking into consideration the heterogeneity of the OC phenotype, advanced strategies are required when trying to mimic the tissue's native hierarchy *in vitro*. Diverse approaches have been applied, which are summarized in the following section.

1.2.2 Engineering Osteochondral Composites

OC unit is a particular gradient integrating cartilage and bone in which chondrocytes and osteoblasts can be recognized as characteristic cells, respectively. Furthermore, OC tissue also represents a gradient of ECM constituents through it. The control over spatial patterning of tissue development represents this way the main challenge for the advance towards engineering functional OC grafts or to model the tissue unit *in vitro*. Regarding this, specific scaffold-based strategies have been proposed, including (I) a scaffold for the bone component, but a scaffold-free approach for the cartilage side, (II) different scaffolds for the bone and cartilage sides, (III) a bilayered scaffold with integrated interface, which can be a gradient or a compact layer. Additionally, hydrogel-based method can be added to the previous scaffold strategies, which consist of (IV) injectable biomaterials applied at the surgical moment, as summarized in table 1.1. Generally, to control the formation of heterogeneous OC interface, different strategies such providing physical cues for stem cells differentiation⁷ have been applied, which are represented in figure 1.3.

Table 1.1 | OC constructs design strategies for TE.

Architecture	Advantages	Limitations	Reference
Two independent layers	Independent cell culture and differentiation	Poor integration between layers	(15, 28, 29)
Bone scaffold supporting cell free monolayer for cartilage	High control over cartilage layer phenotype	Poor integration and absence of transition zone	(30-32)
Bilayer structure with compact interface	Good integration between layers Control of cell migration	Poor communication between subchondral bone and cartilage	(33-37)

	Impaired vascularization in the cartilage part		
Integrated bilayer structure	Good integration between layers Transition zone mimicking calcified cartilage part	Difficult cell culture regarding co-differentiation for osteo- and chondrogenesis	(14, 16, 18, 20, 27, 38-45)
Hydrogel based bilayered structures	Perfect fit and filling of defect High hydrated environment	Poor diffusion of nutrients in several hydrogel systems Poor cell anchorage in high hydrated environments	(46-50)

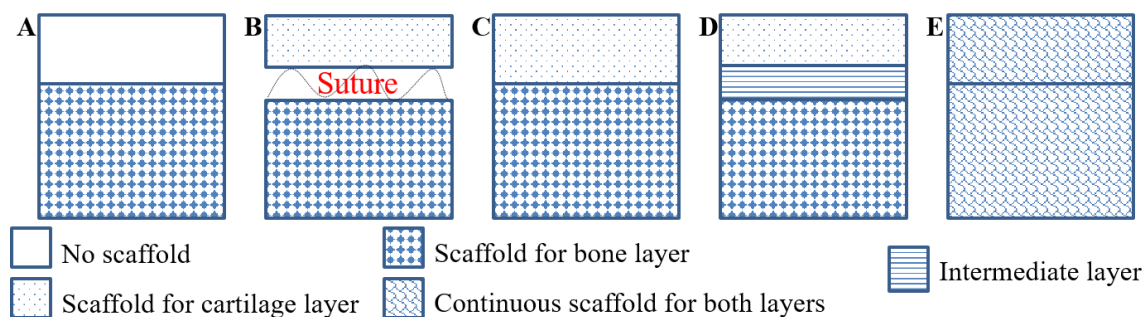


Figure 1.3 | Strategies based on materials engineering strategies to be coordinated with cellular strategies for OC TE. (A) Scaffold-free cartilage layer combined with bony-scaffold; (B) Two separated constructs sutured together, usually involving individual osteo- and chondrogenic pre-maturation; (C) Two different integrated scaffolds merged together; (D) Tri-layer structure composed of two different scaffolds for bone and cartilage and an intermediate layer in the interface; (E) Continuous scaffold for both bone and cartilage layers, which can be different in material properties or cell type.

To accomplish the represented strategies, combinations of fabrication techniques have been applied to achieve scaffolds with diversified characteristics for OC regeneration, namely nanofibers based structures, which can be created by electrospinning⁸, sponges produced by freeze drying⁷; or even agglomerated particles and microparticles⁹. Porous scaffolds can also be created by salt-leaching technique¹⁰, while well-organized matrices can be produced by rapid prototyping technologies¹¹.

In addition to several materials engineering strategies, the spatial controlled delivery of differentiation factors as growth factors (GFs)¹² and gene vectors¹³, and the use of parallel culture medium flow for osteo- and chondrogenic conditioning by custom-made dual-chamber bioreactors¹⁴ or microfluidic devices¹⁵, have been applied.

In any of the previous cases, to build a heterogeneous construct, cells either from different lineages or differently differentiated (ending up in two different phenotypes) are applied at the seeding moment. In coordination with the previous strategies, different cell types from primary osteoblasts co-cultured with primary chondrocytes¹⁶; primary chondrocytes co-cultured with stem cells¹⁷; or co-differentiation of stem cells towards osteo- and chondrogenesis¹⁸, have been tested envisioning the enhancement of OC phenotype by the synergistic action of the different factors produced by each cell type. Envisioning these strategies, both pre-differentiation¹⁹ and *in situ* differentiation⁸ of the stem cells were tested. The full set of previous materials and cells combinations is summarized in figure 1.4.

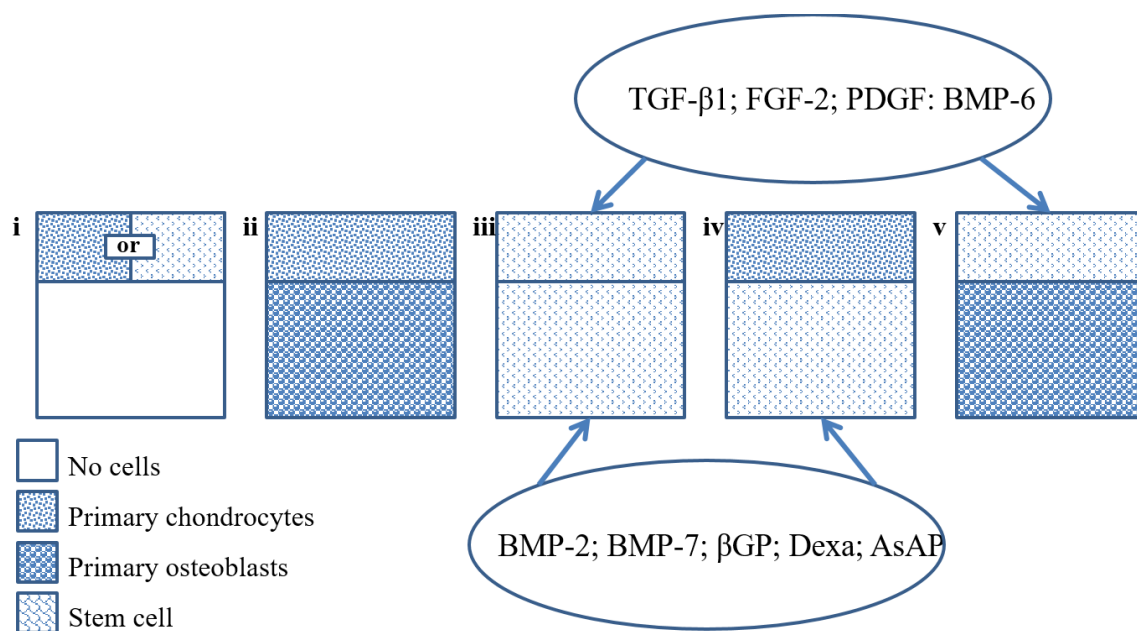


Figure 1.4 | Strategies based cellular approached coordinated with scaffolds for OC TE. (i) cell-free approach for bone region combined with primary chondrocytes or stem cells for cartilage layer; (ii) primary chondrocytes co-cultured with primary osteoblasts; (iii) single stem cell population for co-differentiation; (iv) primary chondrocytes co-cultured with stem cells for cartilage and bone, respectively; (v) stem cells co-cultured with primary osteoblasts for cartilage and bone, respectively.

The challenge of controlling osteogenic and chondrogenic differentiations in a single system has been addressed with the independent pre-maturation of the bone and cartilage parts. A hydrogel phase made of chitosan and a cancellous bone part were seeded with human ASCs (hASCs) for chondrogenesis and osteogenesis, respectively²⁰. After 2 weeks of differentiation period, constructs were sutured together and cultured for further 2 weeks under static or dynamic conditions. The dynamic culture improved the resulting interface connection and enhanced homogeneous nutrient transfer. However, this technique keeps lacking integrity and a robust interface integration between cartilage and bone tissues.

1.2.3 Biomaterials for Bone and Cartilage Regeneration

The rationale of using biomaterials as scaffolds in tissue regeneration is to obtain a temporary 3-D structure for the *in vitro* growth of living cells and its subsequent implantation into the lesion area followed by its biodegradation as newly tissue is being formed. Several natural and synthetic polymers, bioactive inorganic materials, and their combinations have been employed for bone and cartilage TE and regeneration. Polymers have great stiffness and mechanical strength, and natural polymers add advantages such as their resemblance with the extracellular matrix (ECM), specific degradation rates due to the susceptibility to the action of enzymes, and improved recognition by the living body. Bioactive inorganic materials, such as calcium phosphates (CaP) and bioactive glasses, have good biocompatibility, osteoconductivity, and bioresorbability. Despite, they present poor mechanical properties that hinder its use in load-bearing applications. The combination of these different types of materials result in composite structures with significantly enhanced mechanical and biological properties for bone TE. In opposition, cartilage tissue has been mainly engineered using natural/synthetic polymers.

Polymers

Natural polymers, also known as biopolymers, have been extensively used owing their ability to interact with cells and to be susceptible to enzymatic degradation providing space for tissue ingrowth²¹. Naturally occurring polymers most widely explores for bone and cartilage repair/regeneration are: i) proteins (e.g. silk fibroin (SF), collagen and gelatin); ii) polysaccharides (e.g. chitosan, alginate, gellan gum (GG) and derivatives); and iii) glycosaminoglycan (GAG; e.g. hyaluronic acid)²¹.

In comparison to biopolymers, synthetic polymers have several advantages; excellent processing characteristics, excellent mechanical and physical properties (e.g. elastic modulus, strength, and degradation rates), and bioresorbability²². However, many of these polymers present several

disadvantages, such as the possibility of causing persistent inflammatory reactions and not being capable to integrate with host tissues²³. The most used polymers are polyglycolic acid (or polyglycolide - PGA), polylactic acid (or polylactide - PLA), poly lactide-co-glycolide (PLGA), poly (D,L- lactic acid) (PDLLA), poly ethylene glycol (PEG), and poly ϵ -caprolactone (PCL). These polymers have received special attention since they can be self-reinforced to gain better strength properties²⁴.

Bioactive inorganic materials

Inorganic materials often used for bone repair and regeneration are CaP, namely HA ($\text{Ca}_5(\text{PO}_4)_3\text{OH}$) and β -tricalcium phosphate ($\text{Ca}_3(\text{PO}_4)_2$, β -TCP), bioactive glasses, and glass-ceramics owing their bioactivity, biocompatibility and osteoconductivity^{25,26}.

HA is crystalline and is the most stable and least soluble CaP in an aqueous solution down to a pH of 4.2²⁶. Aqueous precipitation^{27,28}, hydrothermal synthesis²⁹, solid-state reaction using calcium oxide, calcium hydroxide or calcium carbonate, and hydrolysis of other CaP, have been methods used to prepare HA microparticles (HAp). The detailed information on HAp synthesis and preparation is well established³⁰. β -TCP is a high temperature phase of CaP, which can only be obtained by its thermal decomposition at temperatures above 800 °C. β -TCP is biodegradable and has been extensively used as bone substitute, either as granules or blocks, or even in CaP bone cements³¹. The resorption capability of HAp and β -TCP is different though their similarity in terms of chemical composition. It is believed that HAp has a slow resorption rate (1 to 2% per year) and may be integrated into the regenerated bone tissue, while β -TCP is completely reabsorbed^{32,33}. Therefore, clinical applications have been performed by combining HAp with β -TCP, which forms the biphasic CaP, improving the bioresorbability and strength of the bone substitutes^{25,30,34}. Nevertheless, these materials are limited to non-load bearing applications due to their poor mechanical properties.

Bioactive glasses and glass-ceramics have been used in bone regeneration due to their capability to react with physiological fluids thus bonding with bone through the formation of HAp layers at the implant interface thus stimulating bone growth^{35,36}. These type of materials are osteogenetic and osteoconductive, while CaP exhibit only osteoconductive properties³¹. It has also been found that reactions on bioactive glass surfaces release concentrations of Si, Ca, P and Na ions, thus inducing intracellular and extracellular responses³⁷. They are also able to improve osteoblast adhesion, vascularization *in vivo*, enzymatic activity, and differentiation of mesenchymal stem cells (MSCs)³⁸. In addition, glasses have shown great potential as reinforcing materials since they fully degrade in aqueous media^{39,40}. Bioactive glasses are brittle materials; this limitation can be solved by the development of glass-ceramics or by the combination with

an additional phase as a polymer, forming a composite^{40,41}. There are different compositions of bioactive glasses, based on silicate, phosphate, and borate, which can be obtained through melt-quenching⁴² and sol-gel process⁴³. The most widely investigated bioactive glass for biomedical applications is the silicate-based glass designated 45S5, also known by its commercial name Bioglass®⁴³. These types of glasses have higher chemical durability and durability limits as compared to others bioactive glasses⁴⁴. By its turn, phosphate-based glasses have unique dissolution properties in aqueous fluids, while borate-based bioactive glasses have faster degradation rates and are able to completely convert into apatite^{45,46}. Bioactive inorganic materials can be doped with trace elements (e.g. strontium, zinc, magnesium, manganese, silicon), which can influence bone health and enhance biocompatibility, while strengthening the mechanical properties of the implants⁴⁷. Besides, minerals and traces of metal elements may provide physicochemical modifications in the produced materials, which can accelerate bone formation and resorption *in vivo*^{48,49}.

Composites

Composite materials embracing a natural/synthetic polymeric matrix and bioactive inorganic materials, as fillers, appeared as a strategy to mimic the human bone, which is a 3-D composite composed of organic, inorganic and cellular phases, strictly assembled to form the natural bone tissue. Composites are the combination of two or more materials, with different compositions and properties, resulting in a single structure with significantly improved mechanical and biological properties. Special interest has been attributed to nanocomposites for bone TE and regeneration due to the nanosized features of the fillers which can intensely improve the tissue bonding capacity of the polymeric matrices, that the individual materials cannot attain thus allowing the production of better biomaterials⁶. The nanoparticles have large surface area when compared to the conventional micro-sized fillers, thus offering improved mechanical properties, while maintaining the osteoconductivity and biocompatibility of the fillers, as well as, cell adhesion and differentiation⁴⁹.

Many combinations of polymers and inorganic materials have been proposed for the production of nanocomposites which final properties will be dependent. As aforementioned, the most common polymers used are of natural origin (collagen, gelatin, silk, chitosan, alginate, hyaluronic acid, and GG)⁵⁰⁻⁵⁶. By its turn, some synthetic polymers (e.g. PEG, PLA, PGA, PLGA, and PCL) have been also used and applied in the clinics. On the other side, nanosized fillers include nanoparticles of CaPs and bioactive glasses, carbon nanotubes, nanofibers, and nanoplatelets. These nanoparticles have been prepared through different processes, namely wet chemical precipitation²⁸, sol-gel synthesis⁵⁷, hydrothermal

synthesis⁵⁸, mechanochemical synthesis⁵⁹, microwave processing⁶⁰, spray drying methods⁶¹, and electrospinning⁶², while nanocomposites have been prepared by simple mechanical mixing⁶³ or co-precipitation⁶⁴.

1.2.4 Stem Cells for Engineering Bone and Cartilage

As stated above, bone marrow (BM) MSCs have been the top choice as a MSC source for bone TE. This is mainly due to their high potential for osteogenic differentiation, which is probably related with their tissue of origin⁶⁵. These cells have been combined with 3-D scaffolds to deliver a myriad of bone TE strategies that ultimately resulted in the formation of bone tissue after transplantation⁶⁶⁻⁶⁹. However, these cells present several limitations. The isolation procedure is painful for the patient as it normally consists of an invasive procedure to aspirate the marrow in the iliac crest. Other methods exist where bone is accessed from patients undergoing hip or knee replacement⁷⁰. These are however exceptions to the rule. Furthermore, the yield of stem cells obtained from BM is low, estimated at 0.001% of colony forming unit-cells per bone nucleated cells⁷¹. This means that these cells need to be heavily expanded to reach therapeutic numbers, which ultimately results in reduced differentiation capacity and therapeutic efficiency⁷². Finally, the potency of each population of BM MSC greatly depends on the donor's age and general condition, which further complicates their applicability⁷³. The quantity of MSCs declines with age; approximately 1 MSC per 10,000 marrow cells are found in a newborn, whereas 1 MSC per 400,000 marrow cells is found in a 50-year-old adult^{74,75}.

While adipose tissue could be considered an alternative source to BM MSCs, ASCs have less chondrogenic potential than BM MSCs.⁷⁶ Although, the infrapatellar fat pad also known as Hoffa's body, an extra-synovial tissue placed in the knee under the patella⁷⁷ that is commonly harvested and discarded in arthroscopic surgeries⁷⁸, was shown to possess a higher percentage of stromal cells than subcutaneous fat.^{79,80} In addition, the regenerative capacity of the cells obtained from Hoffa's body in an OA model was demonstrated⁸¹ thus reinforcing the positioning of this source for cartilage engineering.

Despite an extensive preclinical research and promising clinical results, there are yet some drawbacks related to the harvesting and culture of stem cells to be addressed. Cell seeding number, serum conditions, or even the plastic surface of the expansion systems, can affect cell phenotype. A forced selection happens during cell expansion, which can affect for example MSCs properties by their technical preparation⁸²⁻⁸⁴. Cell culture was not a concern for long time, being poorly controlled over most of the published studies. As a specific example, chondrogenic differentiation requires a 3-D environment, but MSCs are commonly expanded on 2-D plastic surfaces. Furthermore, as two-stage procedures involving

cell culture are expensive and cumbersome, there is an increasing push towards a single stage stem cell treatment, which focuses again on autologous strategies. In this situation, there is some supportive pre-clinical data⁸⁵⁻⁸⁸, but a direct comparison between fresh MSCs concentrates and MSCs expanded *in vitro* is not available⁸⁹. The schematic represented in figure 1.5 summarizes the use of cell-based strategies for regenerative approaches.

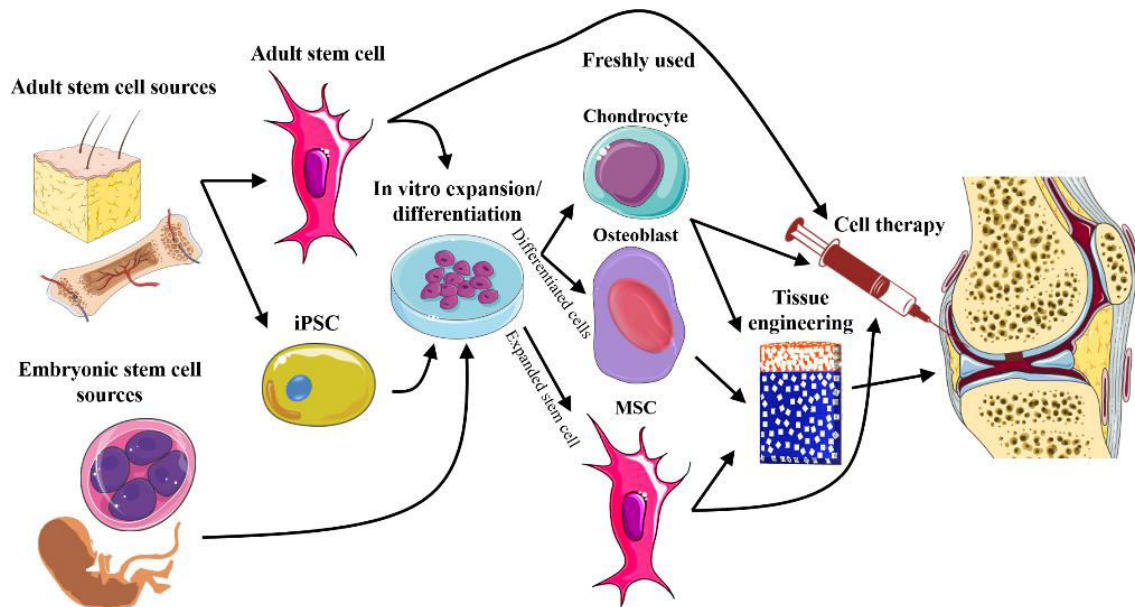


Figure 1.5 | Cell-based strategies for bone and cartilage engineering. Cell isolation has been relying on several sources, from adult to embryonic tissues. After isolation, cells can be freshly used or *in vitro* expanded. During *in vitro* phase, cells can be directly used taking advantage from their stemness, or differentiated. When applied, these cells can be directly injected at the defect site using carriers or not.

1.3 NEURAL TISSUE ENGINEERING

1.3.1 Neocortex Phenotype

The neocortex is composed of six layers of neurons, and those layers differ markedly in neuronal cell type composition, cell density, and connectivity. In humans, neocortical columns are approximately 2 mm tall, have a diameter of 0.5 mm, and contain approximately 60,000 neurons^{90,91}. Furthermore, adult primary neurons cohabit mainly with astrocytes and endothelial cells in the adult cortical brain⁹². These cell types experience inevitably an exchange of secretory bioactive molecules among them, which are not only in

solution but have also to cross tissues with different densities of cells and ECM⁹³. Neuroscientific field has shown that genes are differentially expressed across the neocortical primordium before the arrival of the subcortical afferents. Among those genes are transcription factors expressed in opposing rostral-caudal gradients⁹⁴. So it is pretty well known that gradients of molecules can regulate cortex patterning and axons guidance⁹⁵. However, very little is known about how cortical areas differentiate or what creates these gradients and boundaries. This factor can also be influenced first by the different densities of neurons of each of the 6 layers, second by the heterogeneous population of cells present in the cerebral cortex (figure 1.6)⁹⁶.

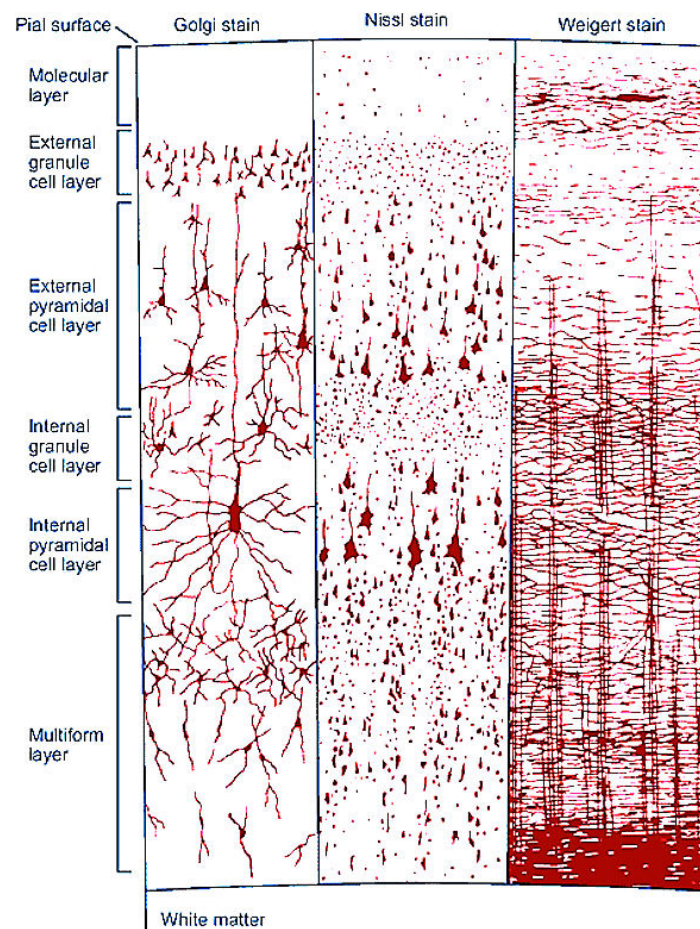


Figure 1.6 | Layered neocortex schematic representation. (adapted from <http://www.humanneurophysiology.com/cerebralcortex.htm>)

Some reports show how oxygen tension^{97,98}, stiffness^{99,100} and GFs^{101,102} play their role, mainly in 2-D cell cultures and *in vivo* models. However, during brain development, the 3-D environment is continuously expanding over time (figure 1.7-A), creating not only one specific value for each one of these factors, but gradients of values and shifts⁹⁹ (figure 1.7-B), as explained above.

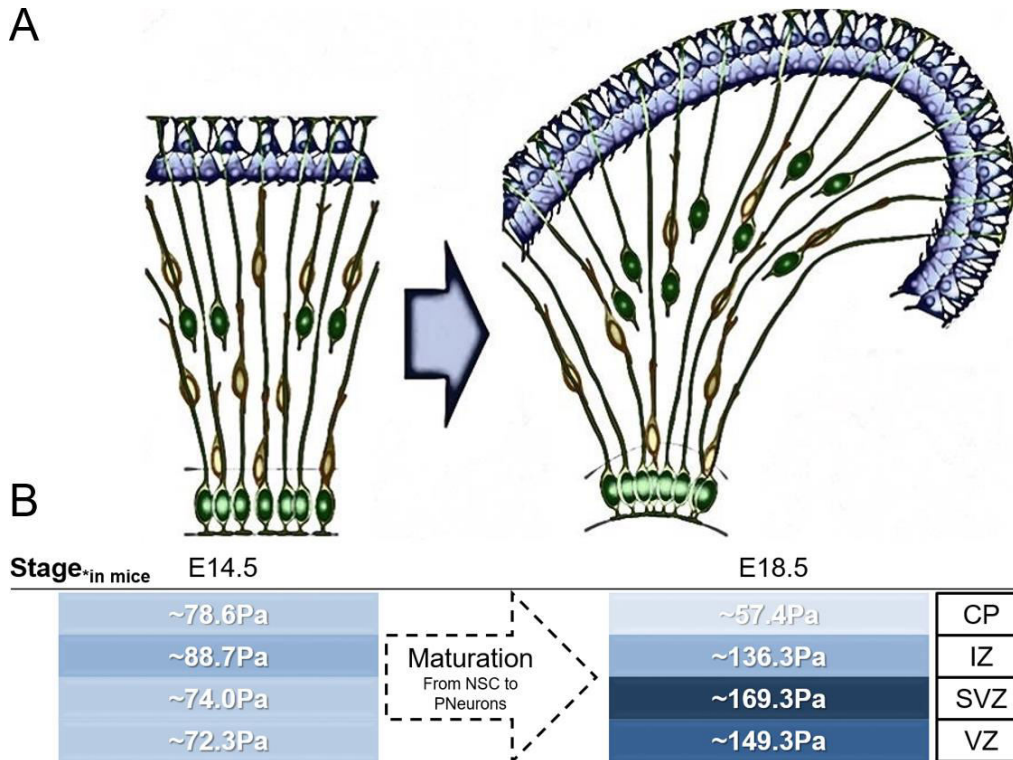


Figure 1.7 | Brain morphological shifts during embryonic development. (A) During brain development, the neocortex experiences an expansion in volume, resulting in patterned layers with varied gradients of factors. (B) Neocortex layers' stiffness shift for example from day 14.5 to day 18.5 of mouse brain embryonic development. Data adapted from Das *et al*, 2015⁹⁹.

These three main stimuli shall play a deterministic effect over neural stem cells (NSCs) differentiation, neurites guidance and neurons phenotype distribution. However, most of the gradients formed during development phase are yet to be determined. Embryonic bodies and organoids have ultimately proved this hierarchical formation by spontaneous self-arrangement during progenitor neuronal cells maturation^{103–106}. Moreover, recent reports demonstrated improved resemblance of degenerative disorders, such as the Alzheimer's disease, in 3-D spheroids when compared to 2-D neural cultures¹⁰⁷. Still, this approach present high variability due to the self-assembling nature of spheroids and the cell microenvironment differs significantly depending on the distance from the core to the spheroid surface¹⁰⁸, which can potentially be controlled using microfluidic and bioreactor technologies.

Alzheimer's disease is the most common form of dementia and it is a good example for which improved *in vitro* models raise the hope to find insights into disease mechanisms and possible treatment strategies. The disorder characterizes by two pathological hallmarks: amyloid- β plaques posits leading to excessive accumulation of amyloid- β peptide triggering neurofibrillary tangles, which are composed of aggregated hyper-phosphorylated tau. Though, to date, no single disease model has serially linked these two

pathological events using human neuronal cells, because there are no models realistically significant serving as platform to study human brain disorders.

Brain models appear nowadays as an essential tool for neuroscience progress. Advances are currently converging biological basic sciences with engineering solutions. First, pluripotent stem cells¹⁰⁹, including human embryonic stem cells¹¹⁰, were cultured *in vitro*. Second, biofabrication and 3-D cell culture methods offer the ability to control patterns in a 3-D surrogate microenvironment and are invaluable tools to examine the physical properties of brain regions^{111,112}.

1.3.2 Engineering Neural *in vitro* Models

In this section, the application of TE approaches is directed for neural *in vitro* models development, instead of tissues substitutes. Understanding the principles that underlie progenitor cells maturation into neurons and its self-arrangement into layers is of major importance for consideration when fabricating a realistic *in vitro* model. Unidirectionally connected by neurites during brain development, these layered pattern is fundamental in biological studies and therapeutics discovery. Such understanding requires the ability to decompose complex developing structures of the brain, such as the cerebral cortex. Studies in humans are complex, provide poor access to all stages of development and lack functional tissue preparations. So, animals can constitute by now the most powerful models of the nervous system. Animal models have yielded important insights in, for instance, the molecular drivers of neural development and activity¹¹³. However, these models are characterized by their high cost, associated ethical issues, poor isolation of variables, as well as reduced significance when translated to the human condition. Therefore, there is a raising demand for relevant *in vitro* models to decompose the brain into specific tissues/regions so that science may enable an improved analysis control for the understanding of the complex electrophysiology of the human brain as well as neuronal regeneration and repair, known as neuroplasticity.

Most of the *in vitro* platforms over the last decades were performed in planar surfaces, which do not sufficiently recapitulate the 3-D aspects of neural connectivity or the microenvironment of the human brain. For example, many of these methods build along a transverse or axial plane contrary to the natural brain architecture which consists of layers in a medial or sagittal plane (Figure 1.8)¹⁰¹. Additionally, to grow NSCs that have the potential to differentiate into diverse neural cell lineages, 3-D aggregation into structures called neurospheres is essential¹¹⁴.

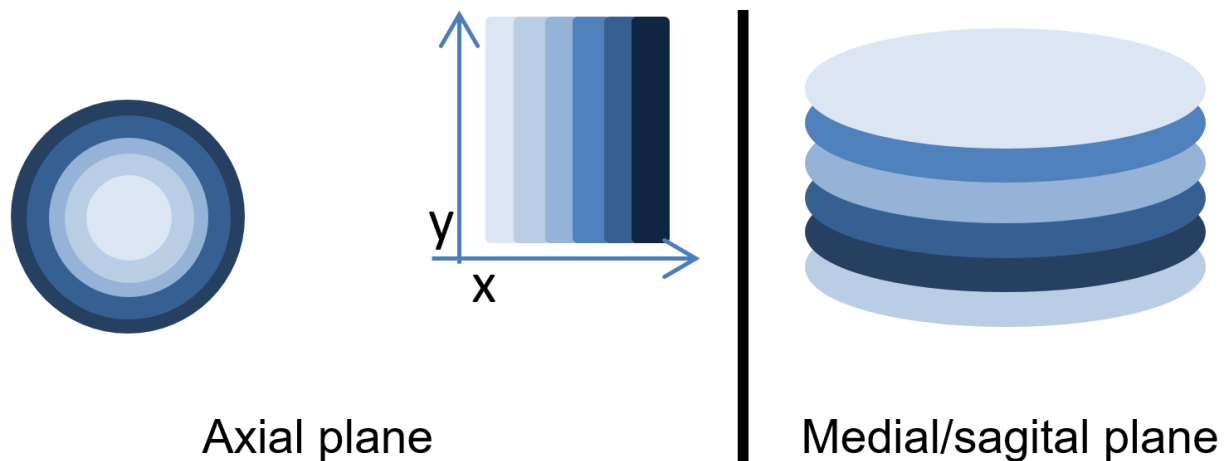


Figure 1.8 | Strategies mainly in two planes. 3-D layers better resemble the *in vivo* environment of cerebral cortex.

Stand-alone, artificial 3-D brain-like models can be used to perform preliminary evaluation of new therapeutic treatments. Furthermore, a functional *in vitro* model can ultimately be used to probe multi-layered neural circuits, enabling a better understanding of physiological learning and memory, as well as traumatic brain injuries and neurodegenerative disorders as Alzheimer's or Parkinson's disease. Progresses have been achieved regarding the 3-D architecture of neurons in culture, but several existing limitations can still be improved by applying TE tools, such as the spatial pattern of 3-D neural environments, programming degradation to mimic degenerative disorders and aging, or to increase diffusion in 3-D *in vitro* tissues by controlling porosity.

1.3.3 Biofabrication and Self-Assembling Techniques for 3-D Neural Models

In vitro models for brain disorders are commonly based on primary animal derived cultures or human neuronal-like cell lines such as neuroblastoma cells^{107,115}. Moreover, multistep strategies have been developed to create the 6-layered tissue surrogates of the cerebral cortex¹¹⁶. For instance, primary rodent neurons were encapsulated in a layer-by-layer assembly approach in concentric 3-D donut shaped constructs, made from composite hydrogel and silk protein¹¹⁷. Micromolding has also been employed to embed primary neurons in 3-D networks of collagen¹¹⁸. The generated 3-D neural surrogate tissues displayed good characteristics in cell density, interlayer neurite connections, as well as biochemical and eletrophysiological functional responses. However, the involved bioengineering operations, beyond of being complicated, they result in an indistinct mixture of neurons and functional patterns, unlike the layered cortex.

A single step bio-acoustic levitational assembly technology was addressed by Charléne Bouyer *et al*¹⁹. Multilayer were formed in a fibrin 3-D microenvironment by the effect of bulk acoustic waves pressure on human stem cell derived neuro-progenitor cells. Because of their larger density and lower compressibility than the surrounding fluid, cells were driven to the node planes of acoustic standing waves where there was minimal pressure. The acoustic frequency was tuned to match the number and spacing of the interlayers to that of native tissues, with an interlayer distance that corresponds to half of the acoustic wavelength. These characteristics made this technique useful to arrange spatially cells encapsulated in hydrogels. However, this method is currently limited to levitate homogeneous or heterogeneous cell populations without spatial difference.

Dynamic approaches can potentially be used to control self-assembling strategies, such as neural spheroids or organoids formation. Microfluidic and bioreactors can even be applied for the spatial control of different neural cell types. Organ-on-a-chip is an additional interesting strategy when mimicking heterogeneous tissue environments, such as the blood-brain barrier.

1.4 DYNAMIC CELL CULTURE SYSTEMS

Bioreactor and microfluidic systems are cell culture environments confined in a reservoir with a shape of a vessel, flask or even channels, connected to inlets and outlets for continuous flowing of nutrients through the cell culture. A bioreactor can be described as a dynamic device or system for culturing cells or tissues in suspension, 2-D or 3-D, under controlled biochemical or mechanical conditions. Microfluidic technique was created as an option for the perfusion of cell cultures, since the compartmentalized nature of microfluidic devices interconnected by microchannels allows perfusing media adjacent to or through a population of cells or 3-D tissue-like construct.

In general, bioreactors and microfluidic systems are designed to perform at least one of the following five functions:

- 1) To achieve uniform cell distribution,
- 2) To keep constant an optimized concentration of gases and nutrients,
- 3) To perform mass transport to the tissue,
- 4) To increase tissue maturation by applying physical stimuli,
- 5) To provide information about the formation of 3-D tissue by attaching sensors and designing transparent chambers.¹²⁰

A bioreactor or microfluidic device incorporating a flow pump to continuously circulate culture medium, is the minimum criteria to define as dynamic a cell culture technique. The pump or motor must be small enough to fit into an incubator and also be usable at 37 °C and in a humid environment. The forces needed for cellular stimulation are very small so it is important to ensure that the pump/motor has the capability to apply small forces accurately. Tissue culture is a continuous, non-steady state process in which the cultivation and tissue specific parameters change with time. Furthermore, the scaffold and chamber shapes are of significant importance for controlling the nutrition flow pattern.¹²¹

1.4.1 Mass Transport: Diffusion and Convection

Static culture systems rely primarily on diffusion, and to a lesser extent natural convection, for transport of oxygen to cells. The depth of media in the static conditions limits the supply of oxygen from the gas phase, which is not a big issue when culturing a monolayer of cells.¹²² However, in a 3-D cell culture approach improving local perfusion of thick tissue constructs remains a significant challenge for 3-D-based devices. Static culture of cell-seeded 3-D scaffolds typically results into localized tissue growth in the construct periphery because of lack on mass transport of fresh nutrients through the tissue.¹²³ Mass transport through the scaffolds can be improved using perfusion methodologies by housing the construct within a flow-through column,^{124,125} or by suspending cell spheroids or constructs within rotary culture devices or spinner flasks, generating a dynamic culture.^{126,127}

The important role of mass transport in a cell culture device is to keep cell metabolism within a physiological range by providing metabolic substrates and removing toxic degradation products. Understanding how device geometry is related to convective or diffusive transport limitations is therefore a key element of the bioreactor and microfluidic systems design.

The rate of diffusion is proportional to the metabolite concentration gradient, being the constant of proportionality the diffusion coefficient, as mathematically represented by:

equation 1.1,
$$D = \frac{kT}{6\pi R}$$

The Stokes–Einstein equation relates the radius of the diffusing particle and temperature to the diffusion coefficient, where k is Boltzmann’s constant, T is temperature and R is the particle radius. The volume of a sphere is proportional to the cube of its radius, $V = 4/3\pi r^3$, so the diffusion coefficient is approximately inversely related to the cube root of molecular weight.

The mass flux is also related to the gradient that can be generated at the interface between cells and medium, according to Fick’s first law of diffusion:

equation 1.2, $J = -D \frac{d\varphi}{dx}$

Fick's equation relates the diffusive flux “J” to the concentration when assuming a steady state (if a system is in a steady state, then the recently observed behavior of the system will continue into the future). The diffusion flux dimension is amount of substance per unit area per unit time, so it is expressed in mol.m⁻².s⁻¹ and quantifies the amount of substance that will flow through a unit area during a time interval. D is the diffusion coefficient and its dimension is area per unit time, so standard units would be m²/s. φ is the concentration in ideal mixtures, of which the dimension is amount of substance per unit volume as mol/m³. x is position, the dimension of which is length, it might thus be expressed in the unit m.

1.4.2 Flow Conditions for Dynamic Cell Cultures

Diffusion is the only driving force to move nutrients and waste in a static culture system. As the size of the scaffold increases, diffusion of nutrients and waste removal to and from the interior of the construct becomes more difficult, leading to necrosis at the core of the scaffold.¹²⁷ Defects requiring TE solutions are typically many millimeters in size for which, in static cell culture systems, it can be quite difficult to enable sufficient fresh medium circulation through the engineering construct.¹²² Scaffolds in such a size range are easily fabricated. However, problems arise when culturing cells on these scaffolds. The main challenge in preparing constructs larger than few millimeters is to obtain a homogeneous distribution of cells, and hence new tissue, throughout the whole 3-D scaffold volume.¹²⁸ This problem is increased by the static culture conditions, which result in scaffolds with few cells in the center of the construct. It has been shown that despite uniform cell seeding, over cell culture period, more cells distribute predominantly on the periphery of the constructs.¹²⁹ The main reasons for this distribution in scaffolds at millimeter scale or more are cell sedimentation, necrosis and, as a consequence of the previous ones, cell chemotaxis. Mineralized bone matrix reaches a maximum penetration depth of 240 μ m when stromal osteoblasts are cultured onto poly(DL-lactic-co-glycolic acid) scaffolds, which is far thinner than an ideal bone graft needed for the clinic.¹³⁰

Perfusion flow performed by bioreactors and microfluidic devices provides several benefits such as stable nutrient supply, waste metabolites washed away and control of oxygen tension distribution. Furthermore, perfusion is one of the key stimulus in vasculature, as it provides shear stress, which affects the cellular morphology and gene expression.^{131,132} So, constant nutrition and oxygen flow supply and metabolic products elimination must be performed when culturing cells up to higher density which is one of the

most important tasks to achieve physiologically meaningful functions for TE and sufficient cell number for *in vitro* constructs development.¹³³

While the first generation of bioreactors for culturing cells was designed simply to pump nutrient through the assembling tissue followed by waste removal¹²⁸, the next wave of bioreactors was designed for maturing tissues such as blood vessels,¹³⁴ cartilage¹²³ or cardiac muscle,¹³⁵ subjecting the emerging tissue to mechanical compression, shear stresses, and even culture medium pulsatile flow. Such stresses lead to improved mechanical properties of engineered tissues such as cartilage.¹³⁶

1.4.3 Flow Shear Stress and Related Stimuli

Flow-derived shear stress provides a physiologically relevant mechanical stimulation that significantly promotes specific protein expression and elicits paracrine effects by increasing the probability of randomly happen secretion, and protein-protein and cell-cell contact.¹³⁷ These events are particularly relevant when culturing complex tissues as interfaces requiring co-cultures. Given the central role of several protein pathways in specific disorders, these stimuli represent a significant dynamic for example for an *in vitro* model of a disorder condition as OA.

Mechanical cues are also crucial for tissue specification and maturation, which can be transduced by the bioreactor chamber design¹²¹ or microfluidic channel geometry, as suggested by a study of the effect of shear stress on mature osteoblasts. Kou *et al*¹³⁸ developed a device capable of applying four different magnitudes of shear stress in parallel channels on one chip. The intracellular calcium intensity peak was proportional to intensity of shear stress, while response time was independent of shear stress for values larger than 0.03 Pa.

While flow rate can be used to modulate media exchange¹³⁹, pore size, interconnectivity and anisotropy can influence different rates of media exchange and shear stress on cells distributed within the construct.¹⁴⁰ Mechanobiological aspects, such as active stretch and tension, are another functional feature that can be added using microfabrication techniques. Although interesting, it has received minor attention in combination with 3-D cell culture. Regarding this challenge, sophisticated devices for 3-D cell culture are one of the main needs for the current RM and TE evolution.¹⁴¹

1.4.4 Bioreactor Designs

The basic principle applied at the first generation of bioreactors for TE was the agitation-based approach. According to this approach, a cell suspension is placed into a container while keeping the suspension in

motion. Gentle stirring is used to provide motion to cells. Due to this, cells do not adhere to the walls and form cell-to-cell interactions. Based on this principle, the main bioreactor designs were created, which are compared in table 1.2 and summarized in figure 1.9.

Table 1.2 | Bioreactor designs compared in dynamics, application, advantages and issues.

Design	Dynamics	Other stimuli	Common application	Advantages	Main issues	References
Static bioreactor	no	no	Cell proliferation	Medium perfusion	Cell sedimentation, lack stimuli, aggregation	¹²² , ¹²⁷
Spinner flask	stirring (magnet)	no	Spheroids formation and osteogenesis	High cell density, uniform cell distribution in constructs	Shear forces, need large amount of medium, heterogeneous sizes of spheroid	¹⁴² , ¹⁴³ , ¹⁴⁴ , ¹⁴⁵ , ¹⁴⁶
Rotating cell culture	Spins the container	no	Spheroids formation, microgravity	Dynamic laminar flow, reduced shear stress	Variability in the size of spheroids formation	¹⁴⁷ , ¹⁴⁸ , ¹⁴⁹ , ¹⁵⁰ , ¹⁵¹ , ¹⁵² , ¹⁵³
Flow perfusion	no	no	Perfusion of scaffolds, osteogenesis,	Enhanced nutrient and mass transfer, homogeneous cell distribution, high seeding efficiency	Not adapted for interfaced tissues or heterogeneous cell differentiations/phenotypes	¹⁵⁴ , ¹⁵⁵ , ¹⁵⁶

Compression bioreactors	Compression	Hydrodynamic shear, hydrostatic pressure, tension, friction, magnetic or electrical pulses	Cartilage, tendon, cardiac tissue, muscle TE	Specific and accurate physical stimuli; improved maturation of specific tissues	Specificity reduces industrial translation or high-throughput applications	157, 158, 159
<i>In vivo</i> bioreactor	Animal movements	Blood circulation	Bone TE	Physiological environment, vascularized tissues	Ethical constraints, poor control over variables	115, 116

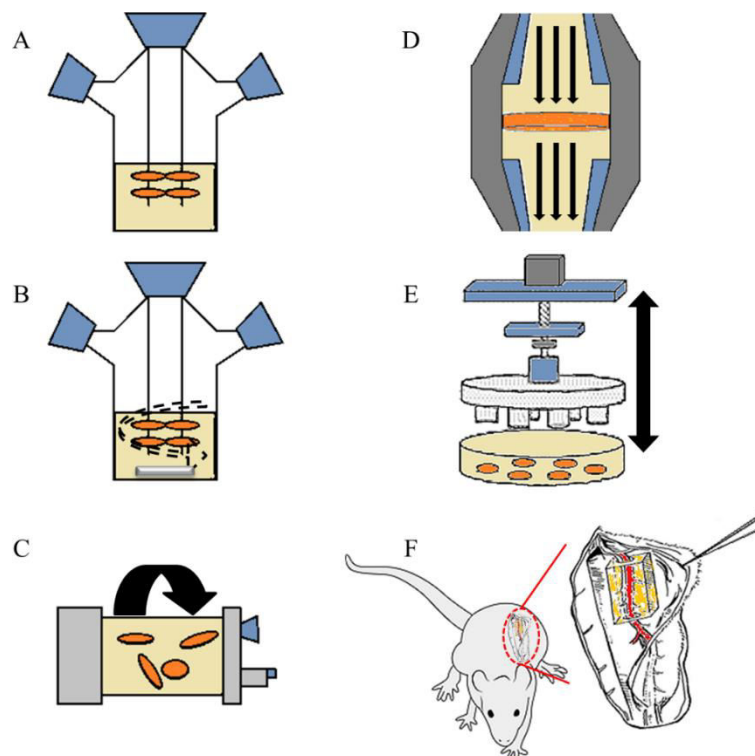


Figure 1.9 | Schematic representation of each bioreactor system for 3-D cell culture. (A) Basic flask bioreactor, (B) Spinner flask bioreactors, (C) Rotating cell culture bioreactor, (D) Flow perfusion bioreactor; (E) Compression bioreactor and (F) *In vivo* bioreactor.

1.4.5 Microfluidic Designs

Microfluidic devices experienced a fast evolution starting in the 1990s¹⁶⁰ and nowadays contribute as versatile platforms in the fields of molecular analysis, laboratory diagnostics, biodefense and consumer electronics.¹⁶¹ Applications of microfluidic systems based on cell and tissue culture have been emerging more recently and TE is taking advantage from micro- and nanofabrication techniques for the development of precise features and high-throughput screening on tissue modelling and drug testing.¹³³ First at 2-D, and during the last 10 years the third-dimension of cell culture has been also revolutionized by the integration of microfluidics.

Microfluidic systems typically consist of devices with channel geometries having characteristic length scaled from tens to hundreds of microns.¹⁶² When microfluidics are designed for cell culture it might encompass from millions to single cells providing a level of flexibility beyond that possible in conventional well plates or even with bioreactors.^{163,164} For example, An interesting technology to study chondrocytes was introduced by Neve *et al*/¹⁶⁵. The developed technique integrates micron-resolution particle image velocimetry with dual optical tweezers that allow for the capture and maintenance of a single chondrocyte in a flow field that can be measured in real-time.

Structural features in microfluidic devices may be designed to provide spatial control over cell behavior, and interactions between cell populations may be controlled through the use of channels, membranes and other features incorporated into these systems.^{166,167} The integration of TE in microfluidic system became known as organ-on-a-chip when several chambers were integrated to allow intercommunications of cell culture/tissue cells mimicking more than one tissue or organ in a single device.¹⁶⁸

Fabrication is typically performed by photolithography by applying a standard technique as Radio Corporation of America (RCA) cleaning, thin film deposition, wet hydrofluoric etching, access hole forming, or chip bonding.¹⁶⁰ Soft lithography and other processing techniques have enabled rapid, simple fabrication of microfluidic devices from a broad range of substrate materials including thermoplastics and thermoset polymers, typically produced in optically transparent formats that may be rigid or elastomeric.¹⁶⁹ Some significant features which make this technology distinguishable are:

1. Microscale resolution and flow conditions match with the cellular structures dimensions and traffic present in the human organism.
2. Spatial control over chemical gradients can mimic the dynamic 3-D biochemical environment existing *in vivo*.
3. Reduced costs as it requires samples in nanoliter volumes.

4. Design of microfluidic devices is compatible with substrates permeable to oxygen enabling cell culture in 3-D.

5. Microfluidics can handle several processes at one time such as culture, replenishment of medium, cell detachment, and subsequent detection. Furthermore, fabrication can use transparent materials allowing microscopic imaging.

One of the main advantages of microfluidic approaches is the spatial fine control over fluids at micrometer-scale, which can be explored to increase the physiological significance of 3-D tissue models. Early examples demonstrate spatial patterning of adhesion molecules¹⁷⁰ and hydrogels^{171,172}, which are still used in microfluidic 3-D cell culture. Today, the most important drivers for the use of microfluidic techniques in 3-D cell culture are:

- (i) The integration of perfusion/flow;
- (ii) The ability of co-culturing cells in a spatially controlled manner;
- (iii) Generation of and control over (signaling) gradients.¹⁷³

Spatial control is essential for the increasing need for more complex tissues *in vitro* modelling, since our body present several interfaced tissues and barriers. These are, for example, determinant for testing drug efficacy as drug molecules either have to cross barriers or be effective to treat a tissue without affecting the adjacent one. Microfluidic fabrication allows patterning surfaces for cells and extracellular microenvironment stratified (co-)cultures with basal–apical access, controlling gradient formation and medium perfusion. In classical culture techniques, the spatial control is usually achieved by a membrane to support surface-attached cell growth dividing the culture well in two independent compartments.¹⁷⁴ The recent trend in microfluidic systems is to use hydrogels interfaced by two channels offering a physiologically more relevant environment. By using laminar flow two or more streams are joined into a single channel flowing parallel to each other without any turbulent mixing, allowing the only mixing by diffusion across the interface. This ability to sustain parallel streams of different solutions in a single microchannel has been applied to pattern cells and their environments.^{175,176} This method can also be used to study subcellular processes by positioning a single cell interfacing two adjacent streams.¹⁷⁷ Furthermore, by patterning a hydrogel between two fluids, stable and predictable linear gradients are formed, which can be controlled by the channel geometry and applied flow rates.¹⁷⁸ To integrate this type of assays into the high throughput drug-screening pipeline, Trietsch *et al*.¹⁷⁹ created a microfluidic platform based on a 96-well titer plate format enabling a double flow perfusion to generate a gradient over an hydrogel. Perfusion flow was maintained by passive levelling between two reservoirs, thereby eliminating

the need for external pumps. This allows high throughput migration assays and gradient formation in combination with stratified co-cultures.

From the microfluidic devices was born the organ-on-a-chip concept, which is based on a microfluidic cell culture device created with microchip manufacturing methods that contains continuously perfused chambers inhabited by living cells arranged to simulate tissue- and organ-level physiology. By recapitulating the multicellular architectures, tissue-tissue interfaces, physicochemical microenvironments and vascular perfusion of the body, these devices could produce levels of tissue and organ functionality not possible with conventional static 2-D or 3-D culture systems. This concept was born for the creation of tools to enable *in vitro* analysis of biochemical and metabolic paracrine activities in between different tissues and can have a huge impact in the future after maturation and optimization of the concept. A summary of different applications based on microfluidic approaches using varied fabrication methods is summarized in figure 1.10.

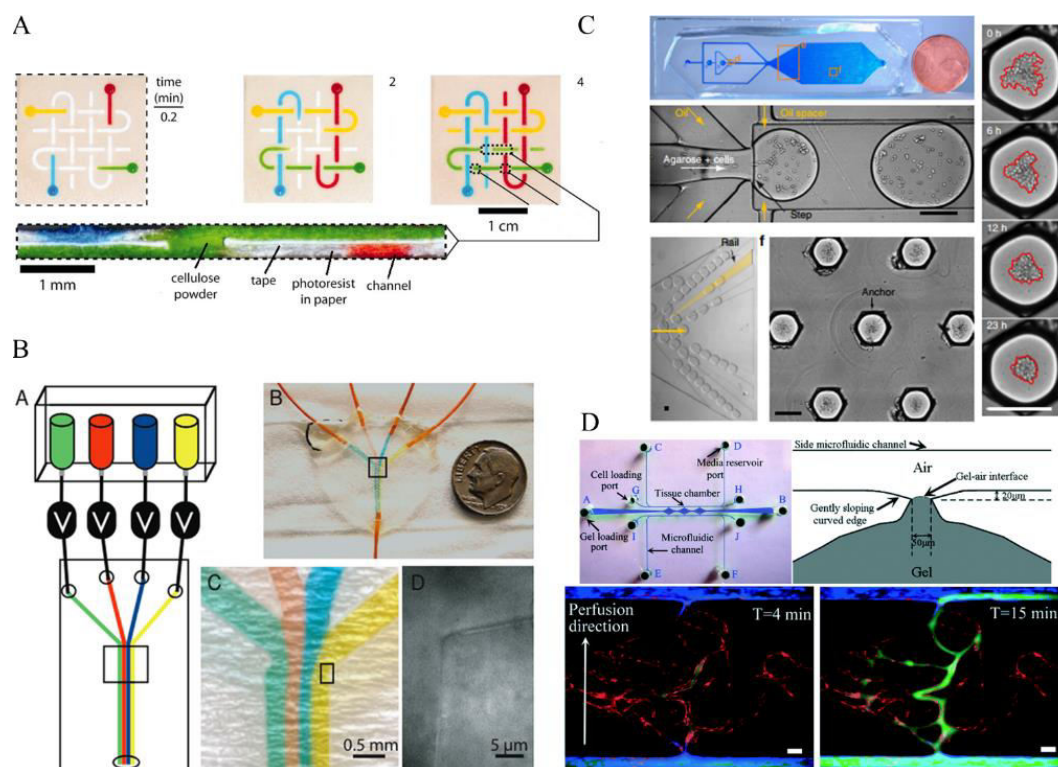


Figure 1.10 | Microfluidic devices designed over several substrates and for broad applications. (A) Crossed microfluidic channels creating interfaces in layered paper-tape-based device. Adapted with permission from ¹⁸⁰ Copyright © 2008 National Academy of Sciences. (B) Microfluidic concept of parallel channels separated by laminar flow from multiple-input that are optically clear on each face, chemically inert, reusable, inexpensive, and can be fabricated on the benchtop in approximately 1h. Adapted with permission from ¹⁸¹ Copyright © 2009 Courson, Rock. (C) Microfluidic device

compartmentalized for 3-D cell aggregates analysis at high-throughput scale. Adapted with permission from ¹⁸² Copyright © 2017, Rights Managed by Nature Publishing Group. (D) Perfusable vascular network interfacing two microchannels creating an organ-on-a-chip system. Adapted with permission from ¹⁸³ Copyright © 2015, Royal Society of Chemistry.

In addition to the physiological relevance, microfluidic systems can potentially improve reproducibility, cost effectiveness and implementation at larger scales for diagnostics and drug screening. For example, the reduced dimensions offer advantages such as reduced consumption of expensive cell material, hydrogels and screening reagents. Well defined heights of microfluidic channels improve imaging quality and speed. Precise metering of liquids with microfluidic techniques enables better quantification of assays. However, moving to a microfluidic reality implies changing several exclusive factors to microfluidic from macroscopic cell culture, such as different culture surfaces, reduced media volumes, and vastly different rates of, and methods for, medium exchange. These unique features slow-down the acceptance and adaptation of the current state-of-art of cell culture techniques to the dynamic microscale. Furthermore, even though there are reports about 3-D cell culture in microfluidic devices, a further push is needed to consolidate this interesting concept for more complex 3-D tissues as interfaces and co-cultures based studies.

1.5 BIOREACTORS AND MICROFLUIDICS FOR OC *IN VITRO* TISSUE MODELLING

Over last decades, strategies to investigate bone-cartilage interactions *in vitro* were mostly limited to cell co-culture well-plates in which bone and cartilage cells were both exposed to the same medium¹⁸⁴ arguably a very distant condition from the *in vivo* environment. Additionally, co-cultures imply the use of trans-wells avoiding direct cell contact which is also crucial for example for interfaced tissues. Using TE techniques, two construct pieces can be independently cultured under chondrogenic and osteogenic medium and joined together after tissue maturation, resulting in an interrupted interface.

Traditional bioreactors have not been frequently explored in the development of skeletal tissues interfaces, but either applied to bone or cartilage tissue development.¹⁸⁵ Regarding this, when co-culturing stem cells in 3-D scaffolds, researchers either need to use costly pre-differentiation operations before cell seeding and keep its phenotype over cell culture or, as a more challenging alternative, simultaneously modulate differentiation down to distinct lineages in a unified culture environment.¹⁵ The classic case of the OC

interface, which consists of a hyaline cartilage layer and an integrated subchondral bone, is a good example of an interface requiring for more complex scaffold design and a dual-environment when cultured *in vitro*. So, when applied to the interfaced OC junction, explants were usually used.

Biological characterization of the OC tissue present in any joint of human body, already revealed the existing and key communication between chondrocytes and osteoblasts across the junction. Furthermore, understanding the mechanical properties of the articular surface is the main focus of interest for graft development, because the OC junction and the subchondral bone are known to confer significant protective mechanical properties to the overlying cartilage.¹⁸⁶ This interface is characterized by a transition of collagen type I to type II and also of collagen fibers orientation. Moreover, several gradients, such as cellularity, cell size, proteoglycans and collagen content characterize the OC tissue interface. Specifically, the subchondral bone has been shown to reduce impact-induced fissuring, chondrocyte cell death and matrix degradation, all of which are hallmarks of pre-osteoarthritis.¹⁸⁷ However, understanding OC phenotype and related disorders through specific communications require an *in vitro* culture system that supports integrated native or engineered osseous and chondral components of an OC unit. The current studies of chronic joint disorders have already revealed the influence of subchondral bone changes in the etiology of osteoarthritis (OA), but these changes have not been effectively reproduced *in vitro*.^{188,189}

Recent developments in the bioreactor field, as the creation of systems adapted for the maturation of interfaces and able to spatially control gradients, will open up new possibilities to foster interfaced TE as it is the case of the OC tissue unit. Dual-chamber bioreactors have been tested for OC TE. Chang *et al*.¹⁹⁰ cultured a gelatin-infused sinbone block to generate OC constructs in a dual-chambered bioreactor for the production of hyaline cartilage within the gelatin portion of the scaffold while the bony portion was acellular. Mahmoudifar and Doran¹⁹¹ used a similar dual-chambered bioreactor for the maturation of two sutured polyglycolic acid meshes seeded with ASCs. After 2 weeks of culture, both layers were cellularized, but showed statistically undifferentiated GAG content. The main limitations of the reported systems are related to lack of mechanical stimuli. While these two reported dual-chambers are under flow perfusion, limited medium diffusion and cell homogenization through the 3-D structures can occur. The current systems need to provide more robust outcomes in terms of cell viability in the core of the construct, differentiation control, and efficient cell homogenization in the 3-D space, resulting in a greater reproducibility of the matured tissue.

OC *in vitro* models based in microfluidic devices were recently reported. Goldman and Babino¹⁹² created a microfluidic dual-chamber device for the control over the osteo- and chondrogenic phenotypes. Bovine MSCs were encapsulated in agarose, then casted against micromolds of a serpentine network and

stacked to produce tissue constructs containing two independent microfluidic channel networks. Constructs receiving differentiation media showed differential chondrogenic and osteogenic gene expression, which was confirmed at the protein level as collagens I, II, and X. The control group under basal culture medium corroborated the results by showing homogeneous expression of the same biomarkers measured in lower concentrations at both the mRNA and protein level.

Shi *et al*¹⁹³ compared a strategy based on a gradient generated by a microfluidic device with a conventional approach where two pieces of ASCs-laden hydrogel were cultured under osteogenic and chondrogenic conditions and joined together. The microfluidic system allowed generating a gradient of differentiation mimicking the OC interface. Although the dynamic cell differentiation methods using the microfluidic device consumed more cell culture media than the static method, the microfluidic system continuously supplied new nutrition and transported the wastes produced by cell metabolism out of the device generating a non-toxic environment. In addition, the flowing media could be collected and reused because much of the nutrients in the flowing media are usually not consumed during the continuous flow of culture media.

Lately, a technological approach using a high-throughput platform was designed and osteoarthritic condition in a OC model was for the first time assessed *in vitro* to validate the system (figure 1.11). Lin *et al*¹⁹⁴ developed the platform adapted for interfaced OC tissue in a well plate format. This approach represents a transition design in between bioreactors and microfluidics. The system consists in a single bioreactor formed by the inset and lid in the context of a 24-well plate. The device was designed to accommodate the biphasic nature of an OC plug by creating two separate compartments for the “chondral” and “osseous” microenvironments. The two microenvironments are independently controlled and the medium flow through each row of wells. The authors have shown that MSC-based chondral and osseous tissues respond to IL-1 β in a manner that the changes in one tissue compartment are communicated to the other along the OC axis.

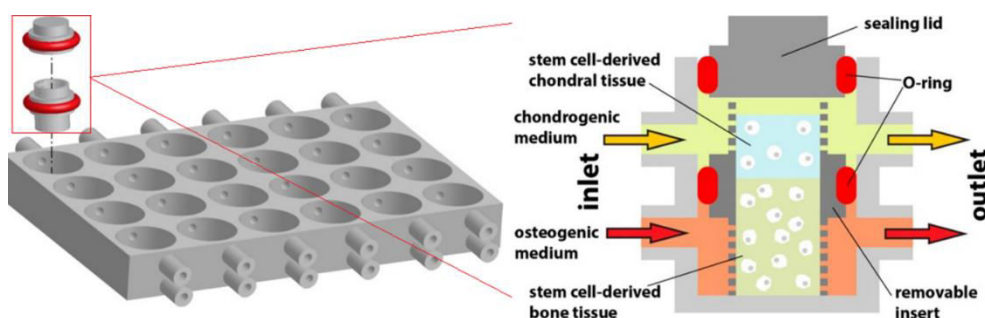


Figure 1.11 | Bioreactor systems adapted for high-throughput concept. Represents a transition from bioreactors to microfluidics concepts as a high-throughput device for 3-D interfaced

tissues. Adapted with permission from ⁹⁷, <http://pubs.acs.org/doi/abs/10.1021%2Fmp500136b> Copyright © 2014 American Chemical Society.

While bioreactors are now seeing its concept turned to the field of tissue modelling, microfluidic systems have been tested more often for this goal. However, the main difference of both concepts is that bioreactors were firstly developed for tissue production to be used as grafts for implantation, and are now useful platforms for *in vitro* tissue modelling, while microfluidic devices can only be used for this last purpose as a drug testing or diagnostic tool.

1.6 BIOREACTORS AND MICROFLUIDICS FOR NEURAL *IN VITRO* TISSUE MODELLING

TE technologies are based on the biological triad of cells, signaling molecular pathways and ECM. Nowadays, TE lead a transition from 2-D to 3-D cell culture techniques in order to achieve more physiologically relevant tissue substitutes and models, but culture techniques are still limited. Typically, current 3-D cell culture techniques do not yet allow meeting the multicellular complexity of tissues, do not offer fine control over gradients and require medium exchange at discrete time points instead of in a continuous manner because they rely on static environments.¹⁷³ Researchers have lastly attempted to create more realistic neural tissue *in vitro* models using 3-D constructs¹¹⁶, micro-chambers¹⁹⁵ and microfluidics¹⁹⁶.

Bioreactors have not been extensively used for neural *in vitro* modelling, but for *in vitro* neural tissues maturation. In an undirected organoid differentiation, such as the techniques developed by the Knoblich group¹⁰⁵, human induced-pluripotent stem cells (iPS) were suspended and grown in an ECM-like environment, such as Matrigel, using spinning bioreactors to increase neural cell density. In a different approach, a custom-designed bioreactor was planned to perfuse a platform that utilizes forced intercellular convection to enhance mass transport, thereby continuously delivering nutrients and withdrawing waste from thick constructs. Applying this micro-perfused chamber, laminar perfusion resulted in a 700 μm thick construct of neural-astrocytic in an equal ratio at cell density approaching that of the 50,000 cells/ mm^3 in the brain.¹⁹⁷

A simple method also using a bioreactor was developed by Pasca et al¹⁹⁸ based on a materials-free approach. For deriving dorsal forebrain in 3-D, intact colonies of human induced pluripotent stem cells were lifted, followed by neutralization and culture exclusively in suspension, without an extracellular matrix

or culture. These spherical cultures grew up to 4 mm in diameter, containing equal proportions of deep and superficial layers of cortical neurons as well as non-reactive astrocytes, and after approximately 9–10 months mature to resemble postnatal stages¹⁹⁹.

Similarly, miniaturized spinning bioreactors were used to control organoids maturation, patterning the forebrain and deriving either into midbrain or hypothalamus²⁰⁰ (figure 1.12). Instead for maturation, this bioreactor-based concept envisioned the use of the system for disease modelling and drug testing.

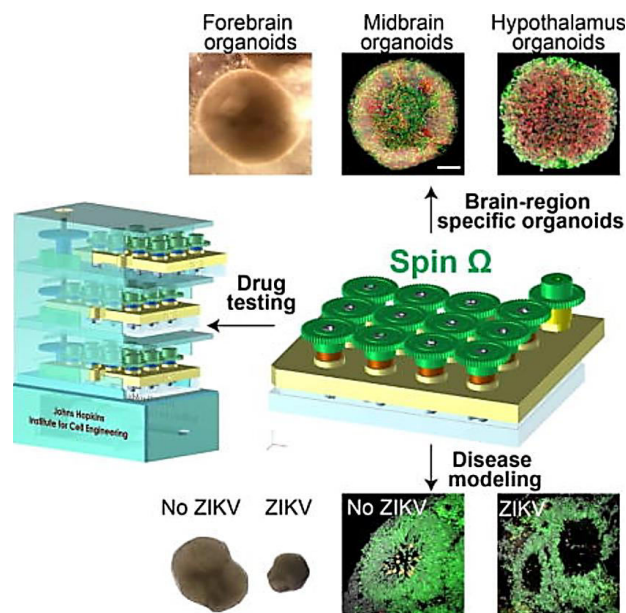


Figure 1.12 | SpinΩ Bioreactor-based Forebrain Organoid Culture System. Reprinted from ²⁰⁰, Copyright (2018), with permission from Elsevier

Top-down approaches, such as microfluidics and organ-on-a-chip, use physical channels to position cell types, create gradients and control the flow of nutrients, and to provide spatial and temporal control of the cellular environment²⁰¹. A microfluidic-based model was designed to control the neurite outgrowth of two subpopulations of primary hippocampal neurons (E18), directing connectivity grown unidirectionally by a specific design of channels shape. The authors found an optimal geometric shape of the microchannels in which the axons coupled two chambers with the neurons in an asymmetric manner. NSCs niches have been mimicked in microfluidic platforms. Diverse cellular conditions were compared, such as cells dispersed in planar or 3-D environments, or even of aggregated in spheroid shape. Additionally, static was compared against perfusion feeding. The different scenarios produced diverse results. While 2-D culture under static conditions benefited the differentiation into neuron lineage, the spheroid culture under perfusion favored the formation of glial cells. The use of a ECM-like environment

to disperse the NSCs in 3-D improved the stem cells self-renewal, resulting in greater proliferation because of a larger surface area. All the conditions were performed in the same microfluidic design, showing control over stem cells proliferation and fate by changing the cell spatial configuration.

In a different microfluidic schematic, anisotropic outgrowth of neurites was achieved in a 3-D system¹¹⁸. Using applying polydimethylsiloxane and fibrillogenesis kinetics of collagen, elastic environments were used to induce the linear growth of neurites by stretching the culture setting.

Increasing the complexity, a microchip model of the blood–brain barrier included endothelial cells on one side of a membrane and neurons, pericytes and astrocytes on the other side, and was used to test the effects of cytokines²⁰². In a different 3-D neuronal approach, neuronal-astrocytic co-cultures under continuous medium perfusion at $2.0\text{--}11.0\ \mu\text{L}\cdot\text{min}^{-1}$ improved viability compared to non-perfused cultures²⁰³, achieving an increased cell density of $10^4\ \text{cells}\ \text{mm}^{-3}$.

A 3-D blood-brain-barrier platform was developed by independently supplying different types of media to separate cell types within a single device (figure 1.13)²⁰⁴. Compared to co-cultures supplied with either one type of medium or a 1:1 mixture, the best barrier properties was achieved when culturing endothelial cells with endothelial growth medium and neural cells with neurobasal medium supplemented with fetal bovine serum (FBS), independently. The measured vascular network permeability was comparable to *in vivo* values $0.45 \pm 0.11 \times 10^{-6}\ \text{cm/s}$ for 20 kDa FITC-dextran, and $0.36 \pm 0.05 \times 10^{-6}\ \text{cm/s}$ for 70 kDa FITC-dextran. Furthermore, these conditions also showed a higher degree of astrocytic contact with the vascular network, and presence of synapses.

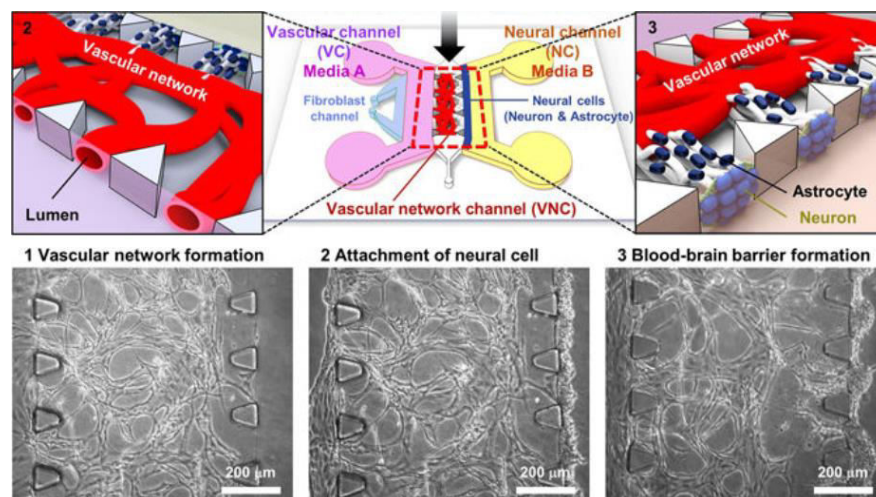


Figure 1.13 | A microfluidic platform for neurovascular unit including blood-brain barrier.²⁰⁴

The perfusable vascular network is formed over a 3-day period.

There have been significant progresses towards 3-D *in vitro* modelling of neural-related conditions. However, there is yet a long way to reach this level of complexity of the cerebral cortex. The native cell high density, interlayer neurites connections, layer thickness of the cortex, biochemical and electrophysiological functional responses, as well as displaying 6 layers of cells presenting their unique characteristics, and the unidirectional growth of neurites as hierarchically distributed in the human brain cortex was not achieved. One additional limitation of the current *in vitro* models of the brain structures is the lack of consideration by mechanical properties change during degenerative disorders²⁰⁵ and aging²⁰⁶.

1.7 GENERAL REMARKS

Strategies to promote a controlled organization of 3-D tissues developed *in vitro* have been emerging over the last decade. Heterogeneous tissues demand for advanced approaches when compared with homogeneous ones. In the case of skeletal interfaces, such as the OC unit, materials engineering strategies have predominantly been employed, while soft patterned tissues, as the brain cortex, have mainly been developed using cellular self-assembling methods. In either OC or neural *in vitro* tissue formation, stem cells are the main building base, performing differentiations or co-differentiations towards multiple defined cell lineages.

A large lot of materials have been tested by TE techniques, even if applied for a specific tissue development. This large screening of materials is by now providing interesting outcomes and large amounts of data are available to compare and choose the most interesting set of materials, or blends, for specific applications. Acellular strategies have been tested to restore for example OC defects (OCD), but when applied to *in vitro* tissue modulation, cells have to be combined, which is one more factor to consider when selecting the biomaterial.

Diverse cell sources have been tested for OC application. Although MSCs are known to be key players, alternatives to BM are required mainly because this is an age-dependent and limited cell source. For example, infrapatellar fat pad is an interesting source of stem cells to be applied on knee OC disorders, since Hoffa's body has to be harvested for surgical arthroscopy in most of the cases. ASCs are much more available than BM- MSCs, but the coming years will reveal if iPS and perinatal stem cells like WJ-MSCs, can be used to replace adult multipotent stem cells, overcoming the current ethical and tumorigenic issues, and becoming the cell sources under the focus of the tissue engineers for OC repair. For neural tissue formation, iPS and embryonic stem cells are mostly investigated showing interesting abilities to create organoids with neocortex native-like patterns formation. However, this strategy based

on cell self-assembling has been resulting in variable outcomes, not replicable. Having this in mind, the ideal stem cell source might be dependent on the target tissue and the right strategy dependent for example, on donors age. However, further investigations relating to optimal cell strategy, such as, autologous versus allogeneic, freshly-isolated versus expanded, stem cells versus differentiated cells, as well as the use of pluripotent stem cells in a consistent way and long-term efficacy, are the main challenges still remaining. Beyond the stem cell source, another issue that is a matter of controversy is the dose of cells that should be used. Huge ranges of cell densities have been applied in the different phases of investigation.

Typically, current 3-D cell culture techniques do not yet allow meeting the multicellular complexity of tissues, do not offer fine control over gradients and require medium exchange at discrete time points instead of in a continuous manner because they rely on static environments. Although, bioreactors and microfluidics devices, as a dynamic stimulus element, may be used as an alternative to or in conjunction with molecular GFs for the signaling part of the TE triad.

Bioreactors and microfluidic systems, as mentioned in previous sections, are capable of creating gradients which are of great interest to OC tissue *in vitro* development. Conventional *in vitro* culture systems such as static fed cultures, spinner flasks, rotating wall vessels and flow perfusion fail to provide physiological conditions capable of reproducing functional interfaces. So, the use of *in vivo* models have been the predominant method to test OC grafts and OA-related drugs, yet usually giving the wrong impression of translation readiness. Indeed, most of the cases fail when translated to clinical trials, mainly because of genotypic and morphological differences. Regarding this, *in vitro* models able of mimicking 3-D interfaces by using gradient or bilayer scaffolds in general and OC tissue in particular, based on human cells are still urgently needed. Since the traditional culturing systems are not specifically adapted for engineering 3-D interfaced tissues, new technologies have been emerging over the last 5 years targeting this gap.

When applied to neural 3-D tissues, bioreactors have mainly been used to mature large spheroids or constructs in suspension for high-cell density tissues formation. Perhaps, these achievements may potentially be combined in the future with culture medium gradient formation to perform spatial control of neural cells in a spatially controlled environment.

Microfluidics, as an alternative to bioreactors at the microscale, allows control over fluids in micrometer-sized channels and has become a valuable tool to further increase the physiological relevance of 3-D cell culture by enabling controlled co-cultures, perfusion flow and spatial control of signaling molecular gradients. Furthermore, these devices have been mainly used for blood-brain-barrier models fabrication.

TE field experienced an evolution boost during the 90s, which reached a plateau during the first decade of the new century. However, the rising of nanotechnology and the adaptation of specific bioreactor devices applying principles from robotics, triggered a new phase for Regenerative Medicine field. In addition, the findings about iPS and the control of 3-D printing is largely contributing for further discoveries and developments that are bringing drug discovery paradigm to the 3-D reality and the clinical approaches towards patient-specific therapeutics.

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CHAPTER 2

MARKET ANALYSIS

2. THREE-DIMENSIONAL STATIC AND DYNAMIC CELL CULTURE SYSTEMS: AN EMERGING MARKET FROM A SCIENTIFIC NEED

2.1 Abstract

Three-dimensional (3-D) cell culture systems are an essential need to develop tissue substitutes for regenerative medicine (RM). Moreover, human cell-based 3-D living tissues are currently being considered the most reliable *in vitro* models for drug discovery to reduce the false positive results delivered by 2-D approaches and animal models before the clinical trials. As a consequence, cell culture requires a shift from two-dimensional (2-D) to 3-D strategies. Together with this scientific demand, a new market was born 5 to 10 years ago. However, a transitional phase is still ongoing and researchers are frequently applying the tissue-culture polystyrene well-plates, commonly used for 2-D cell studies, as devices for 3-D cell constructs maturation. So, new strategic approaches should be taken into consideration when market-facing companies come up with changes to the standard implementation. Thus, this study describes the weight of 3-D cell culture systems and its newly born market, analyzing the existing needs, opportunities, segment and size. Strategic roadmaps for cell culture-related startups are discussed, as well as the technological adoption process. Moreover, detailed analysis of the financing system, patent landscape and regulatory demands is also performed. Important examples of the successful cases, which are indeed the current competition, are displayed along with technological trends emerging in the industry. This report is directed to both researchers and investors, due to the current observed absence of standardized processes, the need to demonstrate the potential use of 3-D cell culture emerging devices and their substantial contribution for current pharmaceutical industry issues on drug discovery.

Keywords: 3-D systems; cell culture; drug screening; pharmaceutical industry; *in vitro* models

2.2 Introduction

Cell culture systems evolved with regenerative medicine (RM) in the 1990s for cell therapy approaches but did not kept on with the TE needs. TE applies mainly to 3-D tissues as they exist in our body, while cell therapy uses single cell conditions as a drug administration approach. Tissue culture well-plates

(TCPS) and t-flasks are the most common devices for human cell culture for static cell culture even today and are used in laboratory since the 1950s¹. Nowadays, these plates optimized for 2-D are being replaced by 3-D cell culture-adapted designs for the creation of cell spheroids². Additionally, dynamic systems as bioreactors and microfluidic devices are gaining attention since these technologies enable a more realistic reproduction of the human physiology in an organ-specific context^{3,4}. However, despite the fact that current systems are efficient for 2-D cell culture, 3-D complex cell-scaffold environments present different metabolic requirements, such as improved diffusion of fresh nutrients and secreted molecules⁵. As an evidence of the current limitations, the non-adherent TCPS invented for cells suspensions, such as for hematopoietic cells culture, are being directly adapted to cells adhesion in 3-D scaffolds placed in the wells of these plates⁶. Although these TCPS have been bridging the gap existing between the old 2-D with the emergent 3-D approaches, several problems are rising over the last decade. Firstly, static cell culture systems present an unstable environment due to the accumulation of metabolites and consumption of nutrients, resulting in pH oscillation⁷. Therefore, these changes do not allow to have a representative scenario of native conditions and compromise the natural cell phenotype, which will consequently affect drug screening in pharmaceutical industry⁸. Secondly, interfaced tissues, such as bone-cartilage, tendon-bone, or dermis-epidermis, which present an heterogenic cellular environment, require more than one culture medium in a single environment⁹, which cannot be afforded by the current commercially available systems¹⁰.

Having these in mind, dynamic culture systems based on the use of bioreactors have seen its concept adapted from prokaryotic to eukaryotic cell culture in order to scale-up stem cell proliferation for their cryopreservation in banks and further use in cell therapy while undergoing 3-D technological evolution¹¹⁻¹⁴. Different types of bioreactors have been created to overcome the limitations of static cultures in TE approaches. Mixed flasks^{15,16}, rotating vessels¹⁷⁻²⁰ and perfused cartridges²¹⁻²³ were developed over the last decades. However, different applications present different needs since different tissues present specific stimuli to their cells. When created *in vitro*, the maturation of engineered tissues requires a physical environment matching the natural one. Regarding this, the concept of bioreactor was lately adapted, providing mechanical stimuli for the development of tissues such as bone²⁴, cartilage²⁵, muscle²⁶, tendon²⁷, neural²⁸ and vasculature²⁹. More recently, microfluidic devices were developed combining the fabrication tools of nanotechnology with the concepts of bioreactors and fluid dynamics to perfuse 3-D cell cultures. Although microfluidic systems have been proposed as a key solution for the manufacture of standardized and cost-effective drug screening, bioreactors have been seen as the ideal concept for large-scale TE graft production³⁰⁻³². However, these concepts were reluctantly embraced and implemented into the industry³³.

Adoption of microfluidic devices by Academia was faster than by Industry^{34,35}. While bioreactors and microfluidics are being developed but slowly assimilated by the pharmaceutical and clinical industries, the use of static cell culture devices, specifically adapted for 3-D cell arrangements, only presented a faster rise over the last years². The market segment for TCPS has seen the devices redesigned to offer either scaffold-based or scaffold-free 3-D cell culture platforms (e.g. 3-D gels or fiber meshes incorporated in the well for 3-D cell adhesion or specific well shapes allowing 3-D cell spheroids formation)³⁶.

In this work, we first review key features of 3-D cell culture systems, traditionally employed in research applications. Subsequently, we describe and critically discuss examples, potentials, and challenges of the current cell culture systems to fabricate TE products. Subsequently, market size and patent landscape of 3-D cell culture devices were discussed, being the competition and value chain in the translational paradigm of TE approaches from basic research to streamlined tissue manufacturing analyzed. Moreover, a survey was performed and results discussed to understand the adoption of such systems. Finally, this paper asserts that market projections for 3-D cell culture systems will not be realized unless academic, government and business interests act in harmony to create standards that accelerate the transition of vital technologies from the lab to the marketplace.

2.3 Significance and current limitations of 3-D cell culture

When applied to *in vitro* modelling, 2-D cell culture has been reported to fail while mimicking the native human tissues. 3-D cell cultures, even when in static conditions, provide several advantages compared to 2-D approaches, such as cellular native morphology, improved differentiation, as well as efficient responses to stimuli, since cells in 3-D have larger surface area being surrounded by external factors. Moreover, gene expression and protein synthesis are affected by the 2-D condition, which also impacts drug metabolism. For instance, a 3-D organotypic cancer model presented 0.34 Pearson coefficient compared to human specimens, while a 2-D cell culture model of the same epithelial cells showed a Pearson coefficient of 0.0 compared to human³⁷. It is further pertinent to highlight the fact that the closest replication of a given human tumor is a tumor in another human, with a Pearson coefficient of 0.45. Additionally, in a different, yet relevant study, gene expression patterns between a human burn and mouse models of burn, trauma, sepsis, and infection, revealed a Pearson's coefficient that ranged from 0.14 to 0.28 of correlation, even lower than the 3-D organotypic model developed with human cells. Even though these are studies with no direct correlation, it is of major importance to emphasize that all these features

contribute for a realistic cell function/phenotype, resulting in an improved significance of 3-D *in vitro* models of human cells.

As a consequence, a scientific paradigmatic shift from 2-D to 3-D cell culture is in progress, as evidenced by the evolution of 3-D cell culture citations in scientific literature (figure 2.1), as well as by the bold statements from world's top scientist released in high impact journals as *Cell*³⁸, *Science*³⁹ or *Nature*^{40,41}:

*"Suddenly, the study of cancer cells in two dimensions seems quaint, if not archaic"*³⁸

*"Relative to 2-D substrates, 3-D-matrix interactions also display enhanced cell biological activities (...) more biologically relevant to living organisms"*³⁹

*"Essential cellular functions that are present in tissues are missed by "petri dish"-based cell cultures (...) it is generally recognized that the flat and hard plastic or glass substrates that are commonly used for cell culture are not representative of the cellular environment found in organisms"*⁴⁰

*"In 10 years, anyone trying to use 2-D analyses to get relevant and novel biological information will find it difficult to get funded."*⁴¹

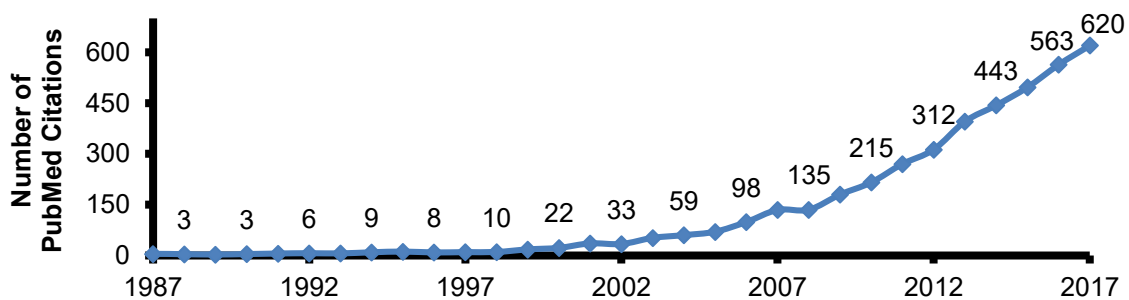


Figure 2.1 | Publications evolution related to 3-D cell culture from 1987 to 2017.

Compilation average of PubMed citations of the terms '3-D cell culture' and two other reports consulted in May 15th, 2017^{42,43}.

An extensive list outlining some of the physiological differences between cells grown in 2-D and 3-D can be found at http://3-CellCulture.com/Just_The_Facts_3-D_vs_2-D_Cell_Culture, which connects each evidence to a publish report.

Interestingly, the current methods to promote the formation of 3-D cell arrangements are based on both static methods, such as hanging drop, forced floating methods by centrifugation, matrices, hydrogels and scaffolds, cell magnetic levitation, and dynamic systems as bioreactor agitation based approaches and microfluidics⁴⁴. But while comparing both static to dynamic conditions, the first presents as main pitfall a limited mass transport that solely depends on passive diffusion within cell aggregates or constructs.

Diffusion is reported to be limited to short distances (100-200 μm) and results in inadequate nutrient supply that is required for maintaining cell viability⁴⁵. In fact, the main clinical challenge that is hampering the industrial/clinical adoption of TE strategies is the poor cell survival at the core of thick grafts⁴⁶. The most evident solution to overcome this problem is the perfusion of such 3-D cell cultures, which is also significant to achieve vascularization⁴⁷, and has been performed in bioreactors⁴⁸ and microfluidic devices⁴⁹. So, while pharmaceutical industry is battling to create the opportunity for 3-D cell culture technologies, it faces some major issues: the low accuracy of predicting complex *in vivo* drug interactions, including toxicity⁵⁰; the poor relevance of 2-D *in vitro* models⁵¹; and the relation cost/time of drug development⁵². Drugs typically take 12 years from the initial discovery stage to licensing approval, and costs estimated at USD 1.52 billion per drug, as previously reported⁵³. About 87% of the molecules entering human clinical trials, after approved during *in vitro* and animal models, are considered false positives⁵⁴. In 2004, Food and Drug Administration (FDA) estimated that, 10% improvement in failure prediction before clinical trials, could save about USD 100 million in development costs per drug⁸. On the other hand, the use of animals in clinical trials is under pressure, being already forbidden for cosmetics testing in the European Union (EU)⁵⁵. Along with the EU, India, Israel, and Norway all have bans in place making it illegal to test cosmetics on animals, and New Zealand's Parliament recently voted unanimously to ban animal testing for cosmetics⁵⁶. Additionally, two examples of the ultimate need in drug discovery and TE are liver⁵⁷ and cancer³⁷ cells 3-D cultures. A monolayer (2-D) of primary hepatocyte becomes undifferentiated and dies within 4 days. Furthermore, it lacks a substantial set of liver-specific functions⁵⁸. In the case of cancer models, it is not a flat tissue in its natural environment and the communication between cancer cells and their cellular and macromolecular environments plays a key role at the gene expression level⁵⁹⁻⁶¹.

The current research for the development of spheroid formation and manipulation and sophisticated bioreactors and microfluidics fabrication makes possible to introduce 3-D cell cultures as opposed to the conventional 2-D TCPS. Recently, magnetic levitation appeared as a tool to achieve spheroid homogenous formation and aggregation⁶². Microfluidic systems are proving to be valuable tools to solve some of these challenges, since these systems allow the use of reduced constructs size, lower culture media volume consumption and shorter trials, leading to faster results⁶³. On the other hand, bioreactors could potentially solve the limitation of engineered grafts viable size. However, many researchers continue using conventional cell culture methods due to unmet needs in basic technology, as compatible detection and readouts, and the currently lack of standard protocols to jump from the conventional 2-D to new 3-D procedures (SI, Survey).

2.4 Market of the 3-D cell culture

RM can potentially reduce the need for long-term care, with latent benefits for the world economy by saving public health agencies money⁶⁴. The global TE and regenerative market reached USD 20.8 billion in 2014 and is expected to perform a compound annual growth rate (CAGR) of 22.3% reaching, in 2019, USD 56.9 billion⁶⁵. The field is considered one of the cornerstones for medical technological evolution and therapeutic solutions⁶⁶ and was key factor on revealing the limitations of the standard cell culture systems. Several of the main topics facing nowadays the need of 3-D cell culture systems are intrinsically connected to RM (figure 2.2).

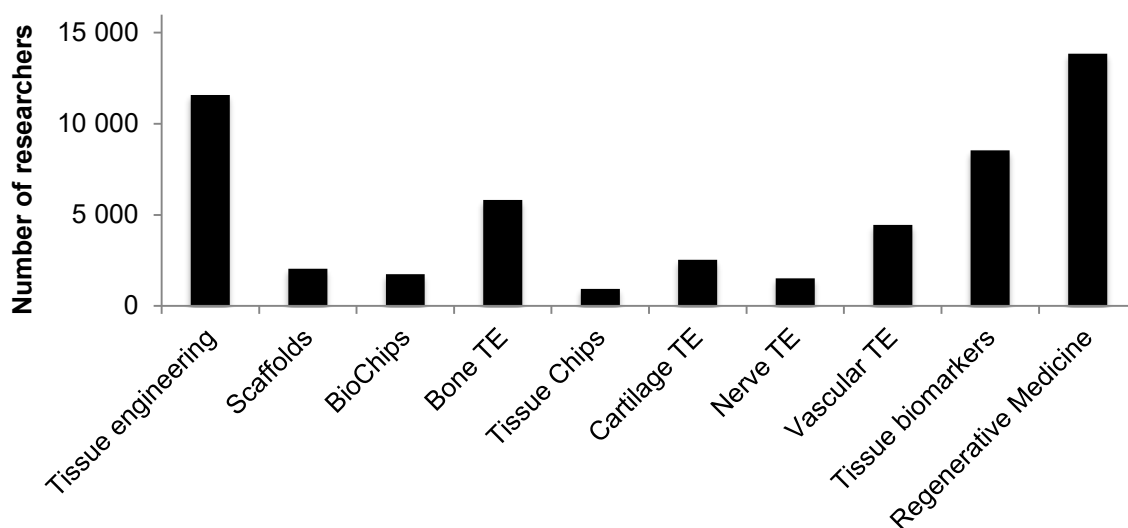


Figure 2.2 | World distribution of researchers per research topic in the field of RM⁶⁷.

The emergent 3-D cell culture systems market niche belongs to the global cell culture market, which is expected to perform a CAGR of 10.7% to reach USD 37.0 billion in 2022⁶⁸. In basic research, the segment of cell-based culture assays and drug R&D was evaluated in USD 13.8 billion in 2017⁶⁹. From this segment, about 17% represents the bioreactors and disposable cell culture systems (combining 2-D and 3-D approaches), which was estimated at USD 2.37 billion in 2017 with a 11.7 % CAGR, even greater than the overall growth of the global cell culture market⁷⁰. The regions of EU and USA represent 58% of this market (figure 2.3)⁷¹.

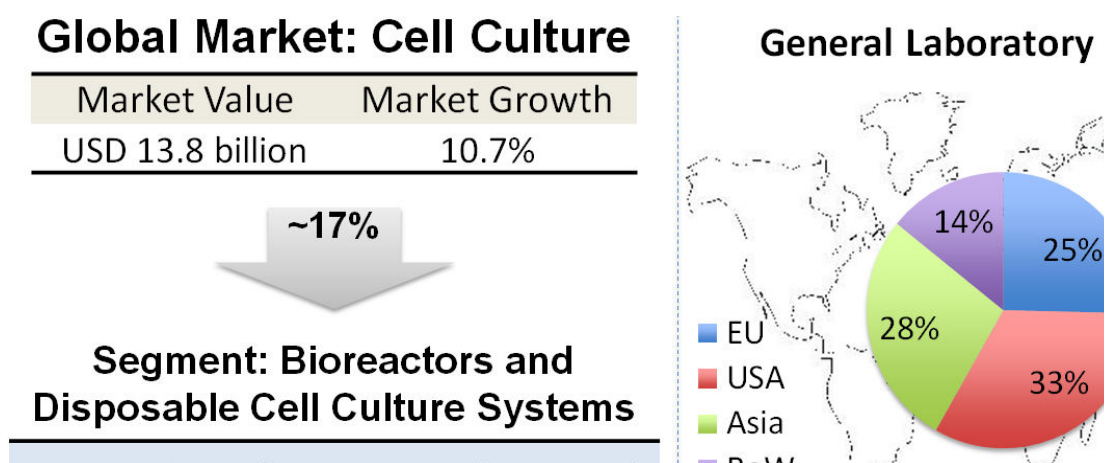


Figure 2.3 | Market identification from the global one to the main niche. Market value and CAGR for the global market of cell culture and for the segment of bioreactors and disposable cell culture systems. General laboratory equipment market distribution represented by percentages.

The specific market niche of 3-D cell culture systems was evaluated at USD 725 million in 2014 and is expected to be an USD 1.69 billion market by 2024, representing an impressive growth of 23.6%^{43,72-76}. As a result of this transition, in the next 10 years, 3-D cell cultures are expected to capture 35.0% of the overall cell culture market⁷³.

The prominent players in the 3-D cell culture market include 3-D Biotek LLC (USA), Becton, Dickinson and Company (USA), Corning Incorporated (USA), Thermo Fisher Scientific (USA), Global Cell Solutions, Inc. (USA), Kuraray Co. Ltd. (Japan), Lonza Group (Switzerland), Merck & Co., Inc. (USA), Sigma Aldrich Corporation (USA), Reinnervate Ltd, a subsidiary of ReproCELL (UK), among others⁷⁶.

An interesting case of a paradigm shift is the 3-D printing technologies market, which is expected to positively influence the future of the 3-D cell culture market. The products of 3-D printing will need to be matured during cell culture, and bioreactors will have an important role on that task⁷⁷. This 20-year-old technology, whose time has finally arrived, has gained the majority of its popularity in the last five years; for instance in 2012, the market for 3-D printed products was estimated as low as USD 777 million⁷⁸, but it is expected to reach about USD 12 billion by 2025, mainly driven by the potential medical uses, according to Lux Research⁷⁹. This expectation means a CAGR of 66% if predictions prevail. Printers alone will be worth USD 3.2 billion, while USD 2 billion will represent formulated materials; USD 7 billion will come from the value of parts produced, which will positively impact medical care and other new needs will rise from there⁷⁹.

2.5 Cell culture systems roadmap for startups

The introduction of a new paradigm creates change, exacerbated by the introduction of 3-D cell culture techniques. The roadmap for the market's adoption of these new technologies has to be analyzed and understood thoroughly before a new strategy can be applied. Since 3-D cell culture devices belong to a market born only 5 to 10 years ago⁸⁰, any innovator trying to bring the new products into the market has higher risk of committing mistakes⁸¹. This explains why paradigm shifts are slower to be taken up by any industry. However, several industries will be influenced by 3-D cell culture systems (table 2.1)^{82,83}. In 10 years, it is expected that a large portion of the 2-D cell culture systems will be replaced by devices designed for 3-D cell culture approaches⁸⁴.

Table 2.1 | Market niches influenced by 3-D cell culture.

Industry	Possible application	Need	Stimulus drivers
Pharma and companies	<ul style="list-style-type: none"> • Drug efficacy screening • Drug toxicity screening • Bioequivalence 	<ul style="list-style-type: none"> • Improved efficacy screening • Improved toxicity screening • Reduced costs and time to market 	<ul style="list-style-type: none"> • Poor correlation of both 2-D and animal experimentation with humans • 88% of false positives from preclinical trials
Cosmetic	<ul style="list-style-type: none"> • Cosmetics efficacy screening • Cosmetic toxicity screening 	<ul style="list-style-type: none"> • Improved efficacy screening • Improved toxicity screening • Reduced costs and time to market • To replace animal models 	<ul style="list-style-type: none"> • Poor correlation of both 2-D and animal experimentation with humans • Animal experimentation starting being forbidden around the World
Biotech	<ul style="list-style-type: none"> • Nutraceuticals development • Bioactive molecules efficacy screening • Bioactive molecules toxicity screening 	<ul style="list-style-type: none"> • Improved production method • Improved efficacy screening • Improved toxicity screening • Decrease media consumption 	<ul style="list-style-type: none"> • Poor correlation of both 2-D and animal experimentation with humans • Low surface area of 2-D systems • Reduced costs and time to market

R&D Centers (Medical field)	• Fundamental research	• Biomimetic systems	• Poor correlation of both 2-D
	• Tissue engineering	• 3-D tissue models	and animal
	• Cancer research	• Biological interfaces	experimentation with
	• Molecular and Cellular biology	development	humans
	• Genetics	• Real time <i>in vitro</i> culture monitoring	• Poor biofunctionality of tissue grafts
		• Functional tissue grafts	• Reduced and limited 3-D tissue models

Analyzing the rank of the top 500 bigger companies in the world (S&P 500), it is possible to observe a profound change: about half century ago, the life-time of a company in this rank was in average of 60 years. Now this time was reduced to 20 years⁸⁵. This reduction means an increased challenge to keep a company competitive, since there is no time in history where so many companies market value overcame the mark of the USD 1 billion⁸⁶. The innovation is nowadays the driving force for this revolution. So, the golden rule for startups is to *innovate or die trying to do it*⁸⁷, and similarly this is the key factor for the success of new 3-D cell culture systems startups to succeed in the prevalent market.

2.5.1 Pricing model

By providing disposable systems to be used in a bioreactor or as a standalone, the new companies can create dependence on the costumer side. This dependence can create a higher revenue flow. However, most of the time a vendor, usually small, will enter the market with a new 3-D cell culture tool only to find out scientists have a little bandwidth to invest their time and money on a promise. However, awareness will bring adoption, and for high-tech systems, costs (budgets), reliability, and efficiency are the main drivers of the pricing model⁸⁸.

After initial market resistance to any new product, which takes many years, some can grow to dominate a market segment that has been unfulfilled by current options⁸⁹. Accordingly, new systems have to be competitively priced, except if they are more effective, and this is still the challenge. If a new product is more expensive than the existing ones, as 3-D cell cultures are when compared to common 2-D systems, time saving, financial advantages and effectiveness represent become key parameters for this new class of products to succeed when entering in the market⁸⁸. Taking this into consideration, product cost by itself is not paramount, but education and training are.

However, the problem exists when scientists compare existing techniques. Today, cell culture systems that include robotic automated medium exchange are more expensive⁶⁸. For example, using 3-D microplates for screening assays can cost from \$8 to \$24 dollars per plate; many scientists will compare that to plates that are priced under \$1 dollar. To survive to this cost differences, the delivered product must fulfill its expected performance, in order to effectively conduct a research with fewer complications and higher success rate⁸⁸. Additionally, money could be saved by avoiding parallel or subsequent processes, and it seems to be the reality of 3-D cell culture compared to 2-D approaches. Considering this, new technology production for 3-D cell culture systems requires a significant higher focus while dealing with marketing strategy.

A good example is the bioreactors systems. These technology is more understandable to the market, but more complex to be implemented, so they have been accepted for longer time but not targeting large segments⁹⁰. Thus, these systems have been selectively adopted by few specific market segments and one of the main factors that could change that, according to 10% of the surveyed costumers, would be the introduction of free trainings by the brand or vendors (SI, Survey). In parallel as mentioned before, price is also one of the main factors and can account for 50.5% of opinions, considering that a price reduction could accelerate the transition from 2-D to 3-D cell culture systems (SI, Survey). Bioreactors have been presenting a wide price range from USD 2,000 for the simplest systems costing over USD 50,000, when it comprises sensors and mechanical stimuli. Although bioreactors can be the key solution for TE standardization, it is expected that static cell culture systems will continue to dominate this market throughout the forecast period⁶⁸.

In general, 3-D cell culture methods still have go over a transition from expensive, messy, laborious, and difficult procedures, to user-friendly, ready-to-use and standard methods at a competitive price. The competition will bring the solution for this technological evolution. Mainly, transparent systems are a potential solution, adapted for assessment by microscopic techniques. Furthermore, it is essential the creation of standard operating procedures for high-throughput approaches and automation, allowing scaling-up of the method. As an example, cell spheroids were the first approaches tested for 3-D cell culture⁹¹. After being dropped by the scientific community for several decades, specific designed high-throughput well plates were recently introduced in the market, as the hanging drop technique⁹² and the super-hydrophobic U-shaped⁹³ well plates. These devices were more successfully adopted by this new market opportunity when compared to scaffolds-dependent approaches, bioreactors and microfluidic devices, showing clearly the need for a smooth transition from 2-D to 3-D cell culture, before the incorporation of materials or even dynamics in these systems. As commonly said, 'the simpler the better'.

However, this desire of making a product simple is the permanent gap which separates investors from the researchers ^{94,95}.

2.5.2 Financing culture systems

The funding of new technology-based startups is a challenging step for the development of a new product during its translation to the market (maybe the most challenging achievement). However, this is a crucial step for most startups since an increase in scale is required for any product with a worldwide application. If the new product is hardware instead of software, this increase in scale requires substantial investment, which in most cases comes from external sources, as it the case of cell culture systems.

Starting a business, 15 years ago, was primarily a technological problem, costing up to USD 5M to create a technology-based company. Today, the value drop to USD 100k in many cases as resources became popular^{96,97}. This drop creates a more volatile market, meaning that big competitors in a market segment may be born now. The same way, an innovative solution for a market need, follows an identical speed trend. This is the main consequence of the startups time revolution. There is no company in the world immune to this movement⁹⁸. Big companies, both billionaire and centenary, are now being defeated by entrepreneurs making their business rise from their garage or living room. Nowadays, the main question about a new market disruption promoted by a newborn company is not anymore an “if” but a “when”^{86,97}. So, what are the problems and the barriers to get funding raise?

Entrepreneurs, startup companies and their investors have a challenge today, especially when factoring in the regulatory obstacles that the 3-D cell culture systems market will face as it meets the clinical space. First, they must decide if the technology in question represents a solid business opportunity for further development and commercialization. Then, they need to secure the technology, usually an in-license with a technology transfer office or an existing patent portfolio owned by a larger company. Intellectual property is one of the common requirements from investors, who avoid risking money in a new solution easily replicable with no patent protection.

2.5.3 Distribution

Distributors have to be considered in order to penetrate other countries and continents. Among the main distributors, such as Sigma-Aldrich and Thermo Scientific, there are many other regional distributors around the world. These are essential to achieve the needed cash flow. However, as a starting challenge, a new tech as the 3-D cell culture systems, offered by startups limited in resources, dealing with large

distributors to reach skeptical initial adoption, results in slow revenue start. Thus, once again, a good marketing strategy is crucial to overcome these barriers.

Large companies with established brand names and distribution channels have a distinct advantage in the research tool and medical device arena. However, new small and medium-sized enterprises (SMEs) are succeeding on a regular basis depending on their technology and its overall cost-performance advantages.

2.5.4 Competition and market share

A startup in this field should pursue a specific, definable, market segment⁹⁹. The challenge faced by startups is the limited resources especially with people and cash¹⁰⁰. Thus, they must focus on the best channel possible; trying to have a multi-channel approach for startups is counter-productive. Once they have penetrated a certain vertical of the value chain, then they can go after another. Along with higher success, comes more funding and startups can take more chances with additional channels after establishing the first one. So, the new company should leverage its technology and products in one key area and large groups and Universities should be targeted first. For example, Contract Research Organizations (CRO) represent a potential key partnership. The growth in the CRO industry has been 9.8% per year and, the top 25 to 50 companies in each market category may account for as much as 70% of the potential business, making it easier to reach the level of targeting customers with a multi-channel tiered strategy if such a partnership would be established¹⁰¹.

2.5.5 Marketing strategy

The marketing strategy should target the industry improvement and researcher awareness using campaigns specifically directed to certain customer segments. The top tier of 20 to 30 customers in each segment should be targeted first^{102,103}. Only a few sales hits in these top tiers will achieve projected forecasts. The new product of a startup should firstly be the only focus, since it aims at an existing, well-defined market, provides the direct contact with the product portfolio and promotes a one-to-one channel. The initial sales will achieve the goals from direct and distributed sales of the product.

Moreover, brand is a major component to be considered and startups should ensure that its marketing communicates, through application notes, the value added to their customers according to 55.8% of the respondents (SI, Survey). First impressions make the difference. Nowadays, even in healthcare related industries, for a new technology to achieve the markets adoption, every single point of contact with the

customer should be considered. Therefore it is important to ensure small aspects to improve brands marketing, such as the logo, website, business cards, presentation and the ability to communicate in a professional and concise way¹⁰⁴. Customers are the base of brand equity and their recommendations have also to be considered. For startups, trustful and clear communications, a strong technology and a promising product must be their brand. A brand is created only after the market opinion has been built and the experience accepted.

Other driving force to raise money is the market approach. Investors are easily convinced by products that were already tested the market itself. The quality of an idea is interesting for an investor depending on market adoption, which is evaluated by the ratio of successful sales per total offers to clients. If a 3-D cell culture technology is not possible to sell before proofing its quality, the prototype should firstly be offered to a reliable group of potential clients to get their opinion. When evidently positive, this transition will result in a larger market adoption creating the needed awareness capturing the attention from entrepreneurs and venture capitalists. Having positive opinions, investors will trust the technology and will become confident on the potential to get profit from it.

2.6 Adoption of 3-D cell culture devices

Key factors influencing the adoption of 3-D cell culture systems by the market are: the number and quality of scientific publications using these technologies in research and medical procedures, measured by their impact factor; the customer or chain of distribution considerations; and the pressure from the regulatory authorities over the pharmaceutical industry to use specific technologies resulting from the increasing number of publications, showing higher reliability when compared to the state-of-the-art.

For some years now, 3-D cell culture has been used by researchers and reported in scientific research (either as a developed result or as a method), with an early adoption by cancer and stem cell research fields. Assuming there are five phases of the growth curve (i.e. innovators, early adopters, early majority, late majority, laggards), it appears that skin is the most mature in the early/late majority phase. This fact is related with the large number of skin-related disorders and to the cosmetic market. Animal models were ban for cosmetics testing, pushing the development of 3-D *in vitro* skin models. Liver is in the early adopters phase in four of the top 15 pharma, with the rest still in the innovators phase⁷⁴. Other applications envision moving from innovators to early adopters in roughly 3 years.

The first adopters are the researchers themselves, from academia, where complexity is the priority over efficiency, creating a risk-directed mindset. On the opposite side of the spectrum is the pharmaceutical

industry, which takes longer to incorporate new systems in the standard operations procedures (SOPs) of their R&D departments but represents the bigger player of the supply chain (figure 2.4). Other potential customers for these new culture systems include CROs, the companies that perform the trials for pharmaceutical industry, biotech, hospitals and private laboratories. The majority of research devices are supplied to these groups.

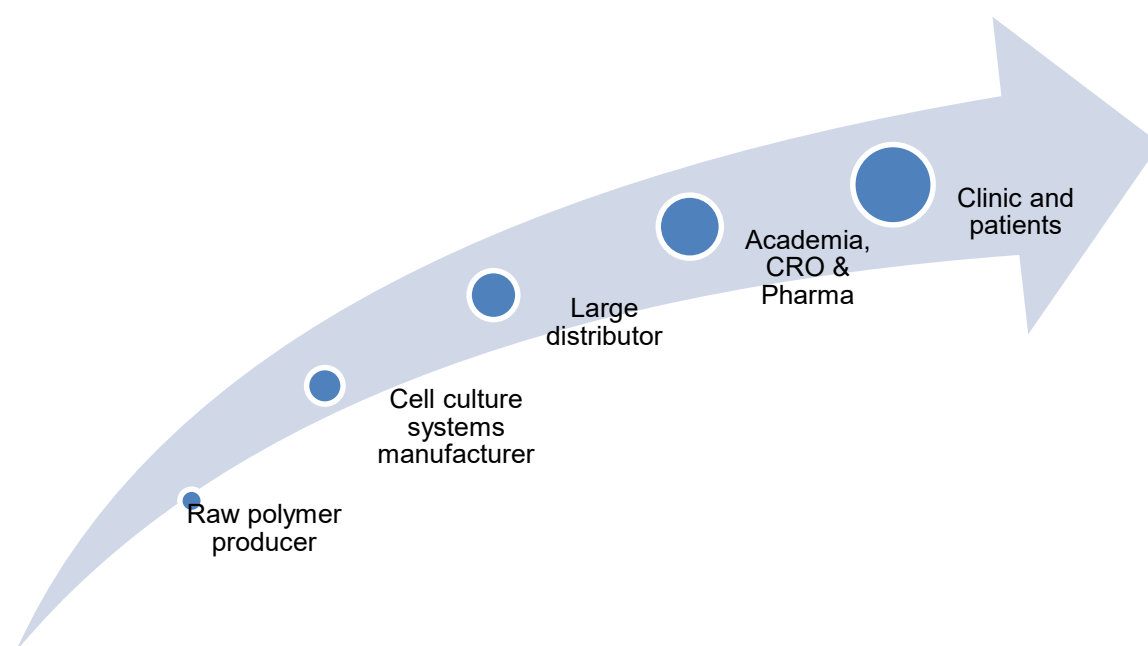


Figure 2.4 | 3-D cell culture systems market value chain considering drug testing as main application. From the raw material producer to the final drug consume in the clinic after drug testing.

3-D cell culture systems adoption is mainly dependent on the creation of industry and regulatory standards, as well as new internal pharmaceutical companies' drug development processes adapted for 3-D cell culture. However, despite the improvement these technologies can bring to the field, their adoption has been slow, since new technologies ask for the standardization of new working protocols. Although 3-D cell culture has demonstrated great potential, critical challenges still need to be overcome and are slowing down the uptake of 3-D cell culture systems. From the technical perspective, cell recovery and analysis are the main limitations that need improvement (57.8%). These systems are yet considered too expensive (23.0%) and still face the validation of adapted SOP which only exist for 2-D cell cultures (49.7%, survey in SI). Moreover, reproducibility between batches of biomimetic scaffolds and the limited ability to scale the constructs up are current concerns¹⁰⁵.

2.7 Patent landscape of 3-D cell culture systems

Licensing a patent or portfolio of patents related to a technology is nowadays one of the main sources of profit from devices developed in academia. 3-D cell culture technologies patent landscape offers an important space to big entities, as well as, for SMEs. The patent landscape related to 3-D cell culture technologies is very open, involving important academic applicants as well as SMEs (figure 2.5). It includes over 2500 patent families and involves over 1000 patent applicants⁴³. The IP landscape and market for 3-D cell culture are currently structuring and should both evolve in the coming years. The IP scene related to 3-D cell culture is dominated by American and Asian applicants. As a consequence, the USA, China, Japan and Korea represent the main countries of filings⁴³. However, Europe seems to represent a new interest for patent assignees, reaching now a patent publication level close to the publication level observed in China⁴³.

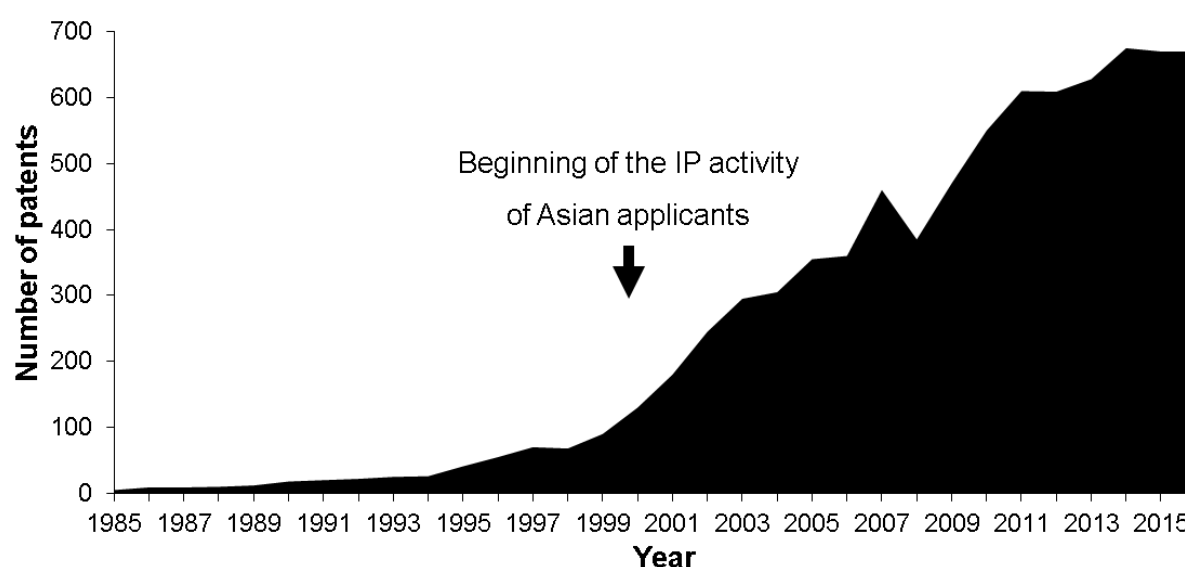


Figure 2.5 | Evolution of the number of patent publications worldwide for 3-D cell culture technologies, for the period of time comprised between 1985 and 2016⁴³.

While Asian applicants focus their IP strategy on a national scale, American applicants tend to develop their technologies in the USA, as well as, in Asia. Europe seems to catch the interest of American applicants, while European applicants do not show a strong patenting activity in the domain of 3-D cell culture ⁴³. A significant number of applicants in 3-D cell culture technologies are academic, and only a few are companies. Interestingly, key technologies are not hold by a few patent applicants and the IP sphere of 3-D cell culture is rather open⁴³.

2.8 Regulatory demands

The transition of a technology from the laboratory to the market does not require FDA approval unless it has some clinical application. In the case of a technology with clinical application, 3-D cell culture are generally considered medical devices, usually classified in the category of clinical chemistry and clinical toxicology devices¹⁰⁶. So, the unnecessary clinical trials make these technologies much less expensive than any product classified as a drug.

As a new brand delivered to the market, the new technology will require registration for CE or UL for European or US coverage, respectively. CE is a mandatory conformity developed for certain products sold within the European Economic Area, since 1985. Underwriters Laboratories (UL) is a regulatory testing, inspection, certification, auditing and validation. The UL Mark is the most accepted certification mark in the United States. Additionally, the China Compulsory Certificate (CCC) Mark indicates compliance with Chinese laws.

In specific scenarios, the introduction of regulation in a particular field can create opportunities for other markets. This is the case of the European prohibition for the use of animals in cosmetics testing. The introduction of such rule has left the cosmetics industry clamoring for 3-D *in vitro* models, consequently increasing the need for cell culture devices. This need will induce pressure over the regulatory entities to simplify the approval and standardization of new technologies and procedures for drug discovery purposes. Furthermore, drug developers and chemical manufacturers also want to use more sophisticated cell-based assays for toxicity testing, since they expect similar regulations in their field in the near future.

Looking into the future, even if the technical challenges described above are overcome; there are still some issues to be solved before shifting the academic know-how into clinical products that benefit society. One of these issues is related with the need to accelerate the standardization and certification of 3-D cell culture devices protocols, because a prolonged delay in this standardization would make market entry even more complicated. Another upcoming issue may be observed in the legal landscape, as the infringement and protection of intellectual properties around 3-D cell culture systems interweave more intensely. Thus, an early standardization and regulation of 3-D cell culture protocols for both, static 3-D cell culture plates and dynamic approaches could be the best preparation to cope with tomorrow's changes.

2.9 Competition and Cases of success

There has been a boom of 3-D formats and the field is willing to adopt and critically evaluate these technologies. There are more than 150 types of 3-D culture systems in the market, including scaffolds, gels, spheroids, organoids, bioreactors, and microchips. Among these, scaffold-based devices are by now the most predominant in the scientific domain, but not in the market side. This predominance is being influenced by the growth of the TE field, which takes advantages from the hybridization of materials engineering with cellular biology.

Several scaffold-based products were introduced in the market. Products range from purely hydrogel systems, as Corning Matrigel®, Trevigen Cultrex® gels, QGel, Lonza/TAP Biosystems RAFT, Celenys Biomimesys®, to fully synthetic scaffold inserts for multiwell plates, as Reinnervate Alvetex Scaffold, 3-D Biotek 3-D Insert, Synthecon NanobioMatrix Scaffolds⁷⁶. Among the scaffold-free approaches, recent advancements in spheroid generation and assaying have led to resurgence in interest in these simple yet powerful tissue models. Spheroids can be generated using a variety of techniques, including hanging-drop plates provided by InSphero or 3-D Biomatrix, low-attachment plates from Scivax, Corning, Thermo Scientific or NOF America, in rotary vessels produced by Synthecon and magnetic levitation developed by n3-D biosciences⁷⁶. For instance, Thermo Fisher Scientific has developed a culture plate called Nunclon Sphera. This system promotes cell self-aggregation to form a 3-D sphere in the well¹⁰⁷.

Market players in this segment range anywhere from electronic to glassware players and huge multinationals to small regional players. This greatly fragments the market, leaving fertile ground for mergers and acquisitions. A recent example includes the Corning acquisition of the majority of BD's (Becton Dickinson) Discovery Labware business (valued at USD 720 million) in November 2012⁷¹. Despite the competition, this market niche has existed for the last 10 years and is not dominated by any other company. About 17 different players are established with substantial market share (table 2.2). However, there are major players in the field of scientific products distribution, such as the already mentioned Thermo Fisher, Sigma, InSphero among others, who are starting to fight or absorb some of the new born companies/technologies.

As the most successful case, the Swiss company InSphero is using the hanging-drop technique as the basis for a TCPS made of the conventional polystyrene but with redesigned wells. Unlike typical round-bottomed wells, InSphero's wells have a V-shaped lower part. At its very bottom, the well is flat. In a hanging drop of culture medium, cells settle and grow as spheroids in a way that enables microscopy. To arrive at the design, the initial concept was generated from pipette tips. Cutting them off, drops injected from the top of the pipette were hanged at the bottom. In 2011, InSphero began a partnership with

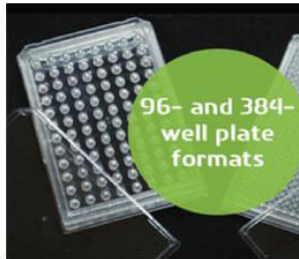
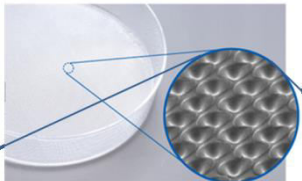
PerkinElmer allowing the plates to be incorporated into PerkinElmer's automated screening instruments, which are nowadays one of the most used by drug development companies. This partnership began as a marketing deal and has generated collaboration on assay development, creating plates that hold pre-formed spheroids of liver for drug toxicity assays¹⁰⁷. The success of the described case is mainly related with the ability to standardize the method, which should serve as a model for other companies in the market.

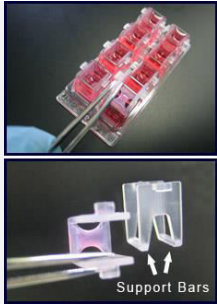
Another academic spin-off that is developing 3-D cell culture is n3-D Biosciences in Houston. This company developed a technology with a working principle totally different from the mentioned above, since the n3-D technology seeds tissue by levitating cells and bringing them together under a magnetic field¹⁰⁷. The use of an external force (magnetic) for aggregation makes the difference when comparing to the previously mentioned technologies, showing spheroids improved homogenization and controlled combination⁶².

From the 3-D cell culture products generated by bioprinting, Organovo is undoubtedly the most known name in the field, but multiple other companies are competing for their space, including Aspect Biosystems, 3-Dynamic Systems, regenHu, n3-D Biosciences, OxSyBio, and Cyfuse Biomedical⁷⁶.

Microfluidics and organs-on-chip devices constitute a different approach which can use one of the techniques mentioned above incorporated in a microscale device, that can precisely manipulate cells, enable electrical and mechanical stimulation and multi-tissue fluidic interconnection, with a faster operation and minimal reagent consumption. By now, only few companies have commercialized organ-on-chip systems. Both EMD Millipore's CellAsic and Mimetas' Organoplate platforms incorporate microfluidic elements into multiwell-plate architecture. Emulate Bio, TissUse GmbH, and InSphero use microfluidic chips to model different tissue systems⁷⁶.

Table 2.2 | The main market players for 3-D disposable cell culture systems.

COMPANY	MAIN PRODUCT	MARKET ENTRY YEAR	WEBSITE/ PAPER REFERENCE	PICTURE
3-D BIOMATRIX	Hanging-drop plates for cell spheroids formation	2010	Https://3-Dbiomatrix.com/	
ORGANOVO	Preclinical <i>in vitro</i> testing services; Bioprints and markets human tissues	2007	Http://organovo.com/	
QGEL	Synthetic extracellular matrix in a 96 well plate	2009	Http://www.qgelbio.com/	
REPROCELL	lps cells and alvetex™ matrix in 3-D well plates	2016	Http://reinnervate.com/	
NANOGAIA	3-d cell spheroids for cancer screening	2010	Http://www.nanogaia.com/	
INSPHERO	3-d microtissues for toxicity and efficacy testing	2009	Https://www.insphero.com/	

INVIVOSCIENCES	Drug screening services and 3-d cell culture inserts	2001	Http://invivosciences.com/	
3-D BIOTEK	3-d cell culture devices	2007	Http://3-Dbiotek.com/	
N3-D BIOSCIENCES	Cell spheroids for biomedical research produced by magnetic 3-d bioprinting	2008	Http://www.n3-Dbio.com/	

ReproCELL Europe Ltd, a new company formed in 2016, is a combination of two companies acquired by ReproCELL in recent years – Reinnervate and Biopta.

In the bioreactors segment, GE Healthcare Life Sciences and Synthecon are the dominant companies in the market share. These are generally dependent on other companies to design and develop their technologies. Their strengths are based on their reputations, current market positions, and their established distribution channels among researchers using their products. Their weakness is that they are generally viewed as being less innovative, particularly in the last years. This makes them vulnerable to a new, improved market disruptor.

The market is extremely sensitive to changes in end-user segments. For instances, pharmaceuticals (which is the major revenue-contributing segment) is focusing on cutting costs and enhancing productivity⁷¹. According to our direct contacts with the Portuguese pharmaceutical companies Bial and Bluepharma, the main need in the process of drug development is not only to have 3-D reliable models, but specifically interfaces able to mimic the native tissue barriers that drugs have to overcome in order to be effective. According to this need, over last years some bioreactors have been patented. A list of some of the main bioreactors patents addressing the needs of TE is listed in table 2.3.

Table 2.3 | Most significant patented bioreactors for TE applications.

BIOREACTOR	SHORT DESCRIPTION	PRIORITY DATE	PATENT
AUTOMATED TE SYSTEM	<ul style="list-style-type: none"> - automated cell culture - simple configuration system - nor adapted for co-cultures of interfaces 	April 8th, 2002	Us2006141623 a1
ROTATIONAL BIOREACTOR IN TWO AXIS	<ul style="list-style-type: none"> - sensorized for one or more metabolites within the chamber - chamber drive capable of rotating the chamber at a first speed about a first axis and a second speed about a second axis - one chamber for all samples, preventing co-cultures with two or more different media 	February 6th, 2003	Us7604987 b2
MECHANICAL STIMULATION BIOREACTOR	<ul style="list-style-type: none"> - electrical stimulation - monitored through one or more optical ports - mechanical loadings: fluid shear, hydrostatic pressure, matrix compression, and rotation - does not present a double-chamber for bilayered structures 	July 20th, 2005	Wo2007/012071
BI-DIRECTIONALLY OSCILLATING BIOREACTOR	<ul style="list-style-type: none"> - bi-directionally oscillating cell culture for homogenization - gas permeable, closed-loop chamber - no adapted for co-cultures 	February 9th, 2007	Wo2008/098165
ANABOLIC MECHANICAL STIMULI BIOREACTOR	<ul style="list-style-type: none"> - mechanical anabolic stimulus for cytomchanics - includes an electro-active polymer actuator - comprises a deformable, water impermeable biocompatible substrate/matrix 	May 7th, 2007	Ep1990402
MULTI-CHAMBER BIOREACTOR WITH BIDIRECTIONAL PERFUSION	<ul style="list-style-type: none"> - multi-chamber bioreactor - bi-directional perfusion - no rotational movements to improve cell homogenization 	August 6th, 2008	Ep2151491 a3
MULTI-CULTURE BIOREACTOR	<ul style="list-style-type: none"> - multi-culture bioreactor tension 	October 6th, 2008	Wo2010/040699

	<ul style="list-style-type: none"> - physically isolated environments but allowing biochemical communication between cells - does not allow obtaining integrated bi- or multi-layered cultured constructs 		
BI-DIRECTIONAL CONTINUOUS PERFUSION BIOREACTOR BONE TE BIOREACTOR	<ul style="list-style-type: none"> - bi-directional continuous perfusion bioreactor for 3-d constructs - mechanical stimuli by flow perfusion shear forces using different pressure gradients 	December 4th, 2008	Wo2010/064943
	<ul style="list-style-type: none"> - maturation of human bone grafts - perfusion of large porous scaffolds - composed by a unique chamber 	March 3rd, 2009	Us2012035742 a1
HIGH-THROUGHPUT SENSORIZED HYDRODYNAMIC BIOREACTOR	<ul style="list-style-type: none"> - high-throughput sensorized - hydrodynamic pressure - shear stress stimuli 	March 30th, 2009	Ep2236597 a1
DYNAMIC LOAD AND RECIRCULATING PERFUSION BIOREACTOR	<ul style="list-style-type: none"> - dynamic load - recirculating perfusion - not adapted for co-cultures 	August 10th, 2010	Cn101899393 a
PULSED FLOW BIOREACTOR FOR BONE AND CARTILAGE TE	<ul style="list-style-type: none"> - one culture chamber, the inner walls of which form a vertical duct to reduce culture media consumption and construct deterioration - does not describe dual-chambers, rotational movements or multi-layered structure design 	February 4th, 2011	Us2014030762 a1
HUMAN BODY LIGAMENT TE BIOREACTOR	<ul style="list-style-type: none"> - mechanical /compression - two-way torsional multidimensional stress loading - not designed for multi-layered constructs 	May 27th, 2011	Cn102796664
PROTOTYPED SET OF BIOREACTOR AND 3-D MATRIX	<ul style="list-style-type: none"> - internal matrices and external chamber are simultaneously manufactured, preferably through a rapid prototyping process - simplified and fast set-up process - not adapted to perform mechanical stimuli nor real time monitoring 	January 5th, 2012	Wo2013103306 a1

TENDON AND TENDON SHEATH CO-CULTURE BIOREACTOR	- tendon and tendon sheath interface culture units - pneumatic tendon sliding mechanism	May 16th, 2013	Cn203269948 u
ROTATIONAL DUAL CHAMBER BIOREACTOR	- dynamic rotational platform for homogenization of cell seeding in two axis - dual-chambers adapted for multi-layered cell co-cultures	March 13th, 2013	Wo2014141136 a1
MULTI-CHAMBERS BIOREACTOR	- dual-chambers for interfaces and co-cultures - chambers adaptable to commercial 6-well tcps for static or pulsed culture media	September 18th, 2014	Wo2016042533 a1

For instances, the patent WO2014141136 A1 refers to a rotational dual-chamber bioreactor, which is able to homogenize 3-D tissue, avoiding cell seeding, taking advantage of rotational movements. Furthermore, the system promotes the creation of interfaces by flowing two different culture media through the same construct. Another patent (WO2016042533 A1) refers to a multi-chamber bioreactor adaptable to standard 6-well culture plates, describing a culture chamber divided in multiple compartments able to create such interfaces (figure 2.6).

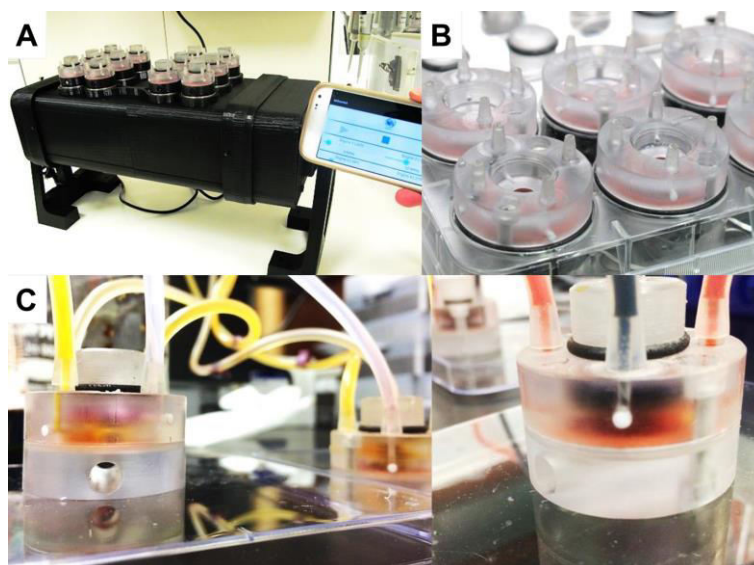


Figure 2.6 | Dynamic dual-chamber bioreactor. A. Rotational dual-chamber bioreactor; B. Dual-chamber adaptable to traditional 6-well plates; C. Interfaced flow created by two perfused dyes.

Most of these technologies have been developed by research groups in academia, generating in much cases, the creation of startups. A boom of startups emerged over the last 10 years, not only in 3-D cell culture systems, but in a wide range of technology-based developments. However, start-ups should not be considered threatening competition, but an opportunity. While start-ups are taking advantage of the current market speed, smooth bureaucracy and bold entrepreneurs, the big companies have their market established, distribution channels, costumer portfolios and expertise. The natural movement is their fusion and not their competition. Summing up, big companies can hire startups, uptake their innovative solutions for their main problems, accelerate creative startups and get profits as: hiring their motivated entrepreneurs; creating acceleration programs as a corporate venture; making fusions or acquiring startups in their exponential phase. Fortunately, if competition breeds innovation, we all stand to benefit.

2.10 Survey analysis

The surveyed population (n=200) is mainly composed by researchers working in academia (93.9%), among them 50.8% are PhD students. 57.9% of the surveyed population is in between 28 and 37 years old and the majority is working in Europe (82.1%).

Positive indicators of future adoption and growth rates, which are dependent on future delineation of standard practices where specific supplier technologies are mapped to specific applications, offer hope of a coordinated landscape with less market friction than the last 10 years. This includes the co-existence of 3-D with 2-D in most research application areas, partially due to change dynamics, as well as researchers' perception of value in comparing 3-D and 2-D results for basic insights. A big jump in the 3-D cell culture market can happen driven by the introduction of assay kits containing all the necessary reagents and specific protocols packaged for laboratory use. Furthermore, free training provided by the vendors, as well as the creation of opportunities to collaborate can entice the consumers, increasing their adoption for more complex 3-D culture systems. The majority of the surveyed population (47.9%) is nowadays using 3-D cell culture systems and 17.6% is planning to start using this new 3-D concept. Among these users, 66.1% started by using 3-D cell culture static plates, 21.0% bioreactors and 12.9% microfluidic devices. The main drawbacks observed in the existing system were considered to be the lack of optimized protocols for 3-D cell culture (50.3%), limitations on experiment monitoring and sample visualization (49.2%) and technical problems as cell recovery and analysis, which are easier in 2-D cell culture plates (57.7%). Solving these problems will potentially impact the 3-D cell culture devices market CAGR.

2.11 Concluding remarks and future trends

The evolution of TE evidenced the limitation of 2-D cell culture systems. Although stem-cell research and cell culture technologies are quickly advancing, viable cell therapies are years away from the market. However, given the impact that RM can have in our life quality, it is easy to imagine how those less familiar with the technology and industry can be frustrated or impatient with its progress. This deceleration is attributable to three main reasons. First, the expectations have become too high for the field's products to reach the clinic due to its fast evolution in the 90's. Secondly, regulatory directives increased the demand for the development of reliable products. Thirdly, the cell culture systems did not follow the evolution of these fields and their requirements. Due to this higher level of expectation and demand, the 2-D-based biological tests performed by the RM field, over the last 30 years, are no longer considered scientifically enough to be prescribe to a patient. This idea started spreading to all drug testing system, in which the pharmaceutical industry used 2-D cell cultures before pre- and clinical trials. The development of 3-D scaffolds was the first step to understand the importance of solving the 2-D cell culture issues by the adoption of 3-D systems. Anticipating this paradigm shift, many companies, offering systems for 3-D cell culture and toxicity screening, were created over the last decade. However, just recently this market is being recognized by academia.

In any case, the replacement of 2-D by 3-D cell culture systems in a large scale can be considered a certainty, but will take time. First, 3-D cell culture will be done by disposable devices, and later bioreactors and microfluidics will do the job by introducing dynamics. These two systems will be used for automated graft production and high-throughput screening, respectively. 3-D *in vitro* models will gain popularity along microfluidics, which can be packaged as a product and sold directly or used for services. With advanced 3-D cell culture systems and bioreactors, together with the evolution of the 3-D printing state-of-the-art, it is expected that RM and TE will experience a new exponential growth phase. Currently, as previously mentioned, the field is no longer only focused on cell therapy and tissue substitutes. It also focusses on 3-D *in vitro* models, which have been applied in drug development. These models are expected to have a huge impact in a near future of the pharmaceutical industry and, consequently, on the 3-D cell culture systems market segment. However, standardization, automation and regulation are urgent.

As a missing piece in the equation for success, communication between scientists and investors should be adapted ceasing the existing knowledge gap between them, enabling startups to raise capital.

2.12 Research Methodology

Most of the data presented in this report has been gathered via secondary and primary research. For this project, as a primary source of information we conducted interviews with experts in the area (academia, industry, medical practice and other associations) to solicit their opinions on emerging market trends. Additionally, a survey was delivered (SI). This is primarily useful for us to draw out our own opinion on how the market will evolve across different regions and technology segments.

The secondary sources of information included annual reports, investor presentations, regulatory documents, industry databases, news releases from company websites, government policy documents, and industry analysts' views. While the focus has been on forecasting the market over the coming years, the report also provides our independent view on various technological and non-commercial trends emerging in the industry. This opinion is solely based on our knowledge, research experience and understanding of the relevant market gathered from various secondary and primary sources of information. When possible, the available data has been checked for accuracy from multiple sources of information.

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2.15 Supplementary Information

Survey (sample size = 200, online)

Table S2.1 | Results obtained from the survey about 3-D cell culture systems.

This survey is directed to everyone performing work related to research applied to the medical field. We want to understand the demanding of 3-D cell culture systems

* Required

Question	Result (%)
<u>Where do you develop your work?</u>	
Academia	93.9
Pharmaceutical company	3.5
CRO company	2.5
<u>What is your role?</u>	
Master student	5.5
PhD student	50.8
Researcher	20.2
Supervisor/PI	5.0
Technician	3.0
Other:	5.5

What is your main field of work/research? *

Fundamental biology	13.1
Bioengineering/Biomedical	62.8
Biotechnology	2.5
Drug discovery/development	2.5
Chemistry applied to medical field	3.5
Nanotechnology applied to medical field	6.0
I am NOT working in a field involving cell culture AND/OR I am NOT aware of 3-D cell culture specificities	4.5
I am NOT working in a field involving cell culture, BUT I AM aware of the 3-D cell culture specificities	5.0

Your opinion about 3-D cell culture (*to analyse your opinion about 3-D cell culture*)

In your opinion, are 3-D cell culture systems important for cell culture in general? *

Yes	95.8
No	0.5
Possibly	3.7

Opinion about 3-D cell culture (*to understand the general advantages and disadvantages seen in 3-D cell culture*)

What applications do you think that 3-D cell culture would have most impact? (check all that apply) *

Stem cell expansion	45.5
<i>In vitro</i> models for drug screening	77.8
Tissue engineering	85.2
My experience or knowledge doesn't allow me to answer this question	3.2

What advantages does one gain by using 3-D cell culture? (check all that apply) *

Cells grow more naturally	63.0
Cell screening will see more accurate 'hits' due to natural cell environment	75.7
Cells will grow more efficiently using less media to reach a population	14.8
Potential to replace animal tests	56.1
My experience or knowledge doesn't allow me to answer this question	4.2

What disadvantages do you see with 3-D cell culture systems? (check all that apply) *

Difficult to use	20.6
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New protocols have to be developed	50.3
Too expensive	22.8
Can't compare to 2-D cell culture experiments	10.6
Technical problems as cell recovery and analysis, which are easier in 2-D systems	57.7
Limitations on experiment monitoring and sample visualization	49.2
My experience or knowledge doesn't allow me to answer this question	9.5
Other:	2.1

Do you have plans on using 3-D cell culture in the future?

Yes, we are currently using them and will continue to do so	47.9
We do have plans to evaluate them in the near future	17.6
No plans to use them at this time	34.6

Choosing a 3-D cell culture system *(to understand which technology is faster reaching the market)*

What kind of 3-D cell culture systems did you start by using or plan to use firstly? *

3-D cell culture static plates/devices	66.1
Bioreactors	21.0
Microfluidic devices	12.9

What is the main reason for your previous answer? (check all that apply) *

Cheaper	25.0
More user-friendly/simple	26.6
More realistic	35.5
More versatile	29.8
More efficient	27.4

What kind of 3-D cell culture systems have you used in your research? (check all that apply) *

3-D cell culture static plates/devices	70.2
Bioreactors	25.0
Microfluidic devices	18.5
I have no experience with 3-D cell culture system yet	18.5

Cell culture systems in the future *(to understand the future market steps)*

What would have to happen for most labs to move from 2-D to 3-D cell culture systems? (check all that apply) *

The price needs to come down	50.5
More and clearer evidence of the benefits of use	52.1
Easier cell recover/isolation at the end of culture period	59.5
Other:	3.7

What would entice a researcher to try 3-D cell culture systems in their lab? (check all that apply) *

Special pricing promotion	31.1
Collaboration opportunity with vendor	39.5
An application note that related directly to their work	55.8
A 100% money-back guarantee	6.8
Free training on these techniques provided by the brand/vendor	10

About you to finish (*for analysis of the population*)

How old are you?

18-27	36.3
28-37	57.9
>37	5.8

Where are you working?

Europe	82.1
America	15.8
Africa	0.0
Asia	2.6
Oceania	0.0

SECTION II

EXPERIMENTAL PART

CHAPTER 3

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1 Abstract

This chapter aims at describing and justifying the selection of the materials and strategies followed to achieve the main objectives proposed by this doctoral project. Moreover, herein it is intended to describe in detail the experimental procedures used throughout the work presented in this thesis. Despite all the following chapters have a “materials and methods” sub-section, herein these are presented in a general perspective, while providing detailed information in all the specific steps followed for each work.

3.2 Materials

TE offers the possibility of combining different materials, with the desired characteristics (extra cellular matrix (ECM)-like). In this context, multilayer structures have been developed targeting OCD and cells self-assembling or patterned constructs have been applied for neocortex *in vitro* models. To support the *in vitro* growth of 3-D complex tissues the mimicry of GAG and fibrous proteins that make up the ECM are of important consideration to design advanced strategies. So, a reliable *in vitro* model has to involve biomimetic components present in a specific tissue unit to constitute a major tool towards the progress of a therapeutic field. To achieve such a challenging need, we believe mainly blended GG, HA, and gelatin, as analogues of GAG, apatite (present in bone), and collagen, respectively, the main components of ECM. Such heterogeneous materials provide tunable solutions to address different goals. Furthermore, incorporating physical cues, such as microparticles, allows adding functional patterns into the 3-D environment. Therefore, this thesis explored three different types of biomaterials: GG, methacrylated gelatin (GelMA), and HAp.

3.2.1 Gellan Gum

GG is a water-soluble exo-polysaccharide (figure 3.1). The GG-producing microorganism was firstly isolated from the Elodea plant tissue which is a natural pond in Pennsylvania, USA¹, by the former Kelco Division of Merck & Company, Inc., in 1978. Further studies revealed that the bacteria was a new strain of the species *Pseudomonas*, and hence termed as *Pseudomonas elodea*².

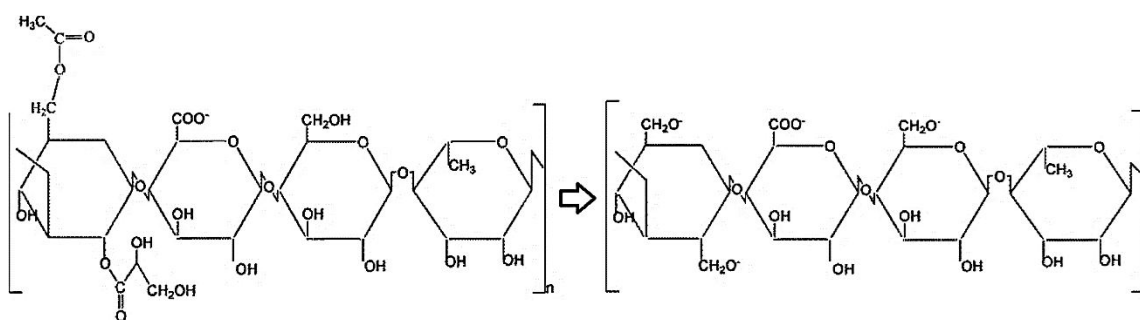


Figure 3.1 | Chemical structure of native (left) and deacetylated (right) GG. The molecular structure of GG consists of a linear repeating tetrasaccharide unit composed of glucose, rhamnose and glucuronic acid (source: www.kelco.com).

In 1992, 14 years after its discovery, the FDA approved GG for use as a food additive³. Specifications for GG were prepared at the 46th Joint Expert Committee on Food Additives in 1996³. These specifications defined GG as “high molecular mass polysaccharide gum produced by a pure culture fermentation of carbohydrates by *Pseudomonas elodea*, purified by recovery with isopropyl alcohol, dried, and milled. The high molecular mass polysaccharide is principally composed of tetracyclic repeating unit of one rhamnose, one glucuronic acid, and two glucose units and is substituted with acyl group at the O-glycosidically-linked esters”.

So far, most of the studies have been focused on the application of GG as a food ingredient^{4,5} and as an additive for various pharmaceutical products⁶. Recently, GG started to be explored as an interesting polymer for biomedical applications, since it can form ionic-crosslinked hydrogels with thermo-responsive behaviour suitable as injectable formulations that jellify *in situ* with a sol-gel transition close to body temperature^{7,8}. Previously, our group explored GG for varied TE applications, such as cartilage^{9,7}, skin vascularization¹⁰ and re-epithelialization¹¹, intervertebral disc regeneration¹², and osteochondral (OC) TE¹³. These different approaches have been proving that GG-based hydrogels are a very promising structures for diversified applications in TE.

3.2.2 Gelatin

Gelatin is a fibrous protein derived from collagen. This polymer has been largely used to build hydrogels¹⁴ or scaffolds¹⁵. Having been used for long time, gelatin has been applied to engineer different tissues, from cartilage¹⁶ and bone¹⁷ to cardiac tissue¹⁸ and muscle fibers¹⁹.

When compared to collagen, gelatin has the advantage of being inexpensive. Furthermore, gelatin is originally hydrolysed collagen (figure 3.2), which reduces its structural variations from different sources²⁰

by breaking its tertiary structure. Furthermore, gelatin retains collagen natural cell binding motifs²¹, such as the tripeptide Arg-Gly-Asp (RGD), as well as collagenase and other matrix metalloproteinase (MMP) recognition sites²². This later feature allows for an improved biodegradability promoting remodulation when implanted *in vivo*²³. Compared to collagen, gelatin has increased solubility and lower antigenicity^{24,25}, allowing for easier processing methodologies and lower immune reactions when implanted.

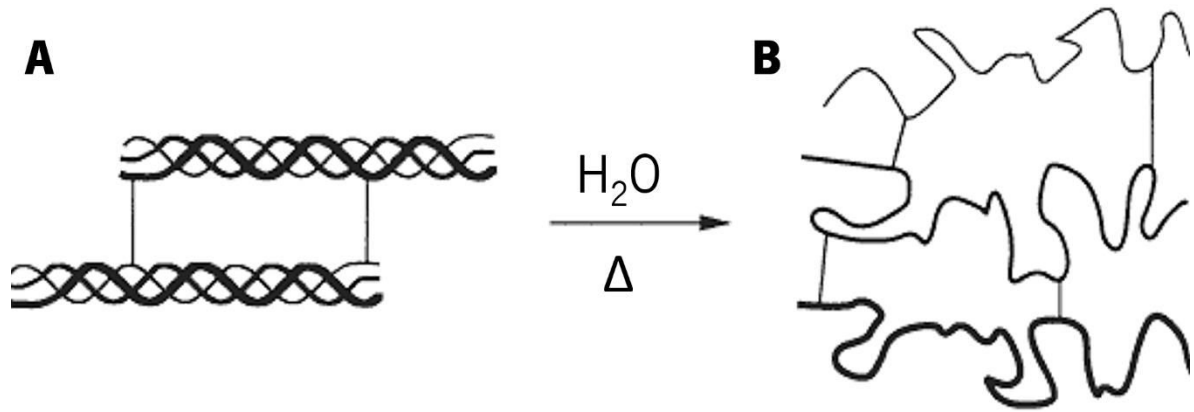


Figure 3.2 | Gelatin as the result of collagen hydrolysis. (A) Represented collagen, and (B) represented gelatin. (source: <http://holowiki.org/wiki/File:Gelatin1.gif>)

A gelatin solution has, on its own, the unique property of gelation at low temperatures^{26,27}. In addition, several chemical reactions have been applied to covalently crosslink gelatin²⁸⁻³¹. Among these reactions, the modification with methacryloyl groups introduced the photo-crosslinking ability which is started by a photo-initiator under exposure to light²⁶. As main advantages, this crosslinking can occur at room temperature (RT), neutral pH, and aqueous environments. Moreover, the chemistry by methacrylic anhydride generally involves less than 5 % of the amino acid residues in gelatin molar ratio²⁶. Specifically, the RGD motifs are not affected/modified by the reaction of gelatin with methacrylic anhydride. Thus, keeping most of the RGD motifs and MMP-degradable sites intact^{32,33}, good cell adhesion properties and biodegradability of the GelMA is granted³⁴.

Taking profit from these properties, GelMA has been used to control temporal and spatial patterns in hydrogels¹⁴. By means of photo-crosslinking. Microfabrication of GelMA hydrogels was achieved at the micro-scale^{35,36} with potential for many different applications ranging from TE, to drug and gene delivery.

3.2.3 Hydroxyapatite microparticles

HA is a naturally occurring mineral form of calcium apatite with the formula $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$. This mineral constitutes up to 50 % in volume and 70 % in weight of human solid phase within the skeletal and dental

tissues³⁷. The stoichiometric apatite $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ has a calcium phosphate (CaP) ratio of 10:6 normally expressed as 1.67.

CaP-based materials have been increasingly used in the past 40 years as bone grafts in the dental³⁸ and orthopedic fields³⁹ because of their biocompatibility³⁷ and bioactivity⁴⁰. Moreover, CaP are also interesting for many biomedical applications, such as gene⁴¹ and drug delivery⁴², but also for broad applications including nutrition⁴³ and photonics materials⁴⁴, due to their biocompatibility, crystalline phase, and density. Applying different fabrication methods have been giving rise to diversified particle shapes and sizes, with diameters varying from 10 nm to 10 mm. Among them, nanoparticles, nanodots, or nanopowder are the most predominant particles due to their spherical high surface area^{45,46} (see figure 3.3). Such particles have been essentially used for the fabrication of pastes and cements for minimally-invasive approaches³⁹.

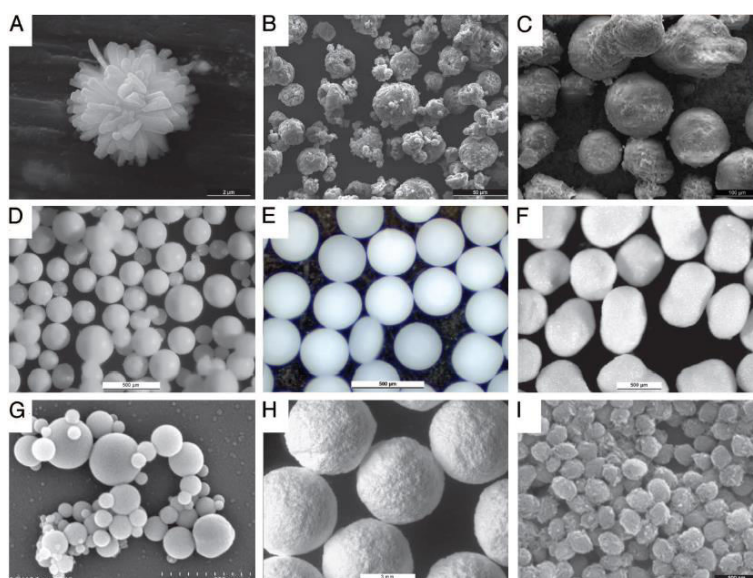


Figure 3.3 | Spherical CaP particles obtained using different methods. (A) Precipitation, dicalcium phosphate (scale bar: 2 μm), (B) spray drying; monocalcium phosphate monohydrate (50 μm), (C) freeze granulation; calcium sulfate dihydrate (100 μm), (D) emulsification; dicalcium phosphate dehydrate (500 μm), (E) drip casting; β -tricalcium phosphate (1,000 μm), (F) extrusion-spheronization; β -tricalcium phosphate (500 μm), (G) suspension plasma spraying; mixture of high-temperature CaP (300 nm), (H) 3-D printing; α -tricalcium phosphate-dicalcium phosphate mixtures (3,000 μm); (I) precipitation in ethylene glycol; β -tricalcium phosphate (500 nm)³⁹.

The production methods can be mediated through different phases, such as solutions, slurries, powders, dispersion media (gas, liquid, or solid), dispersion tools (nozzle, propeller, sieve, mould). Furthermore, at low-temperature are formed crystals such as monetite, brushite or octacalcium phosphate, while at high-

temperature are formed HA, β -tricalcium phosphate or tetracalcium phosphate, can be produced^{39,47}. Specifically, HA can be synthesized via several methods such as wet chemical deposition, biomimetic deposition, sol-gel route (wet-chemical precipitation) or electrodeposition⁴⁸. Yagai and Aoki proposed that HA nanocrystal suspension can be prepared by a wet chemical precipitation reaction (eq. 3.1)⁴⁹:



In TE field, HA has been compared to other CaP doped forms for the healing process in bone defects in non-ovariectomised and ovariectomised rats⁵⁰. The overall bone formation was comparable between HA and strontium CaP, but with topological differences. The bone area was higher in the defect center of the HA group. The biocompatible properties and osteo-inductive capability of CaP have been largely studied⁵¹ and is of interest for bone and OC TE.

3.3 Methods

3.3.1 Materials preparation/modification

Gellan gum methacrylation

Low acyl GG (LAGG) was used in Chapters 4 and 8. GG was methacrylated to form methacrylated GG (MAGG) in Chapter 5. LAGG (Gelzan™, molecular weight: 1,000,000) and methacrylic anhydride were purchased from Sigma-Aldrich.

MAGG was synthesized by reacting GG with methacrylic anhydride. GG (4g) was dissolved in 400 mL of distilled water at 70°C for 1 h, and 8 mL of methacrylic anhydride was added at 50°C. The amount of methacrylic anhydride was calculated to exceed the GG 20x (20 mol of methacrylic anhydride to 1 mol of GG).

The reaction was continued for 6h at 50°C controlling the pH at 8 by adding 5N NaOH solution. It is important to keep the temperature of the reaction constant and as low as possible to keep the reaction's kinetic slow. Since the sol-gel transition of GG is around 42 °C, 50 °C was the temperature selected to perform this modification. Since the reaction kinetic decreases over time, the NaOH flow rate was continuously adjusted to keep the pH constant over 6h.

After 6 h, the reaction was stopped with cold ethanol at -20 °C. The mix was centrifuged at 2000 rotations per minute (rpm) for 15 min and the supernatant discarded. This process was repeated twice centrifuging

for 5 min.

The pellet was dissolved in 400 mL of distilled water, corresponding to the initial volume, and the solution was transferred to dialysis tubing (molecular weight cutoff: 12–14 kDa, Spectrum Labs, Inc.) and dialyzed in distilled water for 4 days, changing water twice a day. The solution was lyophilized to obtain pure MAGG, and it was stored at $-80\text{ }^{\circ}\text{C}$ until further use.

Gelatin methacrylation

Gelatin was modified by methacrylation in the Chapters 5 and 8. A solution of gelatin (from porcine skin, Type A, 300 mw, Sigma) at 10 % (w/v) was prepared by dissolving 2g in 20 mL of phosphate-buffered saline (PBS), at $50\text{ }^{\circ}\text{C}$ for 1 h and covered to avoid evaporation. 0.8 ml of methacrylic anhydride were added per gram of gelatin to the solution at a rate of 0.1 mL/min, counting 16 min down. The beaker was covered with aluminum to avoid light, and the temperature was kept between $42\text{--}48\text{ }^{\circ}\text{C}$ during the addition. Methacrylic anhydride was directly added to gelatin solution under stirring of ~ 160 rpm. The solution was left to react for 2 h while temperature was kept between $40\text{--}45\text{ }^{\circ}\text{C}$ at a slow stirring of ~ 120 rpm.

To finalize the reaction, the solution was added to a pre-warmed dialysis bag (12-14 kDa cutoff) without precipitation of the solute. Dialysis was performed against distilled water in a 5 L beaker for one week keeping the temperature around $45\text{ }^{\circ}\text{C}$ to avoid gelation. Distilled water was changed 3 times at the preparation day, and once a day afterwards.

After 1 week, GelMA solution was removed from dialysis membrane and transferred to 50 mL falcon tube. The solution was frozen in liquid nitrogen or at $-80\text{ }^{\circ}\text{C}$ (this second options requires waiting for 1 day of freezing before drying). Freeze-drying was performed in a freeze-drier for 5 days. Lyophilized GelMA was frozen at $-80\text{ }^{\circ}\text{C}$ until further use.

Hydroxyapatite microparticles synthesis

For the performance of the studies reported in Chapter 4 and 5, HAp was produced by precipitation reaction of calcium hydroxide (Riedel-de Haën, Germany) and ortho-phosphoric acid 85 % (Panreac, Spain) solutions in an aqueous system at RT. The mixing was gently performed at pH 11, adjusted by addition of concentrated ammonium hydroxide (1 M, Riedel-de Haën, Germany), with a continuous flow rate ranging from 8 to 15 mL/min. The ceramic powder formed was sieved in several diameter ranges: less than $63\text{ }\mu\text{m}$, $63\text{--}125\text{ }\mu\text{m}$, $125\text{--}250\text{ }\mu\text{m}$, and $250\text{--}500\text{ }\mu\text{m}$. For the work described in this PhD thesis, particles with less than $250\text{ }\mu\text{m}$ were used.

3.3.2 Materials characterization

Proton-Nuclear Magnetic Resonance analysis

Proton-Nuclear Magnetic Resonance (^1H -NMR) is a widely used technique to identify polymers and analyze their chemical modifications⁵². This technique measures the magnetic resonance of protons which is given by the small magnetic fields created by the movement of electrons. Distribution of electrons in molecules, and hence their resonance frequencies, is distinct in different chemical groups but has proximal resonances in identical chemical groups, enabling its identification. The addition or alteration of a proton in a chemical group, results in the alteration of its magnetic resonance and hence a chemical shift or a new peak appears in the ^1H -NMR spectrum. According to this, functionalization degree can be evaluated by the ratio of the integrated modified and non-modified chemical groups⁵³.

So, to evaluate the degree of chemically modified GG in Chapter 5, the polymer was analyzed by ^1H -NMR spectroscopy. MAGG was dissolved in deuterium oxide (10 mg/ml) at 50 °C for 30 min, and its ^1H -NMR spectra was acquired on a Varian Inova 500 at 50 °C. Chemical shifts at δ 1.45 ppm were referred to the methyl group of rhamnose as an internal standard⁵⁴. The degree of substitution (DS, fraction of modified hydroxyl groups per repeating unit) was determined by the relative integration of the double bond proton peak (I_{DB}) of the methacrylate groups to the methyl protons of the internal standard (I_{CH3rham}), according to eq. 3.2:

equation 3.2:
$$\text{DS} = \frac{\frac{I_{\text{DB}}}{n_{\text{HDB}}}}{\frac{I_{\text{CH3rham}}}{n_{\text{CH3rham}}}} \cdot \frac{n_{\text{OHmonomer}}}{n_{\text{CH3rham}}}$$

where, n_{HDB} and n_{CH3rham} corresponds to the number of protons in the double bond and the methyl group of rhamnose, respectively. $n_{\text{OHmonomer}}$ corresponds to the number of reactive $-\text{OH}$ sites in the GG structure⁵⁵.

2,4,6-trinitrobenzenesulfonic acid (TNBSA) analysis

The reagent 2,4,6-trinitrobenzenesulfonic acid (TNBSA) has been used as a hydrophilic modifying reagent for the detection of primary amines in samples containing amino acids, peptides or proteins⁵⁶. It is an efficient reagent for rapid qualitative and quantitative estimation of these biomolecules.

In Chapter 5 and 8, TNBSA was used to determine the free amino groups in GelMA in order to determine the modification degree. Absorbance and concentration present a linear relationship up to 1 mg/mL. The modification of gelatin by methacrylation is inversely proportionally to the absorbance of the solutions at 335 nm. The determination of the concentration of primary amines was correlated with the substitution

of these amines by methacrylation. The extinction coefficient was used to calculate the total percentage of modification by comparison to amino acid standards as a calibration.

Fourier transformed infrared spectroscopy analysis

Fourier Transform Infrared Spectroscopy (FTIR) is a cost-effective spectroscopic technique that allows the analysis and identification of polymers, chemical groups, and chemical modification⁵⁷. Fourier transformation algorithm allied to IR spectroscopy gives a spectrum of IR absorption per frequency/wavelength of a solid, liquid or gas. It is based on an interferometer that reads the specific absorption and transmission of chemical compounds when passed by IR radiation⁵⁸. The absorption or transmission peaks of a range of incident IR light are converted by a Fourier transformed mathematical processing, producing spectra which represent the “finger-print” of each compound⁵⁸. As a main limitation, similar chemical groups absorb in the IR at similar frequencies, reducing the sensitivity of the technique.

An IR Prestige-21 FTIR spectrometer (Shimadzu) with the potassium bromide (KBr) pellet method or attenuated total reflectance (ATR) technique were used to identify the chemical structure of GG, HAp, and GG incorporating HAp in Chapters 4, 5 and 8. A transmittance spectrum was obtained by performing 32 scans in each spectrum over a range of 800-4400 cm^{-1} and with a resolution of 4 cm^{-1} .

X-ray diffraction analysis

X-ray diffraction (XRD) is a reliable technique to detect the amorphous or crystalline properties of materials. Under X-ray radiation, different levels of crystallinity result in specific X-ray diffractions, which are also determined by the atomic and molecular content of materials. The induction of X-rays diffraction into specific direction are then correlated to crystallinity profiles. So, 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 4 and 8, the X-ray Bragg–Brentano diffractometer (Bruker D8 Advance DaVinci, Germany) employing Cu-K α radiation ($\lambda=0.154056$ nm) at 40 kV and 40 mA, was used to analyze the crystallinity of the HAp Data were collected from 10 to 70° (2 θ values), with a step size of 0.04°, and a counting time of 2 second/step.

Rheological analysis

Oscillatory rheology is a destructive technique that measures the relationship between deformation and flow in materials. It gives the elasticity, viscosity, and plasticity profiles of materials under changes of

strain, frequency, or time⁵⁹. It is also sensitive to any chemical reactions in the flows, such as gelation and polymerization, being of interest for the study of sol-gel transitions. This technique involves the use of an oscillatory rheometer with two plates (a stationary plate and an oscillatory plate), with a material in between, in which a sinusoidal shear deformation is applied and the resultant response is measured.

In Chapter 4, viscometry tests were performed using a Kinexus Pro+ rheometer (Malver, UK). Samples were directly poured from the flask on a cone-plate geometry (40 mm) and the excess of material trimmed with a spoon and paper. A low viscosity oil solution was poured around the GG solution inside the cone-plate to avoid evaporation during the measurements at high temperatures. All data was obtained after an imposed share rate of 100 s⁻¹ and reset the deformation history of all samples. Viscosity data was obtained for single point measurement at shear rates of 0.01 to 0.1 s⁻¹. Additionally, ramps were performed ranging the shear rates from 0.01 to 0.1 s⁻¹. This range, being the typical range of levelling phenomena, was chosen to mimic the shear at the interface of gels mixing at a steady state. The process was sequentially repeated for 45, 50, 55, 60, and 80 °C.

In Chapter 5, rheological measurements were performed using an ARES-G2 rheometer (TA Instruments). All measurements were run with 8 mm serrated plate geometry on swollen samples immersed in PBS during the test using a special solvent dish and conditioned at 37 °C. The swollen sample diameter was always 8 mm, and sample thickness was 1 mm. The rheometer gap was set at 800 µm and a dynamic time sweep was performed with 1 Pa stress, 1 % strain, and 1 rad/s. 5 measurements per condition were performed.

3.3.3 3-D structures preparation and characterization

Preparation of GG-HAp gradient structures

GG was dissolved in two beakers at 90 °C for 30 min in distilled water at 2 % (w/v). HAp was then added to one of the solutions at 10, 20 or 30 % (w/w) and dispersed under stirring. After homogeneous HAp dispersion, 800 µL of this solution was added into a mold of 20x8 mm at 60 °C and topped with a second 800 µL of solution at 50 °C, within 15 seconds after pipetting the first one. Afterwards, hydrogels were obtained by immersing the mold in Ca²⁺ at 0.25 M for 24h for total ionic-crosslinking of the LAGG molecular chains. The hydrogels formed were frozen at -80 °C and then freeze-dried (figure 3.4). After 4 days, cylinders of 6x4 for *in vitro* trials or 5x5 mm for *in vivo* assays were punched from the obtained dried structures (Chapter 4). The structures were submitted to an ethylene oxide treatment for sterilization before *in vitro* or *in vivo* experimentations.

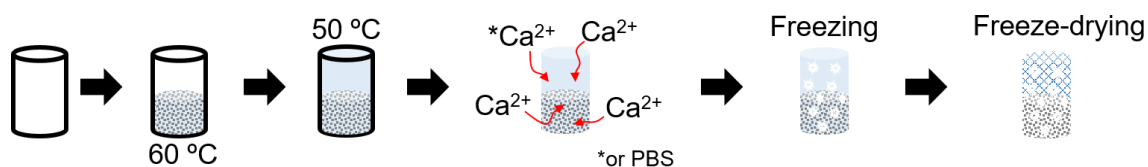


Figure 3.4 | Gradient structures fabrication made of GG-HAp. A colloidal solution of GG with a dispersion of HAp is formed and stabilized at 60 °C before injection in a silicon mold. A second solution of GG is prepared and stabilized at 50 °C and injected on top of the first one. An ionic crosslinking is performed before freeze-drying.

Preparation of MAGG-GelMA anisotropic porous structures

In Chapter 5, 0.5 % (w/v) 2-hydroxy-1-[4-(2-hydroxyethoxy)phenyl]-2-methyl-1-propanone (Irgacure 2959, Ciba Specialty Chemicals) was added to the polymeric solution at 50 °C for 1 h to fabricate the hydrogels. The lyophilized MAGG and GelMA were dissolved (by this order) at 2 % (w/v). In the blends of 3:1 and 4:1 a total concentration of 2 % (w/v) was kept in deionized water under constant stirring. First, MAGG was dissolved at 80 °C for 1 h and then GelMA was added and dissolved at 50 °C. After dissolution, photo-crosslinked hydrogels were obtained by exposure to ultraviolet (UV) light (320-500 nm, 7.14 mW/cm², EXFO OmniCure S2000) for 60 sec. After photo-crosslinking, the hydrogels were immersed in CaCl₂ solution at 0.5 M concentration for 24 h for total ionic-crosslinking of the MAGG molecular chains.

Isotropic structures fabrication:

For all the isotropic structures, silicon molds (10 mm height, 7 mm diameter) were used to hold the hydrogel while freezing at -80 °C or in liquid nitrogen (-196 °C) for a random pore formation. The obtained structures were punched and sliced in cylinders of 3x3 mm.

Anisotropic structures fabrication:

For the fabrication of anisotropic structures with smaller pore diameter, a guided ionic crosslinking was performed before the photo-crosslinking. To guide the ionic crosslinking, cylindrical molds are covered on the bottom part with Parafilm™ and with paper sheet on the top part. The paper sheet is used to adsorb a CaCl₂ solution of 0.5 M feeding the ionic crosslinking of the polymeric solution by capillarity (figure 3.5). The structures were submitted to an oxygen plasma treatment for 3 cycles of 5 min for sterilization before cell seeding, as described by Boscariol *et al*⁶⁰.

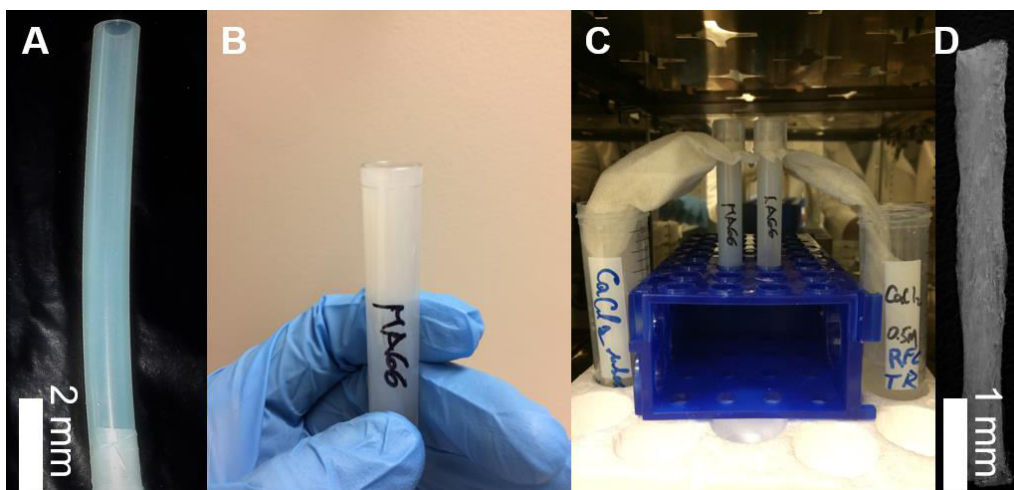


Figure 3.5 | The fabrication steps of anisotropic structures is represented by pictures. (A)

A silicon mold with at least 5 mm length (10 mm was also frequently used) is used to (B) contain the gel solution. (C) A guided crosslinking was applied to achieve the smallest pore size. (D) After freeze-drying, dried structures were obtained before slicing into the desired sizes.

The anisotropic fabrication is preceded by the production of hydrogels as previously described. The anisotropic structures were fabricated using the hydrogels and applying freezing gradient temperatures, reaching the $-80\text{ }^{\circ}\text{C}$ or $-196\text{ }^{\circ}\text{C}$. These temperatures were reached by using dry ice or liquid nitrogen, respectively. To create the temperature gradient, a custom made styrofoam mold involving silicon tube (100 mm height $\times$ 6 mm diameter) was used covering the polymeric solution all around except the top, where a frozen plate at $-80\text{ }^{\circ}\text{C}$ or $-196\text{ }^{\circ}\text{C}$ was directly placed for 2 h. As a third strategy the UV-crosslinking was preceded by a guided ionic crosslinking. After freezing for 2 h and before the freeze-drying, the frozen structures were kept in a freezer at $-80\text{ }^{\circ}\text{C}$ (figure 3.6). The obtained structures were formed by porosity differences in both ends of the cylindrical shaped scaffolds. The portion of the scaffold used for each trial was sliced from the second half of the formed cylinder. In every condition the porosity decreases through the scaffold from the top to the bottom and to avoid that variation the first half of each scaffold was discarded (meaning the half in contact with the frozen metal plate).

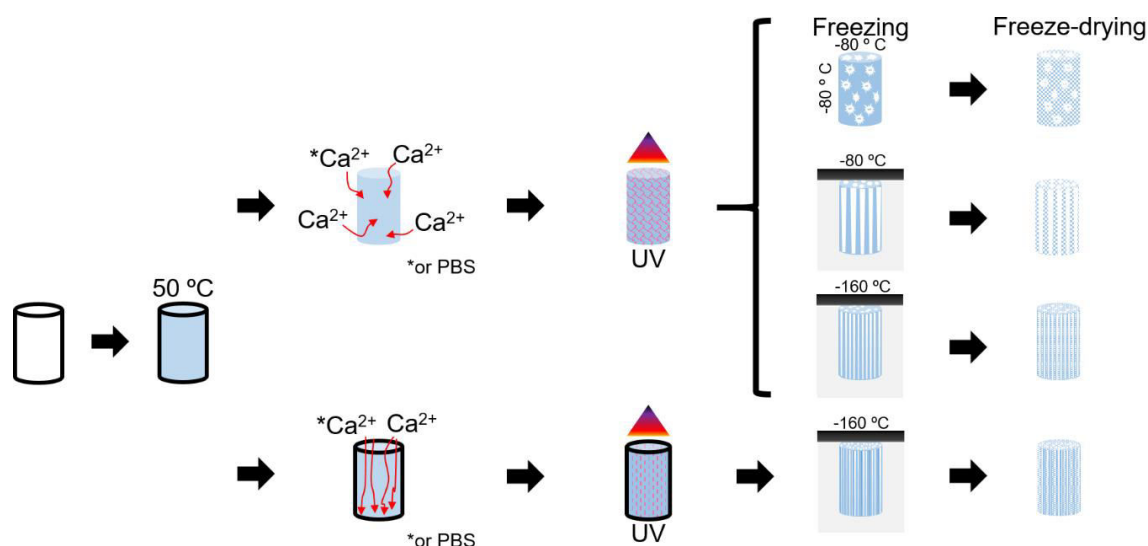


Figure 3.6 | Fabrication and control of oriented porous structures (anisotropic). The control of porous formation was obtained by guiding ice crystals formation. Decreasing the freezing temperature, decreased the ice crystal size and resultant pore. If preceded by a guided ionic crosslinking, this pore was even smaller.

Preparation of LAGG-GelMA-HAp gradient into anisotropic or isotropic porous structures

LAGG and GelMA were dissolved at a total polymer concentration of 2 % (w/v) in two separated beakers. First, LAGG was dissolved at 80 °C for 1 h in distilled water. Then, the solution was cooled down to 50 °C, and 0.5 % (w/v) 2-hydroxy-1-[4-(2-hydroxyethoxy)phenyl]-2-methyl-1-propanone (Irgacure 2959, Ciba Specialty Chemicals) was added to the polymeric solution and dissolved for 1 h. GelMA was then added for dissolution for 1 h at 45 °C. HAp was added at 30 % (w/w) and dispersed under stirring in one of the two beakers with the LAGG/GelMA solution. After complete HAp dispersion, 800 μ L of this solution was added into a mold of 10x7 mm at 55 °C. A second injection of 800 μ L was added on top of the first half at 50 °C within 30s after pipetting the first gel. Afterwards, hydrogels were obtained by immersing the gradients in α -MEM medium without FBS for 24h for total ionic-crosslinking of LAGG molecular chains. For the isotropic structures fabrication, the silicon molds were removed after 1h in medium solution. For the fabrication of anisotropic structures, a guided ionic crosslinking was performed. To guide the ionic crosslinking, cylindrical molds were covered on the bottom part with Parafilm™ and immersed in culture medium solution. After 24 h at 37 °C, the ionic-crosslinked hydrogels were exposed to UV light (320-500 nm, 7.14 mW/cm², EXFO OmniCure S2000) for 60s each side of the mold. After photo-crosslinking, a gradient temperature, reaching the -80 °C, was applied using metallic plates previously frozen in dry ice. To create the temperature gradient, a custom made styrofoam mold involving the silicone mold was used covering the polymeric solution all around except the top, where the frozen plate at -80 °C was directly

placed for 2 h (figure 3.7).

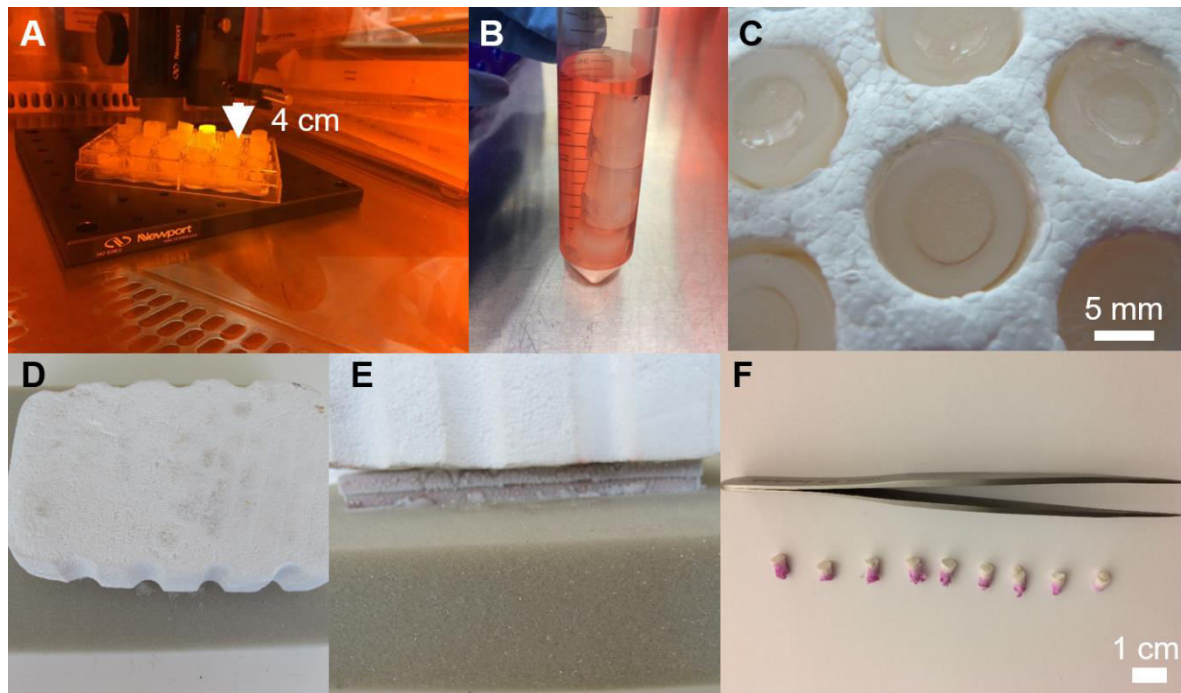


Figure 3.7 | The fabrication steps of anisotropic gradient structures is represented. After pipetting both solutions, the structures were (A) photo-crosslinked under UV-light and (B) ionic-crosslinked. (C) A styrofoam cover was used for thermal isolation of the hydrogels before (D) placing the styrofoam box on top of frozen plates. (E) The copper plate was previously frozen at -80 °C. (F) After freeze-drying, the anisotropic gradient structures were punched and both ends sliced with the desired sizes according to each experiment.

After freeze-drying, the fabricated structures were punched and sliced in cylinders of 4x3 mm. The structures were submitted to 3 cycles of oxygen plasma treatment, 5 min each, for sterilization and surface hydrophilicity cure before cell seeding⁶⁰. Figure 2.8 is a summary of all process until freeze-drying.

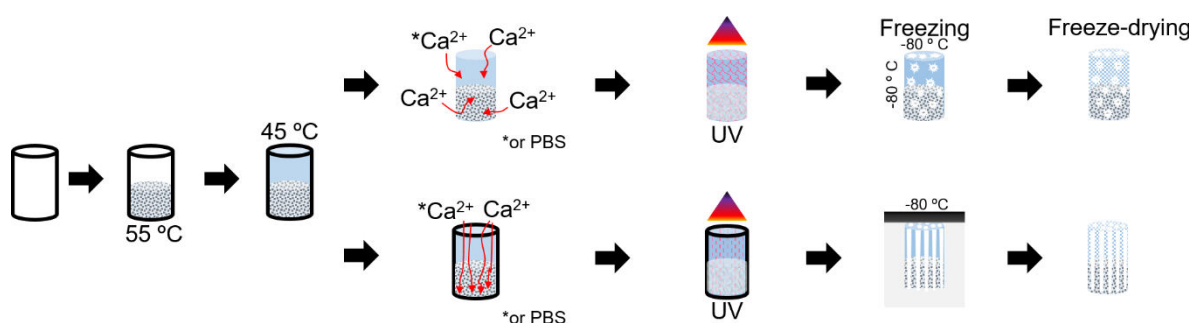


Figure 3.8 | Fabrication of isotropic and anisotropic porous gradient structures. GelMA-

LAGG-HAp and GelMA-LAGG are mixed at 55 and 45 °C since GelMA has a lower gelation point than LAGG, which decreases the effective mixing temperatures for gradient formation compared to pure LAGG gels. To guide the porosity formation, ionic crosslinking and freezing steps are performed in a controlled manner.

Experimental modulation of gels mixing at interface

An experimental modulation has as main objective the tracking and control of determinant variables of a system. In Chapter 4, an experimental modulation was performed to study the phenomenon behind the mixing of two phases of GG, being one of them a colloidal dispersion of HAp. The control and understanding of the mechanisms behind this mixing behavior are important because it results in specific HAp microparticles patterns depending on each phase temperature.

The mixing of two gels was continuously monitored and the corresponding graphs were traced by measuring the color mixing over 15s. Pure GG or GG with dispersed HAp were dissolved in distilled water at 90 °C for 30 min at 2 % (w/v). The gel made of GG and HAp was stained with alizarin red, and the pure GG phase was colored with methylene blue. After stabilization at the target temperature, e.g. 80, 60, 55, 50, or 45 °C, the first gel was injected in a transparent silicon mold (20 x 8 mm) at 0.44 mL/s, followed by the second one which was slowly injected at a flow rate of approximately 0.02 mL/s. A camera was placed for the continuous recording of the mixing of the two gels. Images at 0, 1, 5, 10, and 15 seconds were selected for the analysis. Image J was used to quantitatively evaluate the mixing gels from the RGB histograms of red and blue phases, over time, and at the bottom, interface and top regions of the mold.

Mathematical modulation of gels mixing at interface

A Newtonian fluid of density ρ and temperature T was considered. For simplicity, we assumed that temperature gradients are much lower than $1/\beta$, where β is the volumetric coefficient of thermal expansion. In this limit, the dependence of the fluid density on the temperature may be approximated as $\rho = \rho_0(1 - \beta(T - T_0))$, where ρ_0 is the fluid density at temperature T_0 . The dynamics of the fluid is then described by the following set of equations,

equation 3.3
$$\vec{\nabla} \cdot \vec{v} = 0,$$

equation 3.4
$$\frac{\partial \vec{v}}{\partial t} = -(\vec{v} \cdot \vec{\nabla})\vec{v} - \beta(T - T_0)\vec{g} - \frac{1}{\rho_0}\vec{\nabla}p' + \frac{\mu}{\rho_0}\nabla^2\vec{v},$$

equation 3.5

$$\frac{\partial T}{\partial t} = -(\vec{v} \cdot \vec{\nabla})T + \alpha \nabla^2 T,$$

where $\vec{v}$ is the velocity field, $\vec{g}$ is the gravitational acceleration, $p' = p - p_0$, p is the pressure, $\vec{\nabla} p_0 = \rho_0 \vec{g}$, μ is the dynamical viscosity, and α is the thermal diffusivity. We assumed that μ and α are constants.

We considered a two-dimensional (2-D) simulation box, with the following boundary conditions: the velocity is zero along the boundaries, the temperature is T_0 at the top, $T_1 > T_0$ at the bottom, and periodic along the horizontal direction. In 2-D a stream function ψ can be defined such that

equation 3.6

$$v_x = -\frac{\partial \psi}{\partial y}, \quad v_y = \frac{\partial \psi}{\partial x},$$

where v_x and v_y are the horizontal and vertical components of the fluid velocity, what guarantees that the continuity equation 3.3 is satisfied.

We rescaled the dimensions of the problem using $x^* = x/L$, $y^* = y/L$, where L is the width of the box, $\vec{v}^* = \vec{v}/V$, V is a characteristic (absolute) velocity, $t^* = tV/L$, and $T^* = (T - T_0)/(T_1 - T_0)$, with $0 < T^* < 1$. The adimensional dynamical equations are then reduced to (omitting the asterisks):

equation 3.7

$$\frac{\partial w_z}{\partial t} = -(\vec{v} \cdot \vec{\nabla})w_z + \frac{1}{\text{Re}} \nabla^2 w_z + \frac{\text{Gr}}{\text{Re}^2} \frac{\partial T}{\partial x},$$

equation 3.8

$$\frac{\partial T}{\partial t} = -(\vec{v} \cdot \vec{\nabla})T + \frac{1}{\text{Pr}} \frac{1}{\text{Re}} \nabla^2 T,$$

where the vorticity is related to the fluid velocity and the stream function by

equation 3.9

$$w_z = \frac{\partial v_y}{\partial x} - \frac{\partial v_x}{\partial y} = \nabla^2 \psi.$$

The Reynolds (Re), Grashof (Gr), and Prandtl (Pr) numbers are defined as

equation 3.10

$$\text{Re} = \frac{VL}{\nu}, \quad \text{Gr} = \frac{\beta(T_1 - T_0)gL^3}{\nu^2}, \quad \text{Pr} = \frac{\nu}{\alpha},$$

where $\nu = \mu/\rho_0$ is the kinematic viscosity.

These equations were integrated in a rectangular box of height $L_y = 2L$, starting from an initial adimensional temperature and stream function fields, given by $T = \tanh((1-y)/\Delta + 1)/2$ and $\psi = \delta(1 - \cos(2\pi x))(1 - \cos(\pi y))$, respectively, where $\Delta = 10^{-1}$ and $\delta = 10^{-3}$. So, initially, the temperature is $T = T_1$ at the lower half part of the box, $T = T_0$ at the upper half, and the width of the interface is Δ . The initial ψ insures null velocity at the boundaries and breaks the symmetry in order to trigger any possible instability in the fluid flow.

We used a discretized spatial mesh with $N_x \times N_y = 50 \times 50$ points, and an explicit time integration scheme with a time step $\Delta t = 0.2 / (N_x - 1)^2$ to insure numerical stability. The terms involving the spatial derivatives were calculated through second-order finite differences. At each time step, the new vorticity and temperature fields were determined from Eqs. 3.7 and 3.8, then Poisson eq. 3.9 was solved to obtain the stream function. The velocity field was updated according to eq. 3.6.

To find the temperature regimes that trigger convection, the values of the GG density and viscosity were considered. For the thermal conductivity, we assumed the one of water, since the polymeric phase is 2% weight in the volume of distilled water.

First, we considered a water-like system, at $T_0 = 50^\circ\text{C}$, in a box of size $L = 10^{-2}\text{m}$. We took $\rho_0 = 10^3\text{kg/m}^3$, $\beta = 5 \times 10^{-4}\text{K}^{-1}$, $\nu = 5 \times 10^{-7}\text{m}^2/\text{s}$, $\alpha = 1.5 \times 10^{-7}\text{m}^2/\text{s}$. We fixed $\text{Pr} = 3$ and changed the temperature differences (and consequently the Grashof number), especially around the value $\text{Gr} \approx 10^5$, corresponding to $T_1 - T_0 = 5\text{K}$ (according to eq. 3.10).

We also considered a fluid with the same physical parameters, but with a higher viscosity (10 times higher). For that, we fixed $\text{Pr} = 30$, as we changed the Grashof number around $\text{Gr} \approx 10^3$, which corresponds to the same temperature difference $T_1 - T_0 = 5\text{K}$.

For most cases, we considered $\text{Re} = 1$. But this choice only affects the velocity scale and, consequently, the time scale, keeping the dynamics intact. For larger values of the Grashof numbers, we considered $\text{Re} = 10$, to guarantee numerical stability.

Weight loss

The *in vivo* degradability of the biomaterials is dependent on several factors that include dissolution or erosion, hydrolysis, enzymatic degradation, and phagocytosis. Among these factors in human body,

proteolysis is mainly conducted by MMP enzymes which degrade collagens with high efficiency. Given that, the TE scaffolds should maintain their function during the regeneration process, while the remodulation process occurs at the same rate of scaffold degradation. For these reasons, it is important to assess the stability of the materials *in vitro* before further *in vivo* assessments.

Hydrogel weight loss, along and after exposure to a degrading solution, is the main method to measure degradation. In chapters 5 and 8, two strategies were followed to investigate the stability of the materials herein developed: 1) incubation in PBS to test hydrolysis, or 2) incubation with collagenase-type I to study active degradation of GelMA. Weight loss was calculated according to eq. 3.11.

equation 3.11: $\text{Weight loss \%} = (w_i - w_f) / w_i \times 100$

Where, m_i is initial mass, and m_f is final mass

The degradation profile of the GelMA-MAGG polymeric blend formulations was studied in Chapter 5. Weighed hydrogels (n=4) were incubated in a 13 ng/mL collagenase type I solution at 37 °C for 7 days. The samples were removed, freeze-dried and weighed again to determine mass loss.

In chapter 8, GelMA and LAGG polymers degradation was assessed over 14 and 30 days, respectively. Weighed hydrogels (n=6) were incubated in a 24 ng/mL collagenase type I solution at 37°C. The samples were removed, the excess of water adsorbed, and then freeze-dried and weighed again to determine weight loss.

Water uptake

Water content is a quantitative measure of the water existing in a material whereas water uptake gives the profile of water absorption of a material along time.

The percentage of water uptake of the fabricated structures in Chapter 5 and VIII was determined in the same assay in which weight loss was evaluated. Therefore, at each time point, the percentage of water uptake was calculated following eq. 3.12, dividing each sample wet mass after immersion in dH₂O (pH=7.4, T=37 °C, under agitation) by the final dry mass.

equation 3.12: $\text{Water uptake \%} = m_{\text{wet}} / m_{\text{dry}} \times 100$

Where, m_{wet} is wet mass, and m_{dry} is dried mass

To study the water uptake kinetics of the hydrogels developed in Chapter 5V, samples at 1 % (w/v) were

immersed in 1 mL PBS (Gibco, with monovalent ions) at 37 °C. At different time points, the hydrogels (n=4) were removed from the solutions and quickly blotted on a filter paper. Their wet weight was measured and compared to the initial wet weight.

Alizarin Red Staining

Alizarin red S, an anthraquinone derivative, is usually used to identify calcium deposits either in *in vitro* cultures or in tissue sections to detect mineralization⁶¹. Calcium and Alizarin Red S form a birefringent complex in a chelation process.

The Alizarin red staining was carried out for HAp detection in fabricated structures in Chapters 4 and 8. A solution of 2 % w/v of alizarin red S (Sigma, USA) was prepared in ddH₂O and the pH was adjusted to 4.1~4.3 with ammonium hydroxide 10 % (v/v) (Sigma, USA) solution. The samples were incubated in this solution until a red/purple color developed. After the complexation, samples were washed in PBS and visualized with a visible light microscope.

Scanning electron microscopy analysis and Energy-dispersive X-ray spectroscopy

Scanning Electron Microscopy (SEM) is an imaging techniques that gives high resolution 2-D images of a sample⁶². SEM provides surface images of a sample by scanning it with a beam of electrons. By scanning sample's surface with a focused beam of electrons, the atoms at various depths scatter electrons producing diverse type of signals which are detectable by the SEM equipment, and transformed in a 2-D image. Samples can be scanned at low to high vacuum, at cryogenic to elevated temperatures, as well as under moisture conditions. SEM provides qualitative information regarding sample's surface morphology, including microstructure, porosity, pore size, wall thickness, topography.

Energy-dispersive X-ray spectroscopy (EDS) is a technique that performs elemental or chemical analysis by interacting an X-ray excitation and a sample. The technique is usually combined with SEM to provide high-energy beam of charged particles such as electrons. Using an incident beam of X-rays or electrons, ground state electrons are excited to higher levels. When the energy is released back to ground level, a signal characteristic of each atom is emitted and measured by the energy-dispersive spectrometer.

In chapter 3, the 3-D gradient structures were analyzed by EDS (SEM/EDS, NanoSEM-FEI Nova 200, USA) to assess the bioactivity of the structures shown by apatite formation. Prior the microstructure imaging by SEM, specimens were coated with gold using a Fisons Instruments Coater (Quorum/Polaron E6700, UK) at 18 mA for 120 s. SEM was performed to evaluate structure wall thickness and surface morphology.

Hydrogels translucency assessment

Translucency is a superset of transparency. By definition, a translucent material allows light to pass through, but does not follow Snell's law macroscopically. So, a translucent material can scatter electrons at either of the two interfaces where there is a change in the index of refraction, or internally. This property was evaluated in Chapter 5 since translucency in hydrogels is relevant to allow efficient photo-crosslinking and microscopic analysis for the follow-up of *in vitro* and *in vivo* studies⁶³.

The hydrogels' optical translucency was qualitatively and quantitatively evaluated. The qualitatively evaluation was performed macro- and microscopically against white paper sheet with the text "BAMM Lab & 3B's" to evaluate the light distortion occurred through the hydrogel. The quantification was determined by visible light spectrophotometry at 495 nm and normalized to PBS. Wet paper in PBS was used as a negative control. Five measurements per condition were performed.

Micro-computed X-ray tomographic analysis

Micro-computed tomography (micro-CT) is a non-destructive imaging technique to scan objects, which provides high resolution 3-D images of a sample, resultant from the digital projection of 2-D transaxial images, and is based on the attenuation of X-rays passing through a material⁶⁴.

The obtained data set can be then processed by a set of software procedures. This technique provides relevant qualitative and quantitative information regarding sample density and microstructure, including porosity, pore size and pore wall thickness.

The microstructure and architecture of the fabricated structures developed in chapters 4, 5 and 8, namely the porosity, mean pore size, and interconnectivity, were assessed by micro-CT analysis after 3-D reconstructions. Dry samples of each formulation were scanned using a high-resolution micro-CT (skyscan 1072, Skyscan, Belgium; and SkyScan 1272, Bruker, USA). HAp profile was traced, and mean pore size and interconnectivity were quantified. 3-D projections of the specimens were performed. The 3-D gradient structures were acquired with a SkyScan 1272 scanner (v1.1.3, Bruker, Boston, USA), with a pixel size ranging from 9 to 18 μm . Approximately 400 to 550 projections were acquired over a rotation angle of 180° or 360°, with a rotation step of 0.45° or 0.68°. Data sets were reconstructed using a standardized cone-beam reconstruction software (NRecon 1.6.10.2, Bruker). The output format for each sample was bitmap images. Representative data set of the slices was segmented into binary images with a dynamic threshold of approximately 100–255 for hard ceramic phase analysis, and 30-255 for soft polymeric phase analysis (grey scale values – optimized per sample and analysis). Adjusts to these thresholds were

performed according to samples formulation and materials sensitivity to X-rays. Then, the binary images were used for morphometric analysis (CT Analyser, v1.15.4.0, Bruker) and to build the 3-D models (CTvox, v 3.0.0, Bruker). When needed, samples were vertically oriented in DataViewer (v1.5.2.3, Bruker) before proceeding to CT Analyser and CTvox.

Computed microscopic analysis was also performed by Fiji®⁶⁷, which is an image processing package distribution of ImageJ⁶⁸, bundling plugins for scientific image analysis. In Chapter 5, measurement of pore diameter was assessed using FIJI® software to measure the pore diameter of bright field images collected by optical microscopy (n = 5). More than 20 pores were measured per sample for each formulation.

Dynamic mechanical analysis

When a scaffold is implanted in an OCD, it will be exposed to cyclic loads with different frequencies. Besides the static mechanical properties, it is important to know the performance of the scaffolds under dynamic loading. Dynamic mechanical analysis (DMA) is a helpful tool to analyse this *in vitro* to predict the behavior *in vivo*.

In Chapters 4, 5 and 8, the viscoelastic measurements were performed using a TRITEC8000B dynamic mechanical analyzer (Triton Technology, UK) in the compressive mode. Samples were cut into cylindrical shapes of approximate 8 mm diameter and 5 mm thickness and immersed in PBS until equilibrium was reached (37 °C, overnight). After equilibrium at 37 °C, the DMA spectra were obtained during a frequency scan between 0.1 and 10 Hz. The experiments were performed under a constant displacement amplitude (50 µm), always at 37 °C. A small preload (0.005 N) was applied to each sample to ensure that the entire structure surface was in contact with the compression plates before testing. The distance between plates was equal for all structures under test (n=4).

Bioactivity assay with simulated body fluid

The concept of bioactivity is defined as one of the characteristic of an implant material which allows it to form a bond with living tissues⁶⁵. So, a bioactive material is one that elicits a specific biological response at the interface of the material, which results in the formation of a bond between the tissues and the material. This way, the aim of the bioactivity assay is to confirm the possible formation per se of an apatite layer on the surface of the materials when immersed in a simulated body fluid (SBF) solution, which is relevant for the bone-bonding performance.

In 2003, conventional SBF with the refined recipe was proposed to the Technical Committee ISO/TC150 of International Organization for Standardization as a solution for *in vitro* measurement of apatite-forming

ability of implant materials. This solution has ion concentrations nearly equal to those of human blood plasma as it is shown in the table 3.1. Therefore, the SBF has been used for the *in vitro* assessment of bioactivity of biomaterials.

Table 3.1 | Nominal ion concentrations of SBF in comparison with those in human blood plasma. ⁶⁶

Ion	Ion concentration (mM)	
	Blood plasma	Simulated fluid
Na ⁺	142.0	142.0
K ⁺	5.0	5.0
Mg ²⁺	1.5	1.5
Ca ²⁺	2.5	2.5
Cl ⁻	103.0	147.8
HCO ₃ ⁻	27.0	4.2
HPO ₄ ³⁻	1.0	1.0
SO ₄ ³⁻	0.5	0.5

The SBF solution (Na⁺ 142.0, K⁺ 5.0, Ca²⁺ 2.5, Mg²⁺ 1.5, Cl⁻ 147.8, HCO₃⁻ 4.2, HPO₄²⁻ 1.0, SO₄²⁻ 0.5 mM) was made by dissolving reagent-grade NaCl, NaSO₄, NaHCO₃, KCL, K₂PO₄·3H₂O, CaCl₂ and MgCl₂·6H₂O in distilled water at 36.5 °C. The solution pH was settled to 7.4 by addition of tris-hydroxymethylaminomethane and hydrochloric acid. The GG-HAp structures were soaked in 10 mL of SBF solution using 50 mL polystyrene flasks. The temperature of the solutions was maintained at 36.5°C, as described by Kokubo et al⁶⁶ in order to closest resemble the human blood plasma. Each structure was kept for periods of 12 h, 1, 3, 7, and 14 days. At each time point, the structures were removed and rinsed in distilled water, frozen at -80 °C, and then freeze-dried for 4 days. All measurements were performed in triplicate.

Bioreactor development

Dual-chambers design and fabrication

A dual-chamber bioreactor was designed to integrate two compartments interconnected by a central hole where a membrane or a 3-D structure can be placed. The dual-chambers were fabricated in polycarbonate

to be robust and autoclavable. Medical-grade silicon O-rings were used to seal all the interfaces of the assembled pieces. The design of the dual-chamber is provided in figure 3.9.

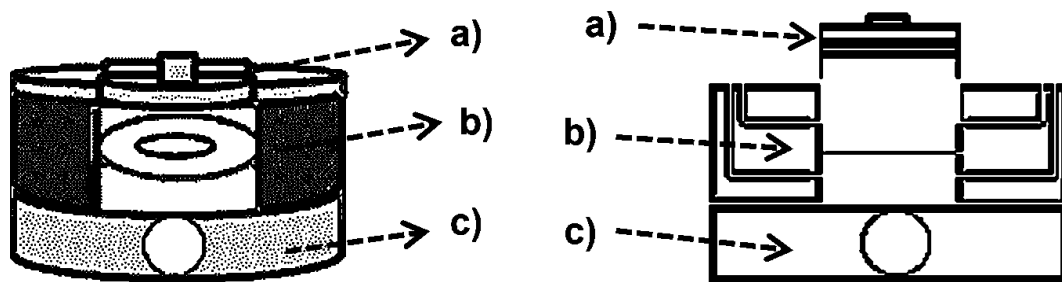


Figure 3.9 | Perspective view of the dual-chamber (with open view for the inside). (a) top cap, (b) middle part where two compartments are connected through a central hole, (c) bottom part with a transversal hole for an optional introduction of a magnet.

The sizes of each compartment were designed to fit a standard 6-well tissue culture plate, as represented in figure 3.10.

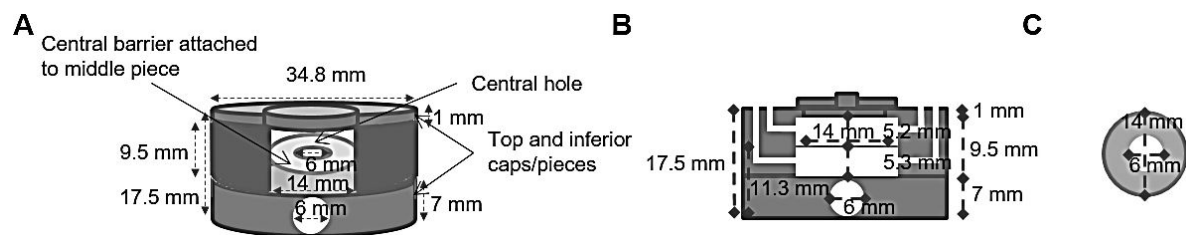


Figure 3.10 | Dimensions of the dual-chambers. (A) Perspective view, (B) lateral section view, (C) top view of the central hole.

Rotational platform design and components

A rotational platform was designed to create a dynamic cell culture device to which the dual-chamber bioreactors can be magnetically attached. The platform is made of polylactic acid and 3-D printed (figure 3.11).

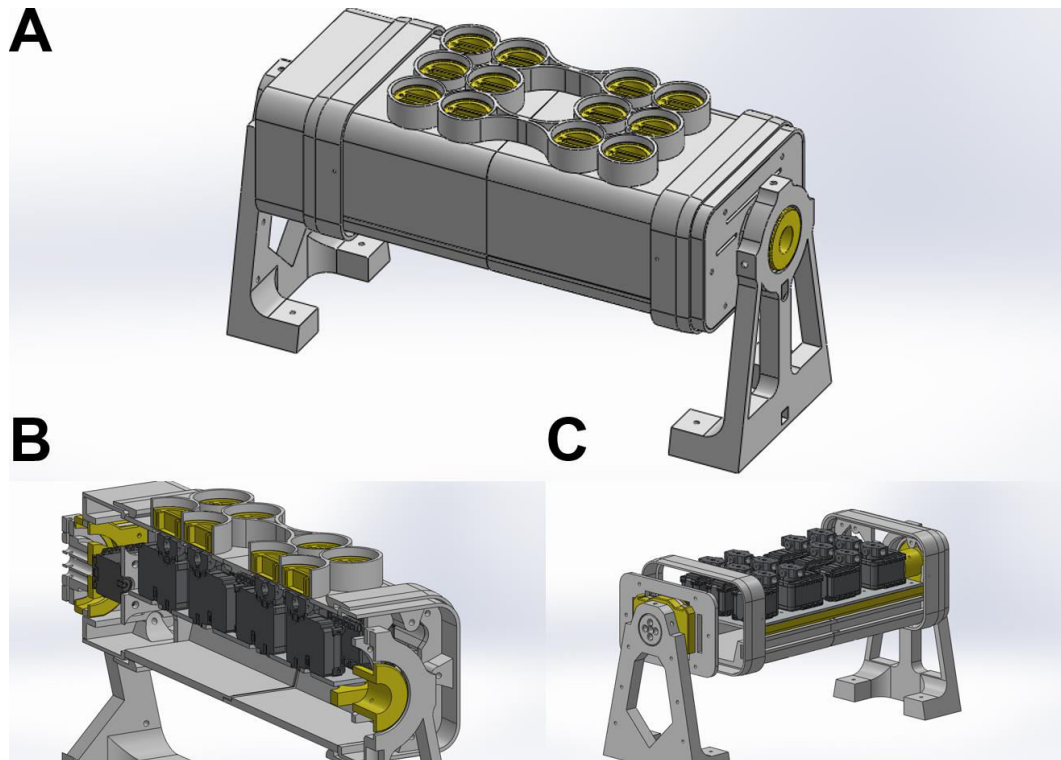


Figure 3.11 | Dynamic rotational platform. (A) Perspective view, (B) Vertical cross-section, (C) scheme of a perspective view of the core.

A robotic system was assembled to coordinate the rotational movements in the horizontal and vertical directions. A servomotor plate coordinates the mechanical rotations of 12 servos commanding the dual-chamber, and a 13th servo which is responsible for the vertical turning. An Arduino® is programmed to provide an interface by Bluetooth®, which controls the servomotor plate. The control of the rotational movement was programmed to provide 15 different rotations per minute, while the control of the vertical turning was programmed to set a delay of rotations in seconds, minutes or hours, and its speed. Simultaneously, the interface software provides a feedback on the temperature of each motor in use. This software was designed for Microsoft (figure 3.12) and Android (figure 3.13).

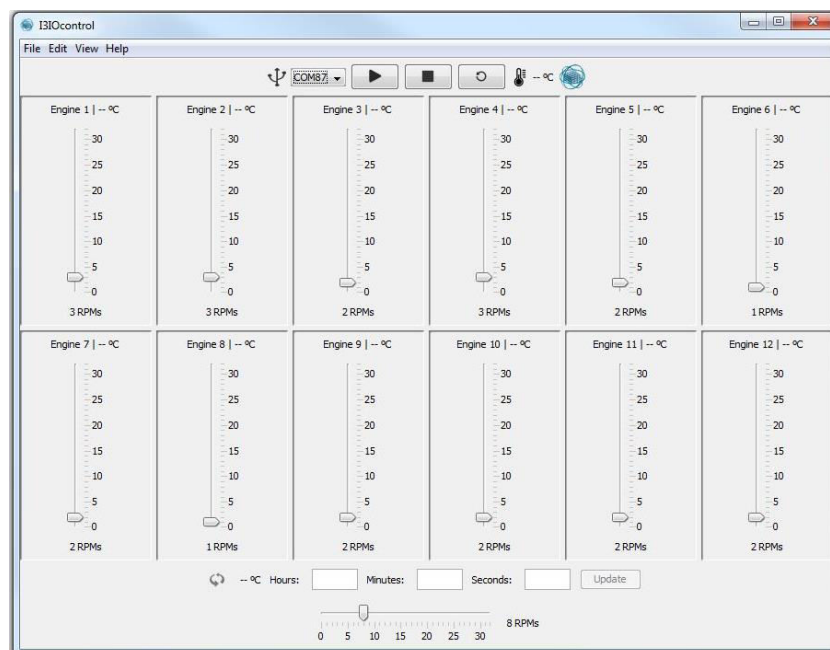


Figure 3.12 | Microsoft-compatible software to control the rotational platform dynamics.

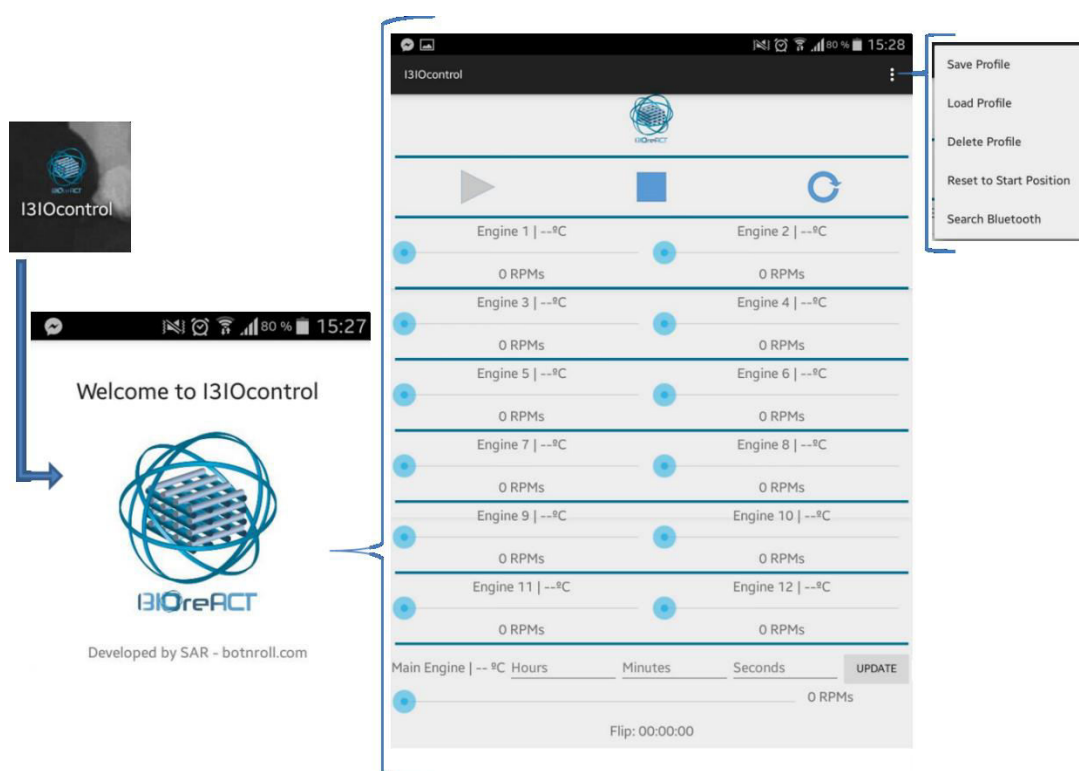


Figure 3.13 | Android-compatible application to control the rotational platform dynamics.

Microfluidic device for 3-D interfaces design

A microfluidic device made of poly(methyl methacrylate) (PMMA) was designed to control gradients at a 3-D interface. To achieve such control, two microfluidic parallel channels with a central barrier were

designed. This barrier is interrupted in the region where the 3-D structure is placed allowing the channels to interconnect (figure 3.14).

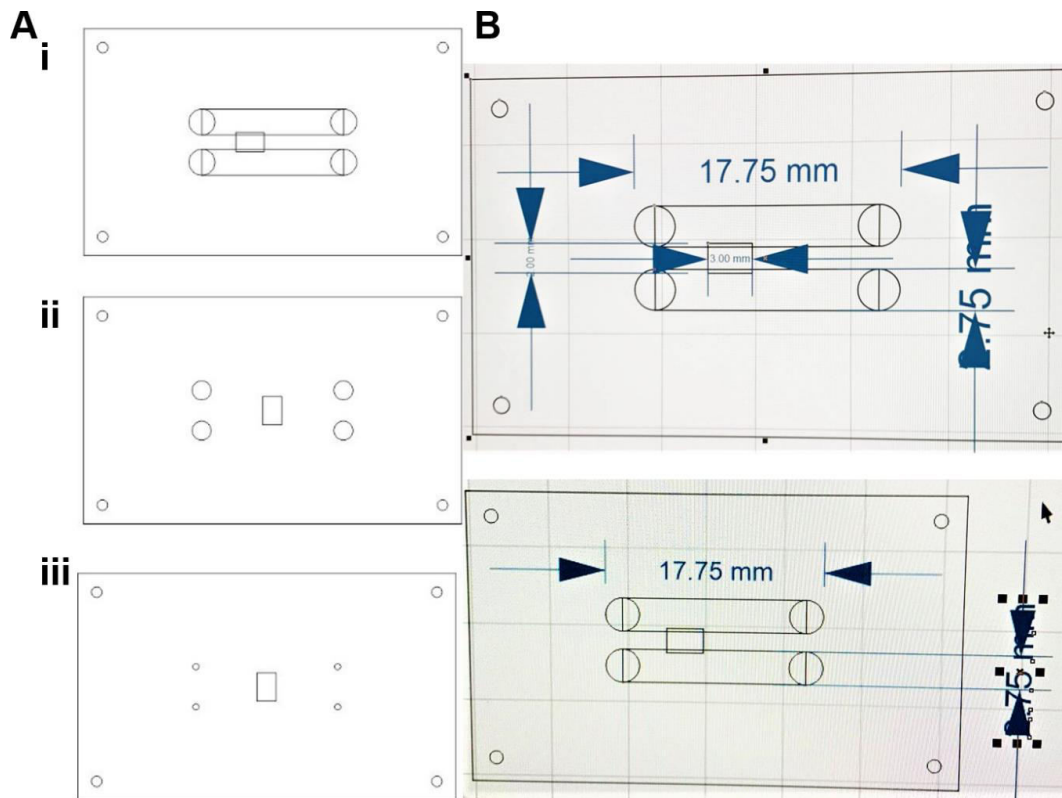


Figure 3.14 | Schematic of a microfluidic system adapted to create interfaces and perfused co-cultures. (A-i) Channels layer, (A-ii) intermediate layer, (A-iii) Top layer. (B) Dimensions of channels, central barrier and interconnected zone.

Cell isolation and culture

Isolation and culture of fat pad adipose-derived stem cells

Human cells were obtained from samples from the Hospital da Prelada (Porto, Portugal), with patient's informed consent and under a protocol with the 3B's Research Group, approved by the ethics committees of both institutions.

MSCs can be isolated from all compartments of the knee joint, and might act as a reservoir of cells that contribute to the maintenance of healthy tissue and/or the response to injury (figure 3.15)⁶⁹. MSCs might also act as immunomodulatory sentinels to reduce inflammation. Degenerative changes in OA can be partly attributed to the aberrant or defective activity of these local MSCs populations. Minor tissue-specific differences in characteristics such as cell-surface markers, proliferative capacity and lineage potential exist between MSCs isolated from different tissues within the knee joint⁷⁰.

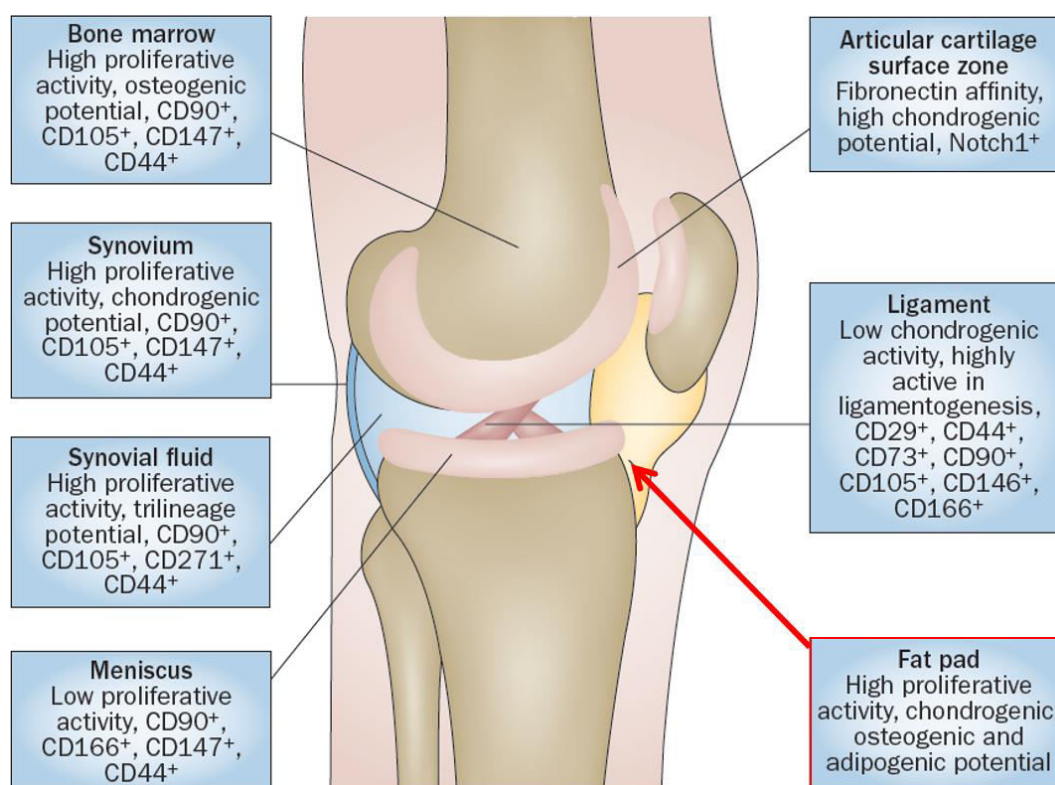


Figure 3.15 | Characteristics, culture phenotypes and cell-surface markers of MSCs isolated from tissues within the knee joint⁶⁹.

Among the niches represented in figure 3.15, this thesis focused on fat pad tissue. The infrapatellar fat pad (IPFP) is a mass of closely packed fat cells surrounded by the fibrous tissue septa. The IPFP, also known as Hoffa's fat pad, is a cylindrical piece of fat that is situated under and behind the patella bone within the knee. Arthroscopic surgeries is performed through routes such as the suprapatellar approach for nail insertion, which are seen as an option to avoid late postoperative knee pain⁷¹. This approach was seen to make it easy to locate the entry point, with lesions only occurring in the Hoffa's body. So, during an arthroscopy, Hoffa's body can be removed because it can be inflamed or damaged, or to better visualize the knee⁷². This way, this tissue can be considered to scientific research as a promising source of ASCs with great potential to differentiate into chondrocytes and osteoblasts (table 3.2)^{73,74}.

Table 3.2. Characterization and phenotypic properties of MSCs in the IPFP tissue

Tissue	Characteristics of resident MSCs	Markers	Reference
Hoffa's body	Highly proliferative, strong chondrogenic, osteogenic and adipogenic activity	Positive: CD13, CD29, CD44, CD90, CD105 Negative: CD34, CD56, CD271, STRO1	Khan et al. (2012) ⁷⁵

Fat pad-ASCs were isolated from human Hoffa's body removed during arthroscopic surgeries on male and female donors with ages between 19 and 32 years, after informed consent, under cooperation protocols established with Centro Hospitalar Póvoa do Varzim and Clínica Saúde-Atlântica. The study has been approved by the Ethical Committee of Centro Hospitalar Póvoa do Varzim, Grupo Saúde-Atlântica and University of Minho.

All the samples were processed within 24 h after the arthroscopic procedure to the knee. hASCs were isolated following an enzymatic digestion-based method with type II collagenase (Sigma). The extracted tissue was placed in PBS solution and washed several times with PBS containing 1 % (v/v) AB mixture, until total removal of blood and cut into small pieces. Tissue digestion was performed by incubation at 37°C in a humidified atmosphere of 5 % CO₂ for 10 h in a 10-20 mL 1:1 mixture of Minimum Essential Media alpha (MEM alpha, Invitrogen) supplemented with 10 % FBS (Gibco) and 1 % AB mixture, with type II collagenase 0.15 % (w/v) in PBS. The digested tissue was filtered and cell suspension centrifuged at 300 g for 5 min. The hASCs were selected by plastic adherence and expanded in α -MEM medium supplemented with 10 % FBS and 1 % of AB mixture.

Isolation and culture of primary cerebral cortex neurons

A neuron is an electrically excitable cell. The neocortex contains about 80 % of excitatory and 20 % of inhibitory neurons⁷⁶, classified as excitatory glutamatergic pyramidal cells and inhibitory GABAergic interneurons⁷⁷.

All experiments were carried out according to animal care standards set forth by the National Institute of Health and were approved by the Institutional Animal Care and Use Committee at Stanford University. Primary cortical neurons were isolated from embryonic day 18 (E18) C57bl/6 mice provided by Jackson Laboratory. Freshly micro-dissected whole mouse cortices were dissociated by trituration after 30 min of enzymatic digestion using Neuronal Isolation Enzyme (Thermo-Fisher Scientific) in Hanks Balanced Salt Solution (HBSS, Life Technologies). The cells were then suspended in neurobasal medium with B27 supplement (Life Technologies) and 500 μ M glutamine (Life Technologies) until seeding/injection. Culture conditions were maintained at 37 °C with 95 % relative humidity, and 5 % CO₂.

Cell-material/construct culture conditions: in Chapter 5, primary neurons isolated from cerebral cortex of mouse embryos at week 18 were embedded in Matrigel® followed by injection of the cells in the isotropic or anisotropic porous structures (10 M cells/scaffold embedded in 50 μ L Matrigel®). The constructs were

cultured in 24-well tissue culture plates for 7 and 14 days in neurobasal medium with B27 supplementation and 500 μ M glutamine at 37°C in a humidified 5 % CO₂ incubator.

Other cell types

- Human Umbilical Vein Endothelial Cells

Human umbilical vein endothelial cells (HUVECs), isolated from the endothelium of the vein of the umbilical cord, are used as a laboratory model system for the study of the function and pathology of endothelial cells (e.g. angiogenesis)⁷⁸.

HUVECs (Lonza, USA) were routinely cultured under the conditions defined by the company. Tissue culture plates surface were covered with gelatin type A (0.7 % w/v) solution for 30 min to 1 h at 37 °C, and washed with PBS. Cells were cultured with EndoGRO-VEGF Complete Media Kit (Millipore, USA) and routinely trypsinized for 5 min at 37 °C with TrypLETM Express (Life Technologies, UK), centrifuged (220 g, 5 min) and resuspended at a density of $\sim 7 \times 10^3$ cells/cm².

- Human Adipose Microvascular Endothelial Cells

Adipose tissue is unique because it has the capacity to continually grow throughout adult life. Thus, it has a high level of angiogenesis to provide its extensive vascularization⁷⁹. Studies have shown that angiogenesis precedes adipogenesis, implying that microvascular endothelial cells influence the proliferation of preadipocytes⁸⁰.

Human Adipose Microvascular Endothelial Cells (hAMECS, ScienCell, USA) were cultured under the conditions defined by the company. Plates were coated as for the HUVECS with gelatin Type A (0.7 % w/v) for 1 h and washed with PBS. Cells were cultured with EndoGRO-VEGF Complete Media Kit and routinely trypsinized for 5 min at 37 °C in TrypLETM Express (Life Technologies, UK), centrifuged (220 g, 5 min) and resuspended at a density of $\sim 7 \times 10^3$ cells/cm².

- NIH-3T3 fibroblasts

The '3T3' designation refers to the abbreviation of "3-day transfer, inoculum 3×10^5 cells." 3T3 cells come from a cell line Swiss albino mouse embryo tissue established in 1962 by George Todaro and Howard Green. The 3T3 cell line has become the standard fibroblast cell line⁸¹.

The NIH-3T3 fibroblasts were expanded in α -MEM medium supplemented with 10 % FBS and 1 % of AB mixture at 37 °C, in a humidified atmosphere with 5 % of CO₂. Culture medium was changed every 2-3 days.

Cell-material/construct culture conditions: A cell suspension of NIH-3T3 was prepared by trypsinization (trypsin/EDTA solution, Invitrogen) and 1×10^5 (per scaffold) were seeded onto the freeze-dried hydrogels formulations prepared as described in Chapter 5. NIH-3T3 constructs were cultured in α -MEM supplemented with 10 % of FBS and 1 % penicillin-streptomycin at 37 °C, in a humidified atmosphere with 5 % of CO₂.

- SH SY5Y neuroblastoma cell line

SH-SY5Y, a human derived cell line originally called SK-N-SH, from which it was subcloned, was isolated from a bone marrow biopsy taken from a four-years-old female with neuroblastoma and first reported in 1973⁸². A neuroblast-like subclone of SK-N-SH, named SH-SY, was subcloned as SH-SY5, which was subcloned a third time to produce the SH-SY5Y line, first described in 1978⁸³. SH-SY5Y cells are adrenergic in phenotype but also express dopaminergic markers and, as such, have been used to study Parkinson's Disease⁸⁴.

SH-SY5Y cells plated (10×10^5 cells/cm²) on 10 µg/ml laminin, cultured in Dulbecco's modified eagle medium/Nutrient mixture F-12 (DMEM/F-12, Thermo-Fisher Scientific), containing 3% FBS (Thermo-Fisher Scientific) and 1% penicillin-streptomycin (Life Technologies). For differentiation into neurons, 10 µM all-trans retinoic acid (RA) was added for 72 h. Differentiation medium was replenished every 48 h.

Cell-material/construct culture conditions: SH SY5Y (1×10^5 /scaffold) were seeded onto the 3:1 freeze-dried formulation described in Chapter 5. Constructs were cultured for 3 days with DMEM-F12 and 5 % FBS (Thermo-Fisher Scientific), 1 % penicillin-streptomycin (Life Technologies) and supplemented with 10 µM retinoic acid for more 4 days at 37 °C, in a humidified atmosphere with 5 % of CO₂.

Osteogenic and chondrogenic differentiations:

The differentiation of BMSCs and ASCs towards the osteogenic and chondrogenic lineages is extensively described in the literature^{85–87} and has been demonstrated to be induced by specifically supplemented culture media. For osteogenic differentiation, factors such as ascorbic acid, β -glycerophosphate and dexamethasone⁸⁸ have been regularly used. As ascorbic acid, proven to increase matrix production⁸⁹, has a very short half-life in the medium⁹⁰, a salt of ascorbic acid, ascorbate-2-phosphate, is used. β -glycerophosphate is involved in matrix mineralization by providing phosphate ions⁹¹ while dexamethasone is a corticoid that commits osteoprogenitor cells to the osteogenic lineage^{92,93}.

In chondrogenesis, glucocorticoids such as dexamethasone upregulate gene expression of cartilage matrix components as aggrecan⁹⁴. Similarly to osteogenesis, ascorbic acid has been shown to stimulate

1,25-dihydroxy vitamin D3 synthesis and cartilage matrix production in chondrogenesis⁹⁵. Hydroxyproline is exclusively present in collagen, which is hydroxylated by ascorbic-acid to constitute a more stable collagen molecule⁹⁶. The absence of hydroxyproline from culture medium was demonstrated to affect chondrogenesis⁹⁷. Another family of factors important for chondrogenesis is the Transforming growth factor (TGF)-isoforms. These cytokines differ in their effects on chondroprogenitor cells. TGF- β 1 is responsible for the initial cell–cell interaction between condensing progenitor cells, and stimulates new matrix synthesis by chondrocytes. TGF- β 2 mediates hypertrophic differentiation of chondrocytes by regulating Indian hedgehog and parathyroid growth hormone expression. Nonetheless, both TGF- β 1 and TGF- β 2 have been reported to possess osteoinductive potential^{98,99}, while TGF- β 3 has been shown to be upregulated in dedifferentiated human chondrocytes¹⁰⁰.

Therefore, mesenchymal progenitors from human BM, or hASCs were cultured for 21 days in osteogenic differentiation medium in Chapters 4 and 8. For osteogenic induction medium, MEM alpha was supplemented with dexamethasone (100 nM), β -glycerophosphate (10 mM), ascorbic acid 2-phosphate (50 mg/mL), 1 % AB, and 10 % FBS. Chondrogenic medium was prepared supplementing DMEM high-glucose with dexamethasone (100 nM), ascorbic acid 2-phosphate (50 μ g/mL), L-proline (40 μ g/mL), TGF- β 3 (10 ng/mL), 1 % AB, and 10 % FBS.

Heterotypic culture in microfluidic device for 3-D interfaces:

Green fluorescent protein (GFP) positive HUVECs (Lonza) and primary neurons were cultured in 3-D oriented porous structures. HUVECs were directly seeded in half of the 3-D porous structure, while primary neurons were embedded in Matrigel® and injected in the other half. The endothelial channel was perfused with endothelium growth medium (Lonza) and the neural channel with neurobasal medium with B27 supplementation and 500 μ M glutamine. The microfluidic device was perfused at a 2 μ L/min at 37 °C in a humidified 5 % CO₂ atmosphere. The culture was performed over 7 days and then the acquired fluorescent microscopic images analyzed with FIJI plugin Directionality.

Co-culture of fat pad ASCs with hAMECs using a dual-chamber bioreactor:

hAMECs were stained with Dil live staining (Thermo Fischer Scientific) and then mixed with ASCs in a ratio of 4:1, respectively. The mixed population was seeded in the top and bottom parts of the isotropic porous 3-D structures placed in the dual-chamber bioreactor. Basal MEM alpha supplemented with 10 % FBS and 1 % A/A was mixed with endothelium growth medium (Lonza) containing 2 % FBS and VEGF in a ratio of 1:4, respectively, and equally perfused in both compartments of the dual-chamber. The

bioreactor chamber was perfused at 5 $\mu\text{L}/\text{min}$ and placed at 37 °C in a humidified 5 % CO_2 atmosphere. After 5 days of perfusion, the cell culture medium in the bottom chamber was changed by the mix of osteogenic differentiation medium (Lonza) with endothelial growth medium (1:4). Constructs were perfused for further 2 days more.

Biological characterization

Live/dead assay

Calcein-AM/Propidium iodide (PI) staining is a method for visualization of cell viability and cell death, especially used when cells are cultured in an environment that absorbs visible light limiting the use of colorimetric methods to determine cell viability (for instance, MTS). Cells are incubated with calcein acetoxymethyl (Calcein-AM), a non-fluorescent derivate of calcein, which is transported through the cellular membrane inside the cells where intracellular esterase from living cells remove the acetomethoxy group and originate green fluorescent calcein. Dead cells do not fluoresce as they do not have active esterases¹⁰¹. For visualization of dead cells, cells are incubated with fluorescent PI which shows enhanced fluorescence when binding with high affinity to DNA. As PI is not permeant through the cellular membrane, PI only binds cytoplasmic DNA if the cellular membrane is disrupted (dead cells). Cell viability within hydrogels or freeze-dried structures was assessed in Chapters 4 and 5 by staining cells with calcein-AM (1 $\mu\text{g}/\text{mL}$, Invitrogen, USA) for 1-2 h and PI (2 $\mu\text{g}/\text{mL}$, Invitrogen, USA) for 20-30 min at 37 °C in a humidified tissue culture incubator with 5 % CO_2 atmosphere. All formulations were studied performing triplicates in three independent experiments.

DNA quantification

In chapters 4 and 8, the proliferation of cells in direct contact with the developed materials was evaluated based on the total double-stranded DNA (dsDNA) content at different culture times. For this, the PicoGreen Quantification Kit (Invitrogen, USA) was used. Picogreen is a fluorescent staining that binds to dsDNA and therefore permit to measure the proportional fluorescence enhancement¹⁰².

The amount of double stranded DNA (dsDNA), that is directly proportional with the cell number, was determined according to the manufacturer's instructions. Before performing the quantification, a complete mechanical disruption of the materials was performed, followed by a thermal shock cycle by incubating the lysates at 75 °C for 1 h and freezing them at -80 °C until further analysis. Supernatant fluorescence was measured at an excitation wavelength of 485/20 nm and at an emission wavelength of 528/20 nm, in a microplate reader (Synergy HT, BioTek Instruments, USA). The quantification of DNA

inhibitors of the formation of HA crystals. The hydrolysis reaction results in the saturation of the extracellular fluid with orthophosphates that induce mineralization¹⁰⁴. Increased levels of ALP activity are thus correlated with enhanced osteogenic differentiation¹⁰⁵.

This enzyme activity quantification has been extensively used to confirm the osteogenic differentiation or osteogenic activity⁸⁸. The ALP activity analysis in Chapter 4 was performed in the same cell lysates used for the DNA quantification following an adapted p-nitrophenol assay^{88,106}. In this assay, p-nitrophenil phosphate was used as an ALP substrate. ALP, if present, hydrolysed p-nitrophenil (colorless) into p-nitrophenol (yellow). In each assay 20 μ L of lysate were incubated with 80 μ L of p-nitrophenil phosphate solution (0.2 % w/w, in diethanolamine, Sigma, USA) in a transparent 96 well microplate, at 37 °C for 45 min. The reaction was stopped using 80 μ L of a 2 M NaOH (Sigma, USA) and 0.4 mM EDTA (Sigma, USA) solution. The optical density of the samples was read at 405 nm. A calibration curve was previously prepared using the p-nitrophenol (Sigma, USA) standards ranging from 0 to 0.2 mmol/mL and used to extrapolate the ALP activity values. These values were then normalized against dsDNA results obtained within the same samples and converted into enzyme activity per hour. Samples without cells were used as control.

Immunocytochemistry

The principle of immunocytochemistry relies on the use of antibodies to target specific antigens in cells. If the antibody is not coupled with a fluorophore, the binding of a primary antibody to an antigen is followed by binding of a fluorescent-labeled secondary antibody to the primary antibody for visualization. This approach can be used to identify specific cell types or the particular expression of a specific marker. Nonetheless, immunocytochemistry is only quantitative if coupled to image analysis methods.

This technique involves preliminary steps after cell culture: a fixation step that preserves the chemical and structural state of the cell components; a cell membrane permeabilization step that enables the antibody to enter into the cell when the antigen is intracellular; a blocking step that blocks unspecific binding of antibodies.

After cell culture within hydrogels or freeze-dried structures, in Chapters 4, 5 and 8, the constructs were first fixed with 10 % v/v of formalin (Thermo Scientific, USA) for 30 to 60 min and then cell-laden materials were cut into thin slices. For cytoplasmic markers, cells were permeabilized with 0.1 % v/v of cold Triton-X 100 (Sigma) for 30 min. Unspecific antibody binding was blocked with 2.5 % w/v of horse serum or bovine serum albumin (BSA) for 30 to 60 min. Samples were then incubated with the primary antibody diluted in 1 % (w/v) BSA/PBS (table 3.3) for 24 h either at RT or at 4 °C. Samples were then washed

three times with PBS and incubated with secondary antibodies Alexa Fluor 488, 594, and/or 647 overnight at 4 °C, and according to the host of the primary antibody. Between antibodies incubations, cells were again washed in PBS and counterstained with 4',6-diamidino-2-phenylindole (DAPI; 0.02 mg/mL, Invitrogen, USA) for 15-30 min for nuclei counter-staining.

Cells were observed using an AxioImager Z1m fluorescence microscope (Zeiss, Germany), a LSM710 inverted confocal microscope (Zeiss, Germany), or with a TCS SP8 confocal microscope (Leica, Germany).

Table 3.3 | Antibodies used to perform immunocytochemistry.

Cells	Phenotype	Antibody (Brand)	Ref	Host	Dilution	Clonality, reactivity
Primary neurons	Microtubules of neurons from central and peripheral nervous system	Anti-TuJ1 (class III β -tubulin, abcam)	ab18207	Rabbit	1:200	Mouse, Rat, Human, Pig, Rhesus monkey, Common marmoset, Dogfish, Catshark
Primary neurons	Glial cells of the central nervous system	anti-GFAP (Cellsignal)	mAb #3670	Mouse	1:200	Human, Mouse, Rat
Primary neurons	Postsynaptic junctions of neurons in the forebrain	anti-PSD95 (abcam)	ab2723	Mouse	1:200	Mouse, Rat, Zebrafish
Human fat pad-ASCs	Osteocalcin expression	anti-osteocalcin (Millipore)	AB10911	Rabbit	1:200	Human Mouse Rat
Human fat pad-ASCs	Sox-9 nuclear protein	anti-Sox9 (Santa Cruz Biotechnology)	sc-166505	Mouse	1:200	Mouse, Rat, Human

Human fat pad-ASCs	RUNX2 nuclear protein	Anti-RUNX22 (Novus Biologicals)	AF2006	Goat	1:200	Human
Human fat pad-ASCs	Bone found in plasma	Anti-Osteopontin (abcam)	ab8448	Rabbit	1:200	Mouse, Rat, Human, Pig
Human fat pad-ASCs	Membranar protein expressed in neurons, among other cells.	APC anti-human CD271 (NGFR) (BioLegend)	345108	mouse	1:200	Human, African Green, Baboon, Cynomolgus, Rhesus

Cell shape and orientation analysis

Cell morphology was observed under microscopy after staining with Phalloidin, which is a rigid bicyclic heptapeptide lethal toxin that binds and stabilizes actin filaments (F-actin) and prevents the depolymerization of actin fibers. Visualization of actin can be attained by incubating previously fixed cells with fluorescent Phalloidin-TRITC (Invitrogen). Accordingly, in Chapter 4 and 8, cell actin filaments were visualized after fixation of cells in 10 % v/v of formalin, followed by incubation with Phalloidin-TRITC (0.1 mg/mL, SIGMA, USA) for 1 h at RT.

When studying neurons morphology and alignment in Chapter 5, instead of phalloidin, anti-TuJ1 was used¹⁰⁷. Anti-TuJ1 is an antibody that binds to class III β -tubulin expressed in neurons of the peripheral and central nervous system. This protein contributes to microtubule stability in neuronal cell bodies and axons, and plays a role in axonal transport¹⁰⁷.

The actin filaments in ASCs and the microtubules in neurons have a polar orientation towards the cell stretching orientation^{108–110}, thus the analysis of the actin cytoskeleton organization is indicative of cell migration direction. Cell alignment was quantitatively analyzed in representative microscopic captions of Phalloidin/DAPI or TuJ1/DAPI stained constructs, at different timepoints, using the public domain FIJI software (NIH)⁶⁷. The images were split into the red and blue channels, and only the red channel, corresponding either to the Phalloidin/TuJ1 stained cytoskeleton in fat pad-ASCs or in neurons in Chapter 4 and 5, respectively, was analyzed after being preprocessed. Since the majority of the structures used

in the different studies are self-fluorescent on the same wavelengths of the applied stainings (from ~470 to ~630 nm), the function “subtract background”, using a 25 pixels filter, to distinguish the microtubules signal from the background was applied. The contrast was enhanced in 0.05 %, followed by a despeckle filter that removes single-pixel noise. The preprocessed images were analysed using the “directionality” plugin which gives as output the main fiber direction and the fiber dispersion¹¹¹. Cell Profiler software was applied to calculate the major and minor axis length of neurons in aligned and random orientations, using an algorithm for the identification of nuclei associated to TuJ1 staining.

Pre-vasculature segments analysis

Angiogenesis analyzer is a plugin for FIJI software (NIH)¹¹² that allows analysis of cellular networks. Typically, it can detect and analyze the pseudo vascular organization of endothelial cells cultured in gel medium. Bright-field and fluorescent images were analysed independently for each area of interest in Chapter 8. The combination and average of both processes allowed reducing the false positive results due to background and artifacts. This methodology resulted in a more robust data for the identification of pre-vascular endothelial segments.

***In vivo* assays**

Subcutaneous implantation

A subcutaneous model was used to determine *in vivo* host response to biomaterials in Chapter 4. All the procedures were in accordance with the national guidelines and international standards on animal welfare as defined by the European Union Directive 2010/63/EU. The animals were screened for good physical condition and were specific-pathogen free (SPF). Food (powdered or pellets) and water were provided *ad libitum* but were withheld overnight pre-operatively. Animals were observed along the time of the assay and weighted by the caretakers. Observations were listed in the individual welfare diary.

In order to evaluate the *in vivo* biocompatibility of the 3D-structures per se, in Chapter 4 inflammatory reaction, materials infiltration and integrity over the 14 days of implantation were analysed. The 3-D structures were subcutaneously implanted in the back of 5 weeks old mice Hsd:ICR (CD-1) with an average weight of 27-32 g at the time of implantation (Charles River Laboratories, Saint-Germain-sur-l'Arbresle, France). Each mouse was anesthetized by intraperitoneal injection of Medetomidine 1 mg/Kg (Domitor INJ 1mg/mL, Novavet, Braga, Portugal) and Ketamine 75 mg/Kg (Imalgene 1000 INJ 100 mg-mL, Novavet, Braga, Portugal). The hair of the mice was shaved at the implantation area, followed by disinfection with iodine (Life Technologies, Carlsbad, CA, USA). In each mouse, two skin incisions (1 cm

length) were made in the dorsal midline. The structures were then implanted subcutaneously at both right and left sides into the respective pockets. Six specimens were implanted. The mice were euthanized 2 weeks' post-surgery with an injection of Eutasil 200 mg/mL (pentobarbital sodium, Novavet, Braga, Portugal) and the implanted materials were retrieved along with the surrounding tissue. The explants were fixed with 10 % (v/v) formalin for at least 5 days at RT and transferred to histological cassettes for paraffin-embedding. Samples were then serially sectioned using a microtome (3.5 μ m thick) and stained with hematoxylin & eosin (HE).

Implantation in osteochondral defects

In Chapter 4, skeletally mature (12-14 weeks-old) New Zealand white rabbits, 2.5 ± 0.25 kg at the time of implantation (Charles River Laboratories, Saint-Germain-sur-l'Arbresle, France), were used. The rabbits were anesthetized with a subcutaneous injection of a mixture of ketamine hydrochloride 25 mg/Kg (Imalgene 1000 INJ 100 mg-mL, Novavet, Braga, Portugal) and medetomidine hydrochloride 0.15 mg/kg (Domitor INJ 1mg/mL, Novavet, Braga, Portugal). Then, both knees were shaved and disinfected with iodine (Life Technologies, USA). An internal para-patellar incision was performed to expose the knee and the patella was dislocated. A deep OC 5 mm defect in the trochlear groove of each knee was done using a drill of 5 mm diameter. The defect was filled with the structures or left empty as control. To finalize, the patella was reduced and the wound was closed. After recovery from surgery, animals were placed in individual cages and fed ad libitum. After 8 weeks, animals were anesthetized as described above, and euthanized through an intra-venous injection of pentobarbital sodium 200 mg/kg (Eutasil 200 mg/ml, Novavet, Braga, Portugal). In each knee, an internal para-patellar incision was made and the patella carefully dislocated to expose the defect region. Macroscopic pictures were taken and the explants of each knee were collected and stored in 10 % (v/v) formalin at 4 °C.

Histological analysis

HE staining is the most common histological staining, generally used for the analysis of tissue morphology. Hematoxylin is a natural cationic dye that stains blue the basophilic molecules in tissues, namely proteoglycans and DNA. Despite being used for the specific staining of nucleus, it can also unspecifically stain the cytoplasm of some cells and the mucin. Eosin Y is a synthetic dye of the xanthene family that colors in various shades of red acidophilic substances, the acidic proteins of the cytoplasm and connective tissue. Being acidic, eosin is an anionic dye, staining positively charged proteins in the

cytoplasm and in connective tissue. Eosin stains erythrocytes bright red/pink and collagen, muscle and cytoplasm varying shades of orange/pink.

In the developed studies described in Chapter 4, a progressive HE staining was chosen. The paraffin sections of the tissue samples were deparaffinized in xylene, followed by hydration in decreasing ethanol series to water. The tissue was immersed in hematoxylin for 8 min and the excess was thoroughly washed in running tap water for 10 min. Afterwards, the tissue was dehydrated through 96 % ethanol, stained with eosin for 2 min and mounted in Depex® (DPX).

Masson's trichrome (MT) staining is a mixture of three different dyes that selectively colors in red the muscle fibers, in light red the cytoplasm, in blue or green the collagen and dark brown to black the cell nuclei. The trichrome is applied by immersion of the fixated sample into Weigert's iron hematoxylin, and then three different solutions:

- Solution A that contains acid fuchsin, Xylidine Ponceau, glacial acetic acid, and distilled water.
- Solution B that contains phosphomolybdic acid in distilled water.
- Solution C that contains Light Green SF yellowish, or alternatively Fast Green FCF. It is used to stain collagen. If blue is preferred to green, methyl blue, water blue or aniline blue can be substituted.

Safranin-O was also used in Chapter 4 for the detection of cartilage since being a cationic stain binds to cartilage proteoglycans and GAG such as chondroitin and keratan sulfate. Histological sections of the explants were automatically stained with safranin-O (0.1 % w/v). Samples were then dehydrated and immersed in xylene, before being mounted. The stained sections were analyzed by microscopy.

Immunohistochemistry

Immunohistochemistry is a method for detection and visualization of specific antigens, with the same principle of immunocytochemistry, but applied to tissues. Relatively to immunocytochemistry, immunohistochemistry involves additional and sequential steps such as: antigen retrieval to unmask the antigen (masked by fixation) and enable antigen-antibody binding, and an incubation step with hydrogen peroxide for blocking tissue endogenous peroxidase activity, which may induce a high and non-specific background when using HRP conjugated antibodies.

In Chapter 4, sections of the explants were deparaffinized, immersed in an antigen retrieval solution and heated in the microwave for 2 min at maximum power. After cooling, the antigens were retrieved in 10 mM sodium citrate buffer solution at 95 °C for 20 min (pH 6.0), and then the endogenous peroxidases were inactivated by using 3 % (v/v) hydrogen peroxide solution. The SNA-lectin immunohistochemical

analysis was performed according to the streptavidin–biotin peroxidase complex system (UltraVision Large Volume Detection System Anti-Polyvalent, HRP; LabVision Corporation, USA). After deparaffinization and rehydration of the specimen slides, the antigens were retrieved in 10 mM sodium citrate buffer solution at 95 °C for 20 min (pH 6.0), and then the endogenous peroxidases were inactivated by using 3% (v/v) hydrogen peroxide solution. Then the slides were incubated in protein blocking solution for 10 min followed by incubation with the primary antibody SNA-lectin for 1 h and at RT. Sections were then sequentially washed with PBS and incubated with the streptavidin–peroxidase complex for 10 min. The immune reaction was visualized by 3,30-diaminobenzidine (DAB; LabVision Corporation, Fremont, CA, USA) as a chromogen. All sections were counterstained with Gill-2 hematoxylin (Merck, Germany). For preparing the negative controls, the primary antibody was omitted. The histological sections were then observed using a light microscope.

Statistical analysis

All formulations tested *in vitro* were studied performing triplicates in, at least, three independent rounds of experiments, and cultures on tissue culture polystyrene well plates were used as a positive control for cell viability.

Throughout the works performed under the scope of this thesis, statistical tools were employed to identify whether statistical differences existed between the parameters measured. GraphPad (GraphPad Software Inc., USA) and Microsoft Office Excel (Microsoft, USA) were used to perform statistical analysis. All results were presented as mean $\pm$ standard deviation (SD) of at least triplicates and then averaged. Biological results were performed at least in three independent experiments (n=3). SD is reported as a measure of sample deviation. When more than two groups and/or treatments were compared at the same time, one-way ANOVA test was applied using Tukey-Kramer method as a post-hoc pairwise comparison test. A p-value < 0.05 was considered statistically significant.

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CHAPTER 4

CONTROL OVER 3-D GRADIENTS

Based on the following publication:

Raphaël F. Canadas, Alain da S. Morais, Pedro Patrício, Luca Gasperini, Carlos A. Vilela, João B. Costa, Nuno A. M. Araújo, Rui L. Reis, Alexandra P. Marques, Joaquim M. Oliveira. Programmed density-based gradients in gels with spatial control over small molecules distribution and mineralization for 3-D tissue interfaces (Submitted)

4. PROGRAMMED DENSITY-BASED GRADIENTS IN GELS WITH SPATIAL CONTROL OVER BIOACTIVE FACTORS FOR 3-D TISSUE INTERFACES

4.1 Abstract

Interfaces play a vital role in nature, where gradients are a building base for distinct yet continuous integrated living tissues in complex organisms. Recently, the integration of heterogeneous interfaced tissues has been approached with the development of three-dimensional (3-D) multi-layered scaffolds. However, these are characterized by dissimilar interfaces, lacking continuous gradients with spatial control of the bioactive agents. Considering this, there is a lack of heterotypic but continuous engineered tissues with spatial control over biomimetic features. Here, we report the development of 3-D HAp gradients in gellan GG hydrogels. Gradients were spatially tuned in distribution and intensity, by programming the mixing of two gels by independently varying their temperatures, volumes and microparticles concentration. The experimental were supported by numerical simulations showing a reproducible control over HAp spatial distribution based on temperature gradients at the interface of the two gel layers. Furthermore, we demonstrated that both, microparticles movement and positively charged molecules diffusion during the hydrogel formation are independent. This way, spatially-controlled bioactive regions were engineered employing a simple, one-step fabrication method. As a proof of concept for OC regeneration, we studied the dependence of ALP activity by fat pad-hASCs cultured in a 3-D pre-designed gradient structure. The results showed that ALP differently responded to specific regions of the structure. Furthermore, this gradient of mineral deposition was maintained *in vivo*, supporting OC regeneration, which can potentially be applied to broad tissue interface modelling and regeneration.

Keywords: 3-D; gradient; interface; tissue engineering; cartilage; osteochondral

4.2 Introduction

In nature, interfaced tissues of gradient transitional phenotypes are critical for organ function¹. These features are frequently observed in the musculoskeletal system composed mainly of structural and mechanical gradients at the interface between hard and soft tissues. OC unit is one of these tissue

interfaces showing a gradient of mineralization from subchondral bone to cartilage with a phenotypic transition of calcified cartilage². This phenotypic transition typically consists of precise spatial arrangements of multiple cell types, matrix components and mineralization in hierarchical structures³. Specific functional roles are associated to these patterns, such as the controlled transmission of mechanical loads across limb joints to minimize stress at the junction of the two tissue types⁴.

To sustain the heterotypic cellular communications required for the proper functioning and homeostasis of such interfaces, a simple but robust control of the cells in the 3-D space is necessary^{1,5}. TE strategies have been proposed to engineer interfaces, which rely on the integration of multiple cell types⁶ and other factors such as bioactive microparticles⁷ or GFs gradients⁸ within multilayered 3-D scaffolds⁹. However, such layered constructs have limitations, usually resulting in dissimilar interfaced tissues due to differences across the supporting materials^{10,11}. Furthermore, while bioactive elements such as GFs and microparticles are important tools for engineering gradients, approaches based on particles are severely limited by poor spatial control of the fabrication methods employed to create the 3-D gradient structures^{12,13}. Therefore, there is an unmet need for controlled 3-D transitional environment using a method able to be translated to a large-scale production.

Here, we combine experimental and numerical methods to explore the possibility of generating 3-D gradient structures with different profiles by controlling the spatial dynamics of mixing of the two GG gels at two different temperatures. We provide evidence that when one of the gels incorporated HAp, in addition to their temperature and volume, the HAp concentration would allow programming the shape and intensity of the gradients. To assess the mixing dynamics when a fluid is injected into a second miscible phase of different density, fluid dynamics was studied focusing the effect of convection over both the polymeric and particle phases. The triggering of the osteogenic differentiation of ASCs from Hoffa's body within the generated 3-D gradient structures was evaluated to conclude about the mediation of stem cells response by the HAp gradient. These results were further validated upon implantation in rabbit OCDs by assessing the formation of new tissue representative of a phenotypic gradient.

4.3 Results

To control and fine-tune the gradient formation, the effect of temperature and viscosity of the gel solutions was analyzed. The viscosity of two GG solutions of the same concentration, one of which had dispersed HAp (figure 4.1A-i), was assessed along a range of temperatures (figure 4.1A-ii). When the shear rate was increased from 0.01 s^{-1} to 0.1 s^{-1} , the shear viscosity decreased. By contrast, the addition of 30% (w/w)

of HAp did not significantly affect the viscosity when changing the shear rate from 0.01 s^{-1} to 0.1 s^{-1} , above 50°C . Generally, while increasing the temperature from the sol-gel transition point at 45°C to 80°C , the shear viscosity decreased. The solution with HAp had higher viscosity at 45°C , but lower above this temperature compared to the solution without HAp (figure 4.1A-iii).

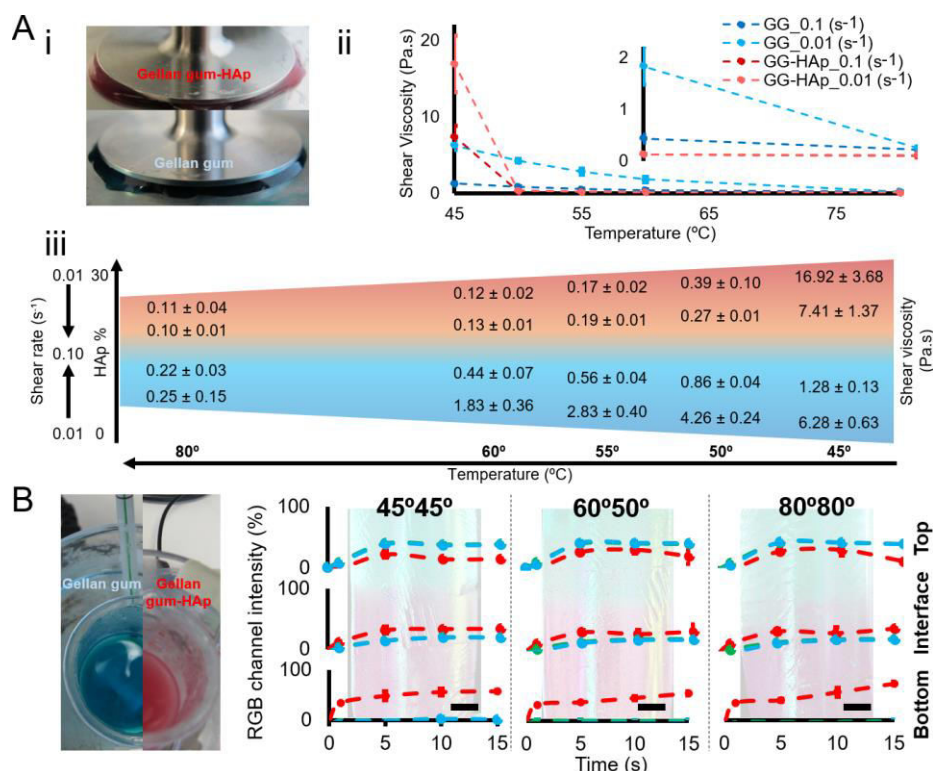


Figure 4.1 | Controlled fluid dynamics for hierarchical hydrogels formation. (A) The shear viscosity of GG solutions was characterized. (i) Two polymeric solutions at 2% (w/v) with and without HAp 30% (w/w) were prepared. (ii) The temperature was varied from 80°C to 45°C and the correlation with the shear viscosity was traced. (iii) Shear rate was tested at 0.1 and 0.01 s^{-1} and the correlation with shear viscosity was measured at several temperatures with or without HAp. (B) The solutions of pure GG 2% (w/v) and GG with dispersed HAp were stained with methylene blue and alizarin red, respectively. The mixture of gels was continuously recorded for 15 seconds to monitor the variation in the three regions of the structure at different temperatures (45°C , 60°C and 80°C). The color changing on the top, middle and bottom regions were traced over time as a RGB histogram using Image J ($n=5$). Scale bar = 1 mm.

To evaluate the influence of the temperature and the associated viscosity in the gel mixed layers, each gel solution was stained with red or blue dyes. Alizarin red was used to stain the HAp concentrated solution, while methylene blue was used as a dye for the HAp-free solution. Methylene blue was selected as a positively charged drug model that can interact with negatively charged GG. The red layer was firstly

injected into the mold, followed by the blue layer on the top of the first one. Changing the temperature of both layers showed no considerable differences in the diffusion of blue and red dyes (figure 4.1B). However, differences were observed in the top, interface and bottom layers. Methylene blue was confined in the top region, showing low mixing with the bottom one. Similar mixing ratios of Alizarin red and methylene blue were observed at the interface, meaning that both gels were mixed in this region. Furthermore, this observation showed that if a positively charged drug similar to methylene blue was incorporated, it would be predominantly distributed in the layer where it was initially incorporated. On the other hand, the Alizarin red stained HAp showed diffusion towards the top region, which was correlated with the lack of charge of HAp allowing the diffusion of the particles to cross the interface. This observation was then corroborated by the results summarized in figure 4.2.

To confirm that the mixing of dyes, at different temperatures, corresponded to the polymer and HAp, the fluorescence of GG-FITC (gel 1) and the autofluorescence of HAp (gel 2) was analysed and traced (figure 4.2A and B). The profile of GG-FITC corresponded to the methylene blue distribution profile. Although the polymeric components mixed at the interface, the interpenetration in the adjacent gel was low. Interestingly, the HAp diffused through the whole hydrogel independently of the mixing of the two gels creating different distribution profiles. When the temperature of the gel incorporating HAp was lower, the interface region increased in size.

To evaluate the HAp gradient formation dependence with temperature, the profile of HAp throughout the structures was quantified by micro-CT and a polynomial regression was adjusted to HAp obtained distribution (figure 4.2B). By changing the temperature of each gel, a different HAp profile was observed. When both gels were at 45 °C, which is the temperature around the sol-gel transition point of GG, a gradient with a high slope of the HAp profile at the interface was formed. When the first gel was at a higher temperature (60 °C) than the second one (50 °C), the formed HAp gradient had an approximately linear profile. When both gels were at similar but much higher temperatures (80 °C), the HAp dispersed through the whole structure before the complete gelation, resulting in an almost homogeneous distribution of the particles along the structure. Note that in the latter case, the time for gelation is longer. The temperature of the two polymeric solutions was changed to assess the effect on the HAp gradient formation (figure 4.2C). The resultant HAp profiles were quantified by micro-CT. Each combination of temperatures generated similar mixing profiles for each dye, but different HAp distribution profiles. Macroscopic observations showed that the mixing of both phases was larger at higher temperature. The formation of a HAp gradient-shaped profile was predominantly achieved when applying different temperatures on top and bottom layers, as shown in the cases of 60°50 °C and 50°60 °C. In the case of

the particles, the convective thermal forces promoted by the difference between the applied temperatures had a direct influence on their distribution, creating different profiles, while GG mainly kept in separated layers.

At lower temperatures (45 °C), the gelation occurred rapidly thus stopping the mixing of the two solutions and decreasing the mobility of HAp, which kept concentrated at the origin. When the temperature of both solutions was increased up to 80 °C, their viscosity decreased resulting in larger interface region allowing HAp to freely diffuse in the whole hydrogel. When the temperature of one solution was increased relatively to the other, their mixing occurred under the influence of a thermal gradient and the distribution profile of HAp was shaped by the gradient of temperature.

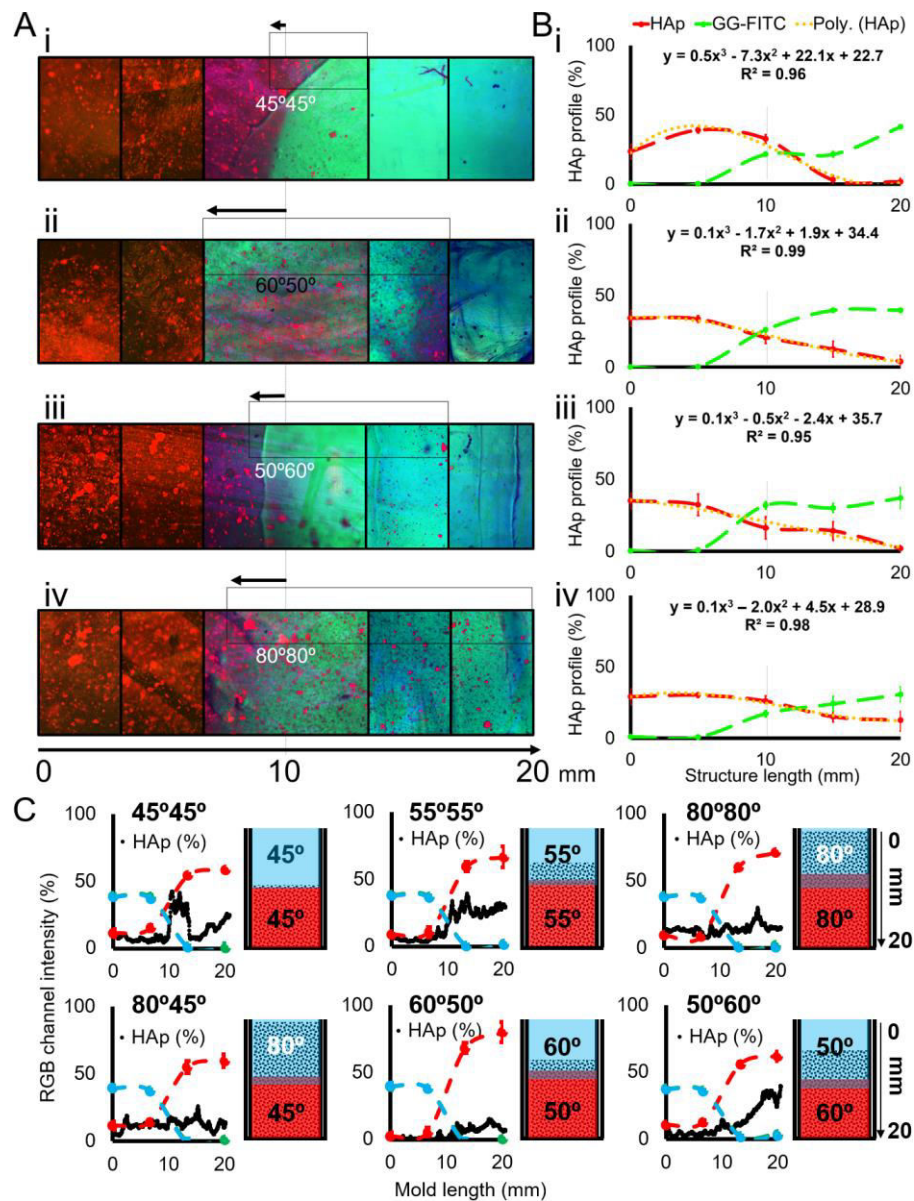


Figure 4.2 | Gradients patterned by HAp controlled dispersion in 3-D structures programmed by gels viscosity/temperature. (A) The mix of both layers at four different

temperatures, (i) 45°-45 °C, (ii) 60°-50 °C, (iii) 50°-60 °C, and (iv) 80°-80 °C, was tracked by fluorescence microscopy using a GG modified with FITC (green) and the auto-fluorescence of HAp (red) (scale bar = 5 mm). A picture of the whole sample was acquired after 3 min of the layering of the two gels (scale bar = 500 μ m). (B) The gradient profile of HAp and the polymeric distribution of GG-FITC were checked and quantified using Image J in 5 representative regions along the hydrogel, 3 samples per condition (n=3). The profiles of HAp and GG-FITC distributions through the 3-D structure were traced for the different mixing temperatures ((i) 45°-45 °C, (ii) 60°-50 °C, (iii) 50°-60 °C, and (iv) 80°-80 °C) and polynomial regressions (yellow dashed line) were calculated according to each profile. (C) Different pairs of temperature were tested to observe the mixed colored phases at different viscosities. By changing the temperature of each phase, e.g. 45°45°, 45°80°, 60°50°, the distribution of dye and HAp were macroscopically registered and assessed by micro-CT, respectively, through the mold height and graphically and schematically represented.

In order to evaluate the role of diffusive convection in the move of HAp in the confined polymeric phase, a study was performed combining experimental modulation (figure 4.3A) and numerical analyses (figure 4.3B). Both approaches documented the formation of HAp circulating patterns in the form of rings in the top layer of the hydrogel, promoted by convective streams. The circulation cells in the numerical modulation overlap with the circulating streams of HAp in the mixture observed during the experimental modulation.

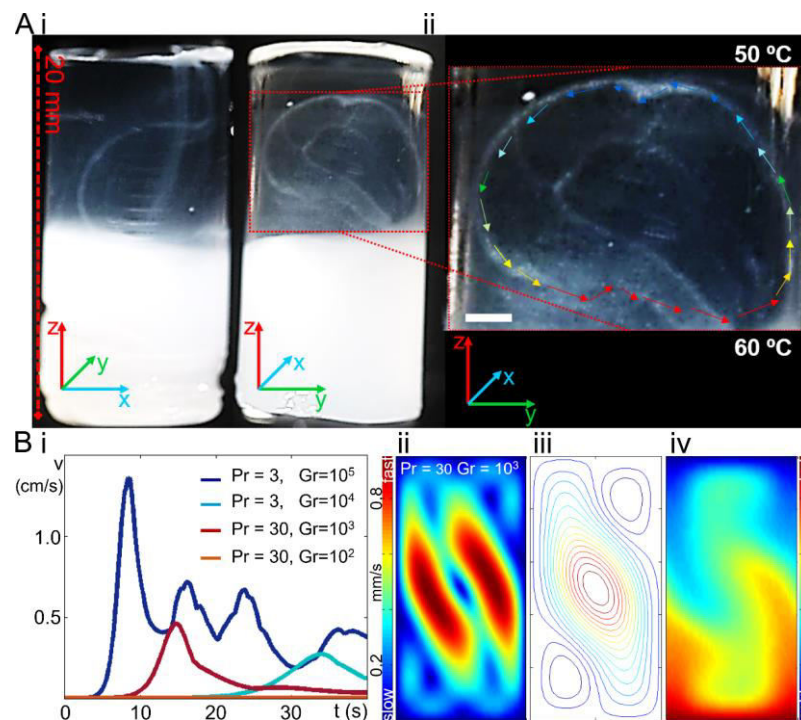


Figure 4.3 | Evidence of convection-driven HAp dispersion in the experimental and simulation models. (A) From the experimental modulation of the condition 50°60 °C, the set of

microscopic observations allowed to capture and schematically represent the HAp distribution (scale bar = 1 mm). (B) Numerical results showed (i) the velocity profile of convective streams in a water-like system (blue lines) and a gel-like system (red lines). (ii) The colored graph for the velocity of simple convection showed the regions of faster streams (red) and regions of slower streams (blue). (iii) The streamlines of convection were traced which reflect their speed according to lines density (higher density of streamlines reflect higher speeds). (iv) The convective streams are promoted by the gradient of temperatures, from hot (red) to cold (blue).

For a colloidal suspension such as GG-HAp, with approximately the same physical properties of water, but with a very different viscosity, it is interesting to note that $Gr \sim 1/\mu^2$ and $Pr \sim \mu$. If the viscosity is 10 times larger, $Gr = 103$ and $Pr = 30$ (figure 4.3B-i). This condition still triggers natural convection. However, if the viscosity of the solution is 100 times larger, then $Gr = 10$ and $Pr = 300$, and natural convection is suppressed (data not shown).

Having observed and modulated the effect of the fluid and HAp dynamics with the temperature of the gels, we selected the condition 50°60 °C to demonstrate the control over the HAp gradient slope, either by changing the volumes of each gel or the concentration of HAp in one of the gels (figure 4.4).

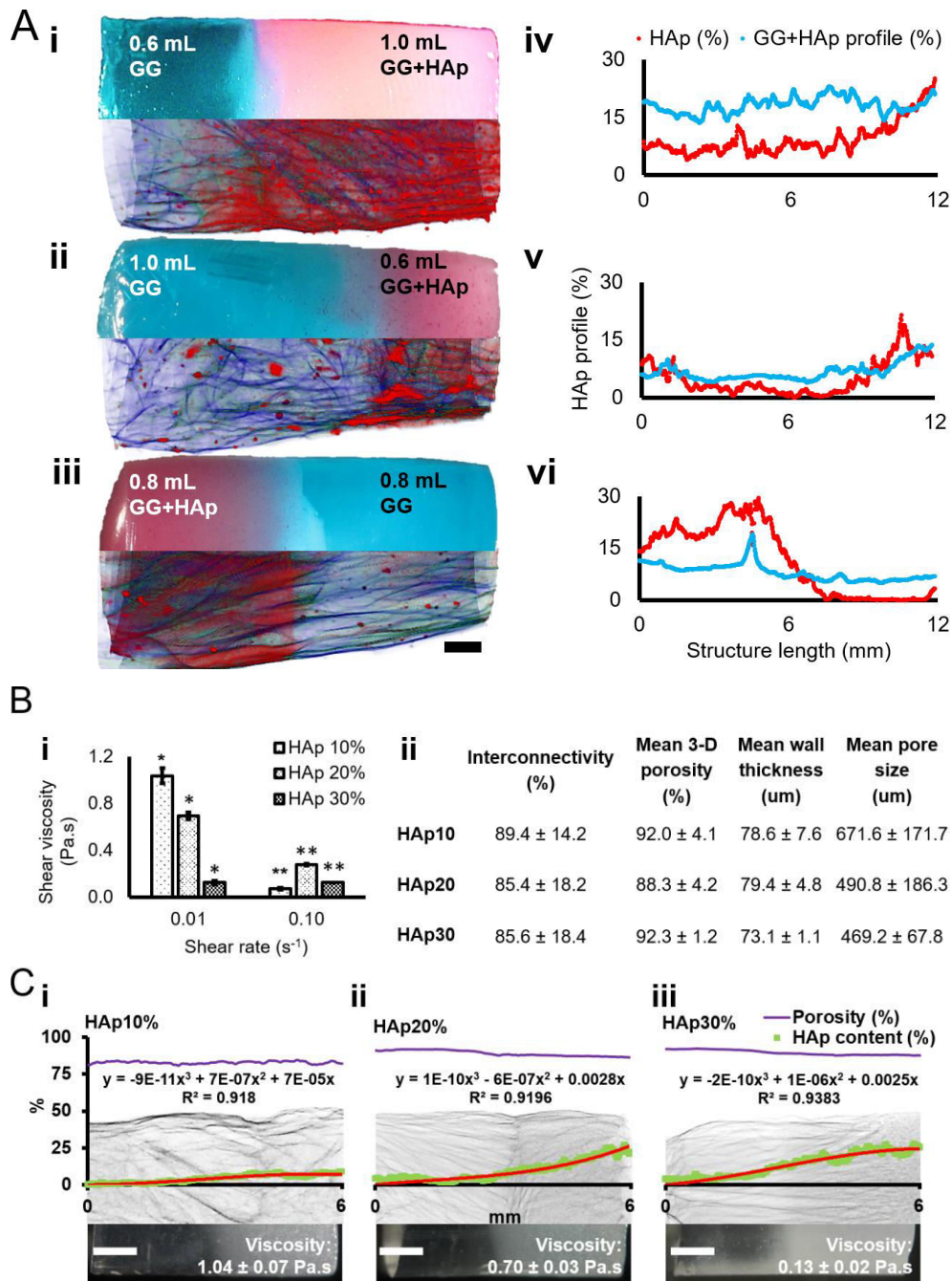


Figure 4.4 | Control of the 3-D gradient by the volume of the gels and HAp concentration.

(A) The gradient formed with gels at 50° and 60 °C was tuned by changing the volume of each layer: (i) 0.6 mL – 1.0 mL, (ii) 1.0 mL – 0.6 mL, (iii) 0.8 mL – 0.8 mL with the inversion of injection steps. The structures were analysed by micro-CT (scale bar = 1 mm) and the profiles ((iv), (v), and (vi)) of the distribution of the HAp (red line) and of polymeric and ceramic phases together (blue line) were traced.

(B) Three different concentrations of HAp (10%, 20%, and 30%), whose (i) viscosity at 0.01 s^{-1} and 0.10 s^{-1} was measured, were considered for the HAp-containing gel to control the formation of the gradient ($n=3$, $*p<0.05$). (ii) The interconnectivity, porosity, wall thickness, and pore size ($n=3$) and the (C) HAp and porosity distribution of the structures were quantified by micro-CT (scale bar = 1 mm). For each profile of

HAp distribution at (i) 10, (ii) 20, and (iii) 30%, a polynomial regression for the different slopes of the formed gradients was traced ($n=5$, $*p<0.05$). Data expressed as (Mean $\pm$ SD).

To assess the ability to control the gradient by changing the volumes of both injected gels, such as 0.6 mL + 1.0 mL, 1.0 mL + 0.6 mL, and 0.8 mL + 0.8 mL inverting the injection of each gel, micro-CT was used (figure 4.4A). When changing the volume of HAp-free solution to 0.6 mL and of HAp-containing one to 1.0 mL, or vice-versa, the microparticles gradient was wider or narrower, respectively (figure 4.4A-iv and A-v). When injecting the HAp-free solution first, keeping the same setting of temperatures, the HAp distribution profile was inverted (figure 4.4A-vi).

To understand the control of the gradient with different amounts of HAp (10%, 20% and 30% (w/w)), we assumed that it would directly correlate with the viscosity of the HAp-containing solutions (figure 4.4B-i). At a steady state condition (0.01 s^{-1}), the increase of HAp content significantly decreased the shear viscosity. This decrease was potentially correlated with a lower interaction between GG chains spaced by HAp. At 0.1 s^{-1} , the viscosity significantly increased up to 20% (w/w) of HAp. The different viscosities of the HAp-containing gels mainly influenced the pore size of the structures as demonstrated by micro-CT (figure 4.4B-ii). Overall, the interconnectivity, porosity and wall thickness were not significantly affected. Although, the pore size decreased with the increasing amount of HAp from 671 to 490 and 469 μm . Interestingly, the HAp distribution was affected by the amount of particles; when the concentration of HAp was increased, the slope of the profile was more pronounced. A polynomial regression was adapted to each condition therefore the exact HAp concentration in each region of the gradient can be predicted (figure 4.4C).

Considering that the optimal HAp gradient control was obtained in the structures formed with a GG+30% HAp gel at $60\text{ }^{\circ}\text{C}$ and a second gel of GG at $50\text{ }^{\circ}\text{C}$, the influence of the generated gradient over their bioactivity was analysed under the SEM and EDS. The structures were soaked in a SBF revealing the formation of an apatite layer, after the period of 14 days. The apatite layer was confirmed in the initial HAp-containing region and disappeared in the HAp residual one showing that the gradient was maintained (figure 4.5A). This was confirmed with the SEM/EDS analysis to quantify phosphorus and calcium in different regions of the scaffold. The absence of phosphorus in the low HAp region could be observed, meaning that no apatite layer was formed, while the high HAp region showed a bioactive behavior after soaking in SBF solution.

Additionally, the correlation between the mechanical features of each region of the 3-D gradient structures was assessed after DMA (figure 4.5B). The values at 1 Hz were highlighted since this is a meaningful

frequency for mechanical stimuli, e.g. the walking step frequency¹⁴. While the loss moduli (G'') were approximately constant along the structure, the energy dissipation ability ($\tan \delta$) decreased from 0.32 to 0.26 was registered for the low- (originally HAp-free) to the high HAp-containing regions, respectively, meaning that the first one has higher capability to disperse energy under mechanical compressive stress.

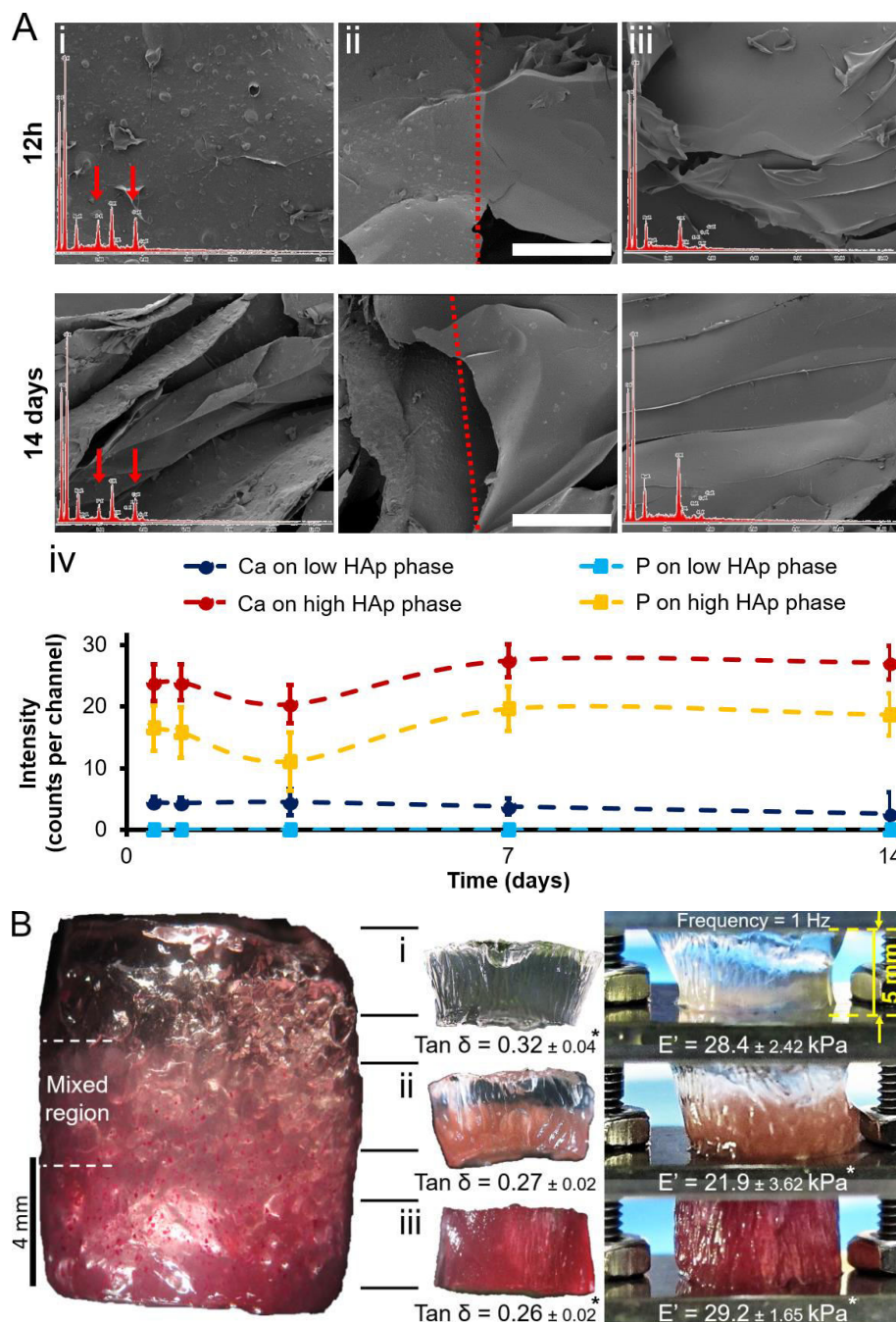


Figure 4.5 | Mediation of the transition from bioactive to non-bioactive, and mechanical properties character by the HAp gradient within the 3-D structures. The structures formed with a GG+30% HAp gel at 60 °C and a second gel of GG at 50 °C were analysed, (A) After being immersed in SBF solution for 12 h and over a period of 14 days at 37 °C, SEM was used to identify the (i) original HAp-containing layer, (ii) the interface region, (iii) the original free-HAp layer, while (iv) EDS allowed to

trace the profile of phosphorus (P) and calcium (Ca) concentrations per region over 14 days (3 counting squared areas per region and per structure; n=3 structures; scale bar = 500 μ m). (B) The dynamic mechanical behavior of (i) top, (ii) middle, and (iii) bottom regions was followed to obtain the storage and energy dissipation ($\tan \delta$) of the respective regions of the 3-D structures (n=3). Data expressed as Mean $\pm$ SD (n=4, *p<0.05).

To assess the cell behavior within the structures formed with a GG+30% HAp gel at 60 °C and a second gel of GG at 50 °C Hoffa-ASCs were seeded onto the scaffolds and cultured for 14 days (figure 4.6A). Cells were able to adhere and distribute evenly within the whole structure (figure 4.6A-ii) and to proliferate along the time of culture (figure 4.6A-iii and A-iv) without relevant cell death (figure 4.6A-v).

The ALP activity increases when osteogenic differentiation is triggered¹⁴. So, to assess the effect of the HAp gradient over cell ALP activity, constructs were cultured in osteogenic and chondrogenic (as a negative control) media (figure 4.6B-i). A significant increase in ALP activity was observed in relation to the control condition in both HAp-rich and -residual regions of the gradient. An increase of ALP activity was also detected in the HAp-rich region in relation to the HAp-residual one (figure 4.6B-ii).

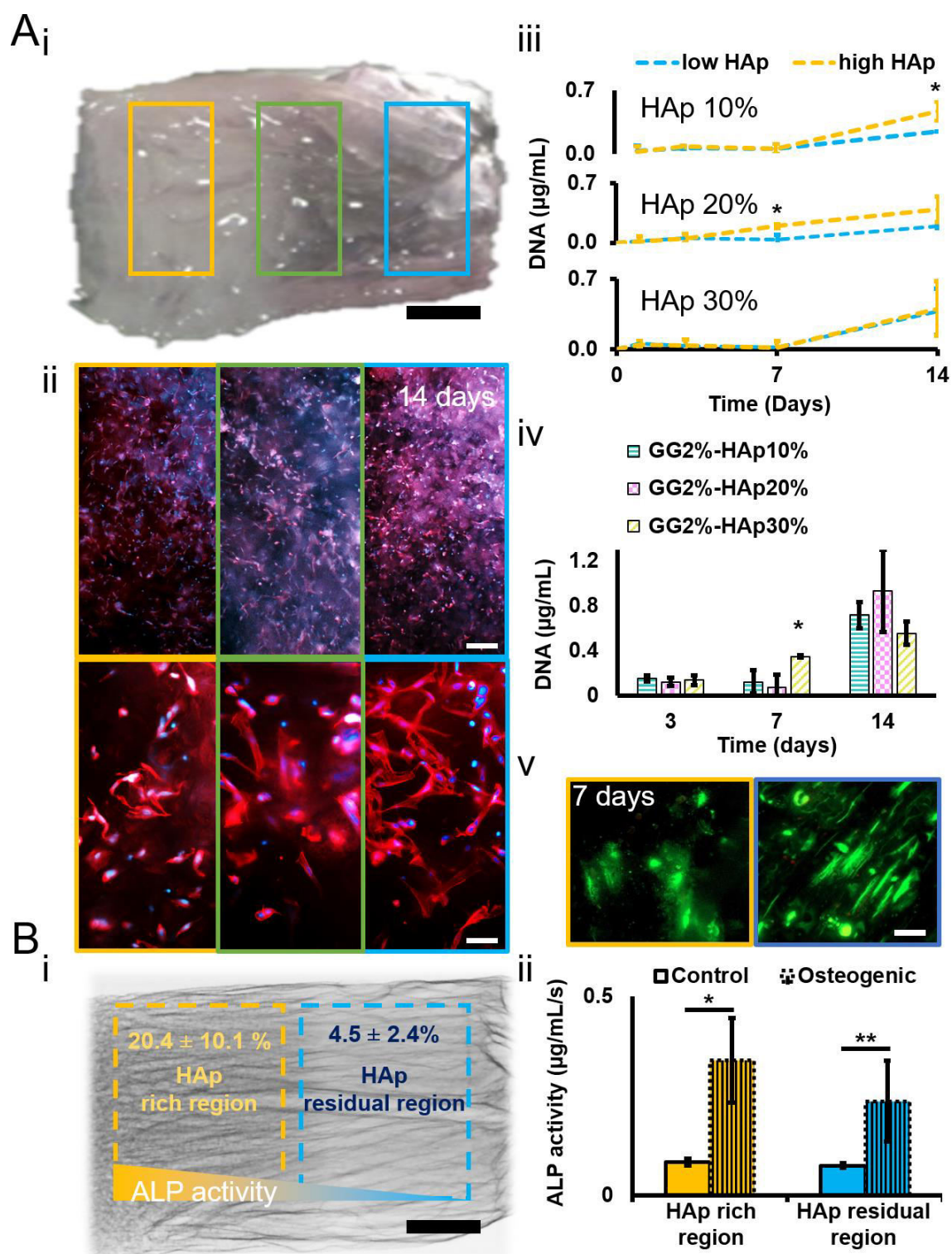


Figure 4.6 | Mediation of stem cells response by the HAp gradient within the 3-D structures.

(A) hASCs stem cells were seeded in the structures formed with a GG+30% HAp gel at 60 °C and a second gel of GG at 50 °C (i) The obtained constructs were divided into three different regions (scale bar = 1 mm). (ii) Analysis of cell distribution after cytoskeleton staining with rhodamine-Phalloidin (red) and nuclei counterstaining with DAPI (blue) (scale bar = 200 μm). Higher magnification images are presented in the lower row of the panel (scale bar = 50 μm). (iii) Cell distribution was also inferred after quantification of

the DNA content in each half of gradient 3-D structures after 14 days of culture. (iv) Overall cell proliferation was determined by quantification of DNA along 14 days of culture. (v) The viability was assessed by live-dead assay using calcein-AM (green) and PI (red, scale bar = 50 μ m). (B) Osteogenic and chondrogenic differentiations were performed over 21 days in the HAp gradient constructs. (i) Both halves with higher and lower concentrations of HAp were exposed to osteogenic and chondrogenic (control) media. (ii) The effect of the HAp gradient on ALP activity was quantified and normalized by the DNA content. Data expressed as Mean $\pm$ SD (n=9, *p<0.05).

To evaluate the level of the inflammatory response provoked by the 3-D gradient structures, a subcutaneous implantation was performed in mice (figure 4.7A). The histological analysis showed no acute inflammatory response and few host cells or tissue infiltrating the 3-D gradient structures. Moreover, the HAp gradient was kept over the 14 days of implantation.

To assess the effect of the gradient structures in the formation of new tissue representative of a phenotypic gradient, as the OC unit from the subchondral bone to the cartilage passing by a calcified cartilaginous interface, the structures were implanted in a rabbit OCD (figure 4.7B). To assess the formation of hard and soft tissues, micro-CT was performed (figure 4.7B-i). After 8 weeks, the implanted structures properly integrated the host tissue as it can be observed at the junction of the neo-cartilage with the host cartilage. Softer tissue was mainly located in the top cartilaginous zone, while in the control the soft tissue was entering the subchondral region.

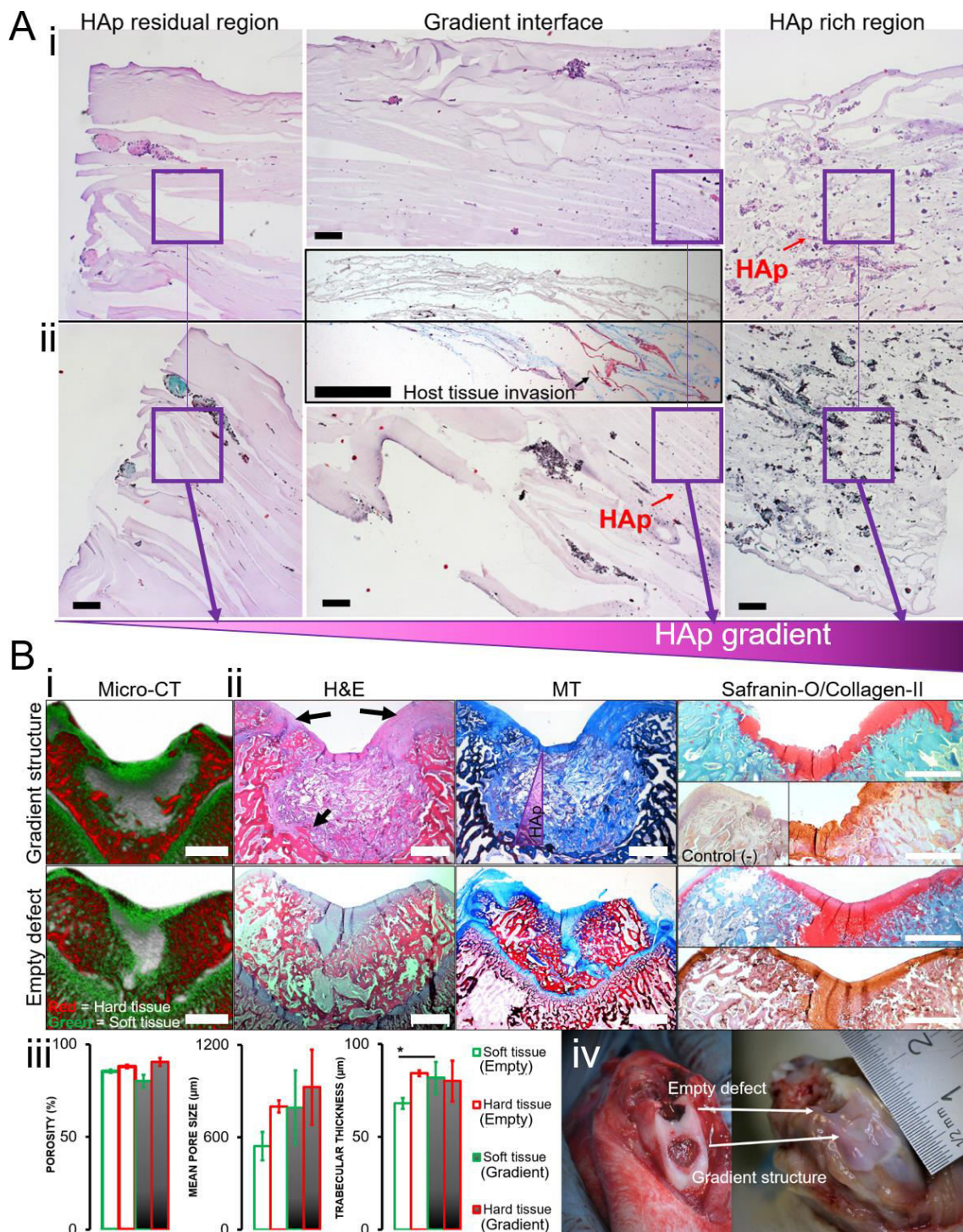


Figure 4.7 | *In vivo* behavior of gradient 3-D structures implanted subcutaneously and in an OCD. (A) The subcutaneous implantation in mice was performed to assess the level of the inflammatory response provoked by the 3-D gradient structures. (i) Hematoxylin and eosin (H&E), and (ii) MT stainings of retrieved samples were performed. HAp-rich, interface, and HAp-residual regions were magnified (scale bar = 200 μm). (B) Acellular 3-D gradient structures were implanted in OCDs in rabbits. Empty defects acted as control. The new tissue formation 8 weeks after transplantation was (i) reconstructed by μ-CT identifying the hard (red) and soft (green) tissues by applying a color histogram

(hard tissue < 100; soft tissue > 100). (ii) Tissue sections were further stained with H&E, MT, and safranin-O, respectively to assess cell invasion into the 3-D gradient structure, and the glycosaminoglycans content of the new tissue. Immunohistochemical staining was performed to assess the collagen-type II content in the newly formed tissue. The empty defect was similarly stained to be compared with the condition under study as a control. Arrows are pointing the integration of neotissue with host tissue. (iii) The explant was analysed by μ -CT to assess porosity, mean pore size and trabecular thickness were quantified in both soft and hard tissues (bars with green and red lines, respectively) of the condition with implanted structures and the control (bars filled in black and white, respectively). (iv) Tissue explants were macroscopically assessed by comparison of the experimental and control conditions. Data expressed as Mean $\pm$ SD. (n=3, *p<0.05, the bar indicates the conditions which are compared to the significantly different condition).

To assess the ECM composition in the newly formed tissue, histological analyses were performed (figure 4.7B-ii). The architecture in the experimental condition was closer to the native subchondral bone, corroborating the observation in the μ -CT assessment. Safranin-O staining showed the prevalent presence of glycosaminoglycan in the cartilage. Furthermore, the deposition of collagen type II was located in the cartilage region, while the empty defect was filled with collagen type II, also invading the subchondral bone. To quantitatively analyse the architecture of the explants (tissue plus remaining implanted structure), μ -CT was performed (figure 4.7B-iii). The mean pore size was superior in the experimental condition, in which the structure was not invaded by fibrous tissue in opposition to the empty control. The 3-D gradient structure supported the OC interface recreation *in vivo*, by defining the localized maturation of cartilaginous matrix in the upper region of the defect (figure 4.7B-iv).

4.4 Discussion

Photolithography¹⁵, chemical bonding¹⁶, and bioprinting¹⁷ have been applied to spatially control bioactive factors in hydrogels. However, the first is limited to thin translucent structures, the second can have cytotoxic side effects because of the applied chemistry, and the last lack resolution at the microscale and may suffer from fiber fusing and collapsing. Furthermore, the previously enumerated methods require sophisticated technologies or techniques, so we propose a simpler way to incorporate bioactive molecules by encapsulation within polymers before their gelation or solidification in 3-D hydrogels. This method avoids biomaterials modifications allowing to keep optimized properties¹⁸. However, the current methods

for hydrogel fabrication lack spatial control over biological agents' distribution, as the precise navigation of microscale objects at high temperature is extremely challenging due to the combination of Brownian motion and strong viscous force¹⁹. We could overcome this issue by understanding fluid dynamics effect over uncharged microparticles location, applying convection to generate a driving force exceeding gravity and viscous friction.

Previously, the mixing of aqueous systems has been shown to be controlled by each solution temperature²⁰, which affects the viscosity of polymeric solutions²¹. In the case of GG, the polysaccharide has its sol-gel transition between 40 °C and 45 °C²², decreasing the viscosity when increasing the temperature from this point on. Two different dyes were incorporated to understand the mixing of two gel solutions at different temperatures. Methylene blue was selected as a model drug^{23,24}, allowing to modulate drug incorporation and investigate its spatial diffusion along the gradient structure. The dye was spatially confined to half hydrogel, meaning that a spatial control is achievable when small molecules are incorporated. This was confirmed when, GG modified with FITC was used to compare the location of the dyes with the mixing of the polymer itself, and similar profiles were observed even under the combination of solutions at different temperatures. Methylene blue was electrostatically bonded to GG, and therefore the electrostatic forces were more relevant than diffusion or convection. Furthermore, the mix of polymeric phases was possibly inhibited by the negative charges of GG, which was previously reported for other polymeric systems²⁵. So, mixing was occurring only in the interfacial zone promoted by convection. In fact, when the first injected layer was warmer than the second one, the interface moved into the layer of higher temperature and when the top (second) layer was warmer than the first one, the interface kept at the half length of the mold.

Interestingly, while both layers showed a low mixing ability, the HAp microparticles could cross the interface region from the bottom to the top layer. Mass transport (of particles) in a fluid can either be diffusive or advective, in which matter or/and heat is transported by the larger-scale motion of the convective streams in the fluid²⁶. A considerable class of spatial patterns in fluids have been structured due to thermal effects, which trigger hydrodynamic instabilities²⁷. For example, well-known instabilities triggered by thermal effects, such as Rayleigh-Bénard convection, are often found in systems with well-defined initial conditions²⁸. In this work, when the temperature difference between solutions increases, the Brownian motion is superseded by convection and the transport of heat matched the transport of matter (HAp). We found that this phenomenon was dependent on the temperature of each GG solution, which relates to its viscosity. So, HAp exhibited a different behavior compared to methylene blue since it was in a crystalline form with no charge. By applying the Rayleigh-Bénard convection in colloidal systems²⁸,

we confirmed that HAp moves freely through the molecular chains of GG driven by the convective streams. Then, we modulated the convective streamlines to achieve a predictable distribution of microparticles for future approaches. We found that the circulation of convective cells in the bilayered structure were dependent on the relation between the thermal gradient and the fluid viscosity. Thus, when the thermal gradient increased, overcoming the friction promoted by the fluid viscosity, the convection streams drive the microparticles in circular motion. This microparticles distribution pattern was programmed by means of controlling each solution temperature and volume, and/or particles concentration, as initially hypothesized, preserving 3-D polymeric organization and interesting mechanical properties for application on interfaces TE, such as soft-to-hard tissue junctions²⁹. Overall, these observations and modulations provide a controllable tool to predict uncharged microparticles distribution in a gel, while having two or more charged small molecules with spatial control per layer.

In the past, when thermal gradients were introduced in the presence of an initial well-defined density gradient, the formation of distinct layered patterns was observed due to double-diffusive convection in colloids³⁰, but rarely translated to biological systems. In this study, we showed the ability to control the formation of microparticles gradients driven by convection, applying the fabricated structures to OC tissue regeneration. In the particular case of the studied system, the formulation resulting from combining GG solutions at 50°-60 °C on top and bottom layers, respectively, was selected considering the calcified gradient characteristic of the OC tissue³¹. The gradient of HAp resulted in structures with bioactive-to-non-bioactive properties, interesting for bone-cartilage interface regeneration, as supported with hASCs cultures and the differences in ALP activity, an *in vitro* bone formation index³². Moreover, this feature was further confirmed in a OC regeneration model. The structures were crucial to keep distinguishable ECM deposition and tissue organization in the bone-to-cartilage transition. The supported formation of hyaline-like neo-cartilage occurred at the same time as the penetration of fibrous tissue into the subchondral bone region was avoided. Nonetheless, the subchondral bone was not regenerated as fast as the formation of neo-cartilage, which is mostly likely due to the relative dimensions occupied/to be regenerated, much smaller for cartilage. Future work, might also address the hypothesis that faster subchondral bone formation can occur if structures of lower polymeric concentration are implanted.

While the present work shows the relevance of HAp gradients for bone-to-cartilage interface regeneration, the proposed methodology to generate 3-D gradient structures can potentially be translated to other interfaced tissues. The inclusion of small molecules, proteins or other bioactive factors, such as gene vectors in a spatially controlled way is of major interest in different contexts. The main challenge to address is related with the effect of high temperatures in the GG system, which can interfere with some

bioactive agents, such as GFs. However, further developments are being performed to modify GG decreasing its sol-gel transition point, allowing to replicate the present system at lower and physiological temperatures.

In conclusion, the present study provides a simple way of controlling microparticles distribution in hydrogels by induced convection. The performed modulation showed how temperature, layer volumes, and particles distribution can be programmed for spatial control of bioactive molecules and particles, creating patterns in 3-D environments with potential utility in fundamental stem cell studies, drug testing, graft fabrication, and of broad interest for interfaces design potentially useful from architecture to energy infrastructure.

4.5 Material and methods

Materials. Unless otherwise stated all the reagents were purchased from Sigma-Aldrich.

Hydroxyapatite microparticles (HAp) synthesis. HAp was prepared by precipitation reaction of calcium hydroxide (Riedel-de Haën, Germany) and ortho-phosphoric acid 85% (Panreac, Spain) solutions in an aqueous system at RT. The mixing was gently performed at pH 11, adjusted by addition of concentrated ammonium hydroxide (1 M, Riedel-de Haën, Germany). The ceramic powder formed was sieved and particles with less than 263 μm were used for the present study.

Rheological analysis. Viscosity tests were performed using a Kinexus Pro+ rheometer (Malver, UK). Samples were directly poured from the flask on a cone-plate geometry (40 mm) and the excess of material trimmed with a spoon and paper. A low viscosity oil solution was poured around the GG solution inside the cone-plate to avoid evaporation during the measurements at high temperatures. All data was obtained after an imposed shear rate of 100 s^{-1} to mimic the perfusion by injection and reset the deformation history of all samples. Viscosity data was obtained for single point measurement at shear rates of 0.01 to 0.1 s^{-1} . Additionally, ramps were performed ranging the shear rates from 0.01 to 0.1 s^{-1} . This range, being the typical range of leveling phenomena, was chosen to mimic the interface of gels mixing at a steady state. The process was sequentially repeated for 45, 50, 55, 60, and 80 $^{\circ}\text{C}$.

Experimental modulation of gels mixing at interface. The mixing of two gel layers was continuously monitored and the corresponding graphs were traced by measuring the color mixing over

15s. Pure GG or GG with dispersed HAp were dissolved in distilled water at 90 °C for 30 min at 2% (w/v). The gel phase made of GG and HAp was stained with alizarin red, and the pure GG phase was colored with methylene blue. After stabilization at the target temperature, e.g. 80, 60, 55, 50, or 45 °C, the first layer was injected in a transparent silicon mold (20 x 8 mm) at 0.44 mL/s, followed by the second one which was slowly injected at a flow rate of approximately 0.02 mL/s. A camera was placed for the continuous recording of the mixing of the two layers. Images at 0, 1, 5, 10, and 15 seconds were selected for the analysis. Image J was used to quantitatively evaluate the mixing gels from the RGB histograms of red and blue phases, over time, and at the bottom, interface and top regions of the mold.

Numerical modulation of gels mixing at interface. We considered a Newtonian fluid of density ρ and temperature T . For simplicity, we assumed that temperature gradients are much lower than $1/\beta$, where β is the volumetric coefficient of thermal expansion. In this limit, the dependence of the fluid density on the temperature may be approximated as $\rho = \rho_0(1 - \beta(T - T_0))$, where ρ_0 is the fluid density at temperature T_0 . The dynamics of the fluid is then described by the following set of equations,

$$\vec{\nabla} \cdot \vec{v} = 0, \quad (1)$$

$$\frac{\partial \vec{v}}{\partial t} = -(\vec{v} \cdot \vec{\nabla})\vec{v} - \beta(T - T_0)\vec{g} - \frac{1}{\rho_0}\vec{\nabla}p' + \frac{\mu}{\rho_0}\nabla^2\vec{v}, \quad (2)$$

$$\frac{\partial T}{\partial t} = -(\vec{v} \cdot \vec{\nabla})T + \alpha\nabla^2T, \quad (3)$$

where $\vec{v}$ is the velocity field, $\vec{g}$ is the gravitational acceleration, $p' = p - p_0$, p is the pressure, $\vec{\nabla}p_0 = \rho_0\vec{g}$, μ is the dynamical viscosity, and α is the thermal diffusivity. We assumed that μ and α are constants.

We considered a two-dimensional (2-D) simulation box, with the following boundary conditions: the velocity is zero along the boundaries, the temperature is T_0 at the top, $T_1 > T_0$ at the bottom, and periodic along the horizontal direction. In 2-D a stream function ψ can be defined such that

$$v_x = -\frac{\partial \psi}{\partial y}, \quad v_y = \frac{\partial \psi}{\partial x}, \quad (4)$$

where v_x and v_y are the horizontal and vertical components of the fluid velocity, what guarantees that the continuity equation (1) is satisfied.

We rescaled the dimensions of the problem using $x^* = x/L$, $y^* = y/L$, where L is the width of the box, $\vec{v}^* = \vec{v}/V$, V is a characteristic (absolute) velocity, $t^* = tV/L$, and $T^* = (T - T_0)/(T_1 - T_0)$, with $0 < T^* < 1$. The adimensional dynamical equations are then reduced to (omitting the asterisks):

$$\frac{\partial w_z}{\partial t} = -(\vec{v} \cdot \vec{\nabla}) w_z + \frac{1}{\text{Re}} \nabla^2 w_z + \frac{\text{Gr}}{\text{Re}^2} \frac{\partial T}{\partial x}, \quad (5)$$

$$\frac{\partial T}{\partial t} = -(\vec{v} \cdot \vec{\nabla}) T + \frac{1}{\text{Pr}} \frac{1}{\text{Re}} \nabla^2 T, \quad (6)$$

where the vorticity is related to the fluid velocity and the stream function by

$$w_z = \frac{\partial v_y}{\partial x} - \frac{\partial v_x}{\partial y} = \nabla^2 \psi. \quad (7)$$

The Reynolds (Re), Grashof (Gr), and Prandtl (Pr) numbers are defined as

$$\text{Re} = \frac{VL}{\nu}, \quad \text{Gr} = \frac{\beta(T_1 - T_0)gL^3}{\nu^2}, \quad \text{Pr} = \frac{\nu}{\alpha}, \quad (8)$$

where $\nu = \mu/\rho_0$ is the kinematic viscosity.

These equations were integrated in a rectangular box of height $L_y = 2L$, starting from an initial adimensional temperature and stream function fields, given by $T = \tanh((1 - y)/\Delta + 1)/2$ and $\psi = \delta(1 - \cos(2\pi x))(1 - \cos(\pi y))$, respectively, where $\Delta = 10^{-1}$ and $\delta = 10^{-3}$. So, initially, the temperature is $T = T_1$ at the lower half part of the box, $T = T_0$ at the upper half, and the width of the interface is Δ . The initial ψ insures null velocity at the boundaries and breaks the symmetry in order to trigger any possible instability in the fluid flow.

We used a discretized spatial mesh with $N_x \times N_y = 50 \times 50$ points, and an explicit time integration scheme with a time step $\Delta t = 0.2 / (N_x - 1)^2$ to insure numerical stability. The terms involving the spatial derivatives were calculated through second-order finite differences. At each time step, the new vorticity and temperature fields were determined from eqs. (5) and (6), then Poisson eq. (7) was solved to obtain the stream function. The velocity field was updated according to eq. (4).

To find the temperature regimes that trigger convection, the values of the GG density and viscosity were considered. For the thermal conductivity, we assumed the one of water, since the polymeric phase is 2% weight in the volume of distilled water.

First, we considered a water-like system, at $T_0 = 50^\circ\text{C}$, in a box of size $L = 10^{-2}\text{m}$. We took $\rho_0 = 10^3\text{kg/m}^3$, $\beta = 5 \times 10^{-4}\text{K}^{-1}$, $\nu = 5 \times 10^{-7}\text{m}^2/\text{s}$, $\alpha = 1.5 \times 10^{-7}\text{m}^2/\text{s}$. We fixed $\text{Pr} = 3$ and changed the temperature differences (and consequently the Grashof number), especially around the value $\text{Gr} \approx 10^5$, corresponding to $T_1 - T_0 = 5\text{K}$ (according to eq. (8)).

We also considered a fluid with the same physical parameters, but with a higher viscosity (10 times higher). For that, we fixed $\text{Pr} = 30$, as we changed the Grashof number around $\text{Gr} \approx 10^3$, which corresponds to the same temperature difference $T_1 - T_0 = 5\text{K}$.

For most cases, we considered $\text{Re} = 1$. But this choice only affects the velocity scale and, consequently, the time scale, keeping the dynamics intact. For larger values of the Grashof numbers, we considered $\text{Re} = 10$, to guarantee numerical stability.

Preparation of gradient 3-D structures for biological studies. Low acyl GG was dissolved in two beakers at 90°C for 30 min in distilled water at 2% (w/v). HAp was then added to one of the solutions at 10, 20 or 30% (w/w) and dispersed under stirring. After homogeneous HAp dispersion, 800 μL of this solution was added into a mold of 20x8 mm at 60°C and topped with a second 800 μL of solution at 50°C within 15 seconds after pipetting the first one. Afterwards, hydrogels were obtained by immersing the mold in Ca^{2+} at 0.25 M for 24h for total ionic-crosslinking of the GG molecular chains. The hydrogels formed were frozen at -80°C and then freeze-dried. After 4 days, cylinders of 6x4 for *in vitro* trials or 5x5 mm for *in vivo* assays were punched from the obtained dried structures. The structures were submitted to ethylene oxide treatment for sterilization before *in vitro* or *in vivo* experimentations.

Computed X-ray micro-tomographic analysis. Structures were analyzed by micro-CT after freeze-drying. Images were acquired by X-ray diffraction, and then used for a 3-D reconstruction. HAp profile was traced, and mean pores size and interconnectivity were quantified. 3-D projections of the specimens were performed. The 3-D gradient structures were acquired with a SkyScan 1272 scanner (v1.1.3, Bruker, Boston, USA), with a pixel size of 14 μm . Approximately 400 to 550 projections were acquired over a rotation angle of 180° or 360° , with a rotation step of 0.45° or 0.68° . Data sets were reconstructed using a standardized cone-beam reconstruction software (NRecon 1.6.10.2, Bruker). The output format

for each sample was bitmap images. Representative data set of the slices was segmented into binary images with a dynamic threshold of approximately 100–255 for the analysis of the ceramic phase, and 30-255 for soft polymeric phase (grey scale values – optimized per sample and analysis). Then, the binary images were used for morphometric analysis (CT Analyser, v1.15.4.0, Bruker) and to build the 3-D models (CTvox, v 3.0.0, Bruker). When needed, samples were vertically oriented in DataViewer (v1.5.2.3, Bruker) before proceeding to CT Analyser and CTvox.

Bioactivity assay with simulated body fluid (SBF). The GG-HAp structures were soaked in a solution of SBF, as described by Kokubo et al. [8]. In order to closest resemble the human blood plasma, the SBF solution (Na^+ 142.0, K^+ 5.0, Ca^{2+} 2.5, Mg^{2+} 1.5, Cl^- 147.8, HCO_3^- 4.2, HPO_4^{2-} 1.0, SO_4^{2-} 0.5 mM) was made by dissolving reagent-grade NaCl, NaSO_4 , NaHCO_3 , KCL, $\text{K}_2\text{PO}_4 \cdot 3\text{H}_2\text{O}$, CaCl_2 and $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ in distilled water at 36.5 °C. The solution pH was settled to 7.4 by addition of tris-hydroxymethylaminomethane and hydrochloric acid. Each structure was immersed in 10 mL of SBF solution using 50 mL polystyrene flasks and kept for periods of 12 h, 1, 3, 7, and 14 days. At each time point, the structures were removed and rinsed in distilled water, frozen at -80 °C, and then freeze-dried for 4 days before SEM/EDS analysis. All measurements were performed in triplicate.

Scanning electron microscopy analysis. The 3-D gradient structures were analyzed by EDS (SEM/EDS, NanoSEM-FEI Nova 200, USA) to assess the bioactivity of the structures shown by apatite formation. Prior the microstructure imaging by SEM, specimens were coated with gold using a Fisons Instruments Coater (Quorum/Polaron E6700, UK) with a current set at 18 mA, for a coating time of 120 s. SEM was performed to evaluate architecture morphology.

Dynamic mechanical analysis (DMA). The viscoelastic measurements were performed using a TRITEC8000B dynamic mechanical analyzer (Triton Technology, UK) in the compressive mode. Samples were cut into cylindrical shapes of approximate 8 mm diameter and 5 mm thickness and immersed in PBS until equilibrium was reached (37 °C, overnight). 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 a constant displacement amplitude (50 μm), always at 37 °C. A small preload (0.005 N) was applied to each sample to ensure that the entire structure surface was in contact with the compression plates before testing, and the distance between plates was equal for all structures being tested (n=4).

Isolation and culture of fat pad adipose-derived stem cells (ASCs). Fat pad-ASCs were isolated from human tissues obtained from Hoffa's body removed during arthroscopic surgeries on male and female donors with ages between 19 and 32 years, after informed consent, under cooperation protocols established with Centro Hospitalar Póvoa do Varzim and Clínica Saúde-Atlântica. The study has been approved by the Ethical Committee of Centro Hospitalar Póvoa do Varzim, Grupo Saúde-Atlântica and University of Minho.

All the samples were processed within 24 h after the arthroscopic procedure to the knee. hASCs were isolated following an enzymatic digestion-based method with type II collagenase (Sigma). The extracted tissue was placed in PBS solution and washed several times with PBS containing 1% (v/v) AB mixture, until total removal of blood and cut into small pieces. Tissue digestion was performed by incubation at 37°C in a humidified atmosphere of 5% CO₂ for 10 h in a 10-20 mL 1:1 mixture of MEM alpha (Invitrogen) supplemented with 10% FBS (Gibco) and 1% AB mixture, with type II collagenase 0.15% (w/v) in PBS. The digested tissue was filtered and cell suspension was centrifuged at 300 g for 5 min. The hASCs were selected by plastic adherence and expanded in α -MEM medium supplemented with 10% FBS and 1% of AB mixture.

Fat pad ASCs were seeded in a concentration of 1×10^6 cells per scaffold and cultured for 3 days before starting cell differentiation in a 24-well suspension cell culture plate (VWR). Cells were suspended in 50 μ L culture medium and seeded in both ends of each structure. After 1h, 500 μ L of culture medium was added. At day 3, metabolic activity was measured before and after dividing each structure in two halves to assess the cell distribution in each layer versus the whole scaffold. Then, each layer of the construct was cultured in osteogenic differentiation medium over 14 days. Osteogenic induction medium was MEM alpha supplemented with dexamethasone (100 nM), β -glicerophosphate (10 mM), ascorbic acid 2-phosphate (50 mg/mL), 1% AB, and 10% FBS. Chondrogenic medium was prepared supplementing DMEM hi-glucose with dexamethasone (100 nM), ascorbic acid 2-phosphate (50 μ g/mL), L-proline (40 μ g/mL), TGF- β 3 (10 ng/mL), 1% AB, and 10% FBS, and was used as a control condition (n=9).

Live/dead assay. Cell viability was assessed after staining with calcein AM (living cells) and PI (dead cells) diluted at 1:600 and 1:1000, respectively, in PBS (Life Technologies) for 20 min. All formulations were studied performing triplicates in three independent experiments.

Cytoskeleton staining. Samples from the *in vitro* assays were fixed with 10% formalin for 20 min at RT and washed with PBS. Red fluorescent phalloidin was used to label F-actin for cytoskeleton staining. The

blue fluorescent 40,6-diamidino-2-phenylindole (DAPI) was used as a nuclear counter staining. After washing, 3-D structures were visualized with a transmitted and reflected light microscope (Axio Imager Z1m, ZEISS).

DNA quantification. The amount of double stranded DNA (dsDNA), that is directly proportional with the cell number, was determined using a fluorimetric dsDNA quantification kit (PicoGreen, Molecular Probes, Invitrogen Corporation, USA) according to the manufacturer's instructions. Before performing the quantification, a complete mechanical disruption of the materials was performed, followed by a thermal shock cycle by incubating the lysates at 75 °C for 1h and freezing them at -80 °C until further analysis. Supernatant fluorescence was measured at an excitation wavelength of 485/20 nm and at an emission wavelength of 528/20 nm, in a microplate reader (Synergy HT, BioTek Instruments, USA). The quantification of DNA was calculated according to a standard curve prepared with concentrations ranging between 0 and 2 mg/mL, relating quantity of DNA and fluorescence intensity. Samples without cells were used as control.

Alkaline phosphatase (ALP) activity quantification. The ALP activity at day 21 of cell culture was determined on the same samples collected for dsDNA quantification as a marker of osteogenic differentiation. ALP activity was quantified by the specific conversion of p-nitrophenyl phosphate (pNPP; SigmaAldrich, MO, USA) into p-nitrophenol (pNP). A buffer solution containing 0.2% (w/v) pNPP was added to the sample in a 96- well plate (Corning Costar, NY, USA). The enzyme reaction was carried out at 37 °C for 45 min and then stopped by the addition of a solution containing 2 M NaOH (Merck, Germany) and 0.2 mM EDTA (Sigma-Aldrich, MO, USA) in distilled water. The absorbance of pNP formed was read at 405 nm in a microplate reader (Synergy HT, BioTek Instruments, USA) and concentrations were read off from a standard curve made using pNP values ranging from 0 to 0.2 mmol/mL. The obtained pNP concentrations were normalized against the DNA concentrations of the same samples and converted into enzyme activity per hour. Samples without cells were used as control.

***In vivo* assays.** All animal procedures were based upon the "3R's" policy, approved by the National Authority Guide for the Care and Use of Laboratory Animals (ethical committee authorization (009/2016)).

Subcutaneous implantation

In order to evaluate the *in vivo* biocompatibility of the 3-D structures per se, in terms of possible inflammatory reaction, materials infiltration and integrity over the 14 days of implantation, the 3-D structures were subcutaneously implanted in the back of 5 weeks old mice Hsd:ICR (CD-1) and with an average weight of 27-32 g at the time of implantation (Charles River Laboratories, Saint-Germain-sur-l'Arbresle, France). Each mouse was anesthetized by intraperitoneal injection of Medetomidine 1 mg/Kg (Domitor INJ 1mg/mL, Novavet, Braga, Portugal) and Ketamine 75 mg/Kg (Imalgene 1000 INJ 100 mg-mL, Novavet, Braga, Portugal). The hair of the mice was shaved at the implantation area, followed by disinfection with iodine (Life Technologies, Carlsbad, CA, USA). In each mouse, two skin incisions (1 cm length) were made in the dorsal midline. The structures were then implanted subcutaneously at both right and left sides into the respective pockets. Six specimens were implanted. The mice were euthanized 2 weeks' post-surgery with an injection of Eutasil 200 mg/mL (pentobarbital sodium, Novavet, Braga, Portugal) and the implanted materials were retrieved along with the surrounding tissue. The explants were fixed with 10% (v/v) formalin for at least 5 days at RT and transferred to histological cassettes for paraffin-embedding. Samples were then serially sectioned using a microtome (3.5 μ m thick) and stained with H&E.

Implantation in an osteochondral defect

Skeletally mature (12-14 weeks-old) New Zealand white rabbits, 2.5 ± 0.25 kg at the time of implantation (Charles River Laboratories, Saint-Germain-sur-l'Arbresle, France), were used. The rabbits were anesthetized with a subcutaneous injection of a mixture of ketamine hydrochloride 25 mg/Kg (Imalgene 1000 INJ 100 mg-mL, Novavet, Braga, Portugal) and medetomidine hydrochloride 0.15 mg/kg (Domitor INJ 1mg/mL, Novavet, Braga, Portugal). Then, both knees were shaved and disinfected with iodine (Life Technologies, Carlsbad, CA, USA). An internal para-patellar incision was performed to expose the knee and the patella was dislocated. A deep OC 5 mm defect in the trochlear groove of each knee was done using a drill of 5 mm diameter. The defect was filled with the structures or left empty as control. To finalize, the patella was reduced and the wound was closed. After recovery from surgery, animals were placed in individual cages and fed ad libitum. Defects were allowed for eight weeks. Animals were anesthetized as described above, and euthanized through an intra-venous injection of pentobarbital sodium 200mg/kg (Eutasil 200 mg/ml, Novavet, Braga, Portugal). In each knee, an internal para-patellar incision was made and the patella carefully dislocated. Macroscopic pictures were taken and the explants of each knee was collected and stored in 10% (v/v) formalin at 4 °C.

Immunohistochemical analysis. Immunohistochemical staining of the explants from *in vivo* trials was performed with mouse anti-rabbit collagen II alpha 1 (Acris Antibodies GmbH, Herford, Germany) and biotinylated Sambucus nigra (Elderberry) bark lectin (SNA-lectin; Vector Laboratories, Burlingame, CA, USA) following the protocol provided by the supplier. After deparaffinization and rehydration of the specimen slides, the antigens were retrieved in 10 mM sodium citrate buffer solution at 95 °C for 20 min (pH 6.0), and then the endogenous peroxidases were inactivated by using 3% (v/v) hydrogen peroxide solution. Then the slides were incubated in protein blocking solution for 10 min followed by incubation with the primary antibody SNA-lectin for 1 h and at RT. Sections were then sequentially washed with PBS and incubated with the streptavidin–peroxidase complex for 10 min. The immune reaction was visualized by 3,30-diaminobenzidine (DAB; LabVision Corporation, Fremont, CA, USA) as a chromogen. All sections were counterstained with Gill-2 hematoxylin (Merck, Germany). For preparing the negative controls, the primary antibody was omitted. The histological sections were then observed using a light microscope.

Statistical analysis. The investigated parameters were expressed as mean $\pm$ SD or confidence interval. Student's t test was used to compare group means, and a p-level < 0.05 was considered statistically significant. Statistical analyses were carried out with Microsoft Excel 2010 and GraphPad Prism 5 for Windows. In all cases, not-paired two-tailed Student's t-tests and nonparametric Mann-Whitney test were used to determine significance of the results.

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4.7 Supplementary Information

4.7.1 Supplementary Results

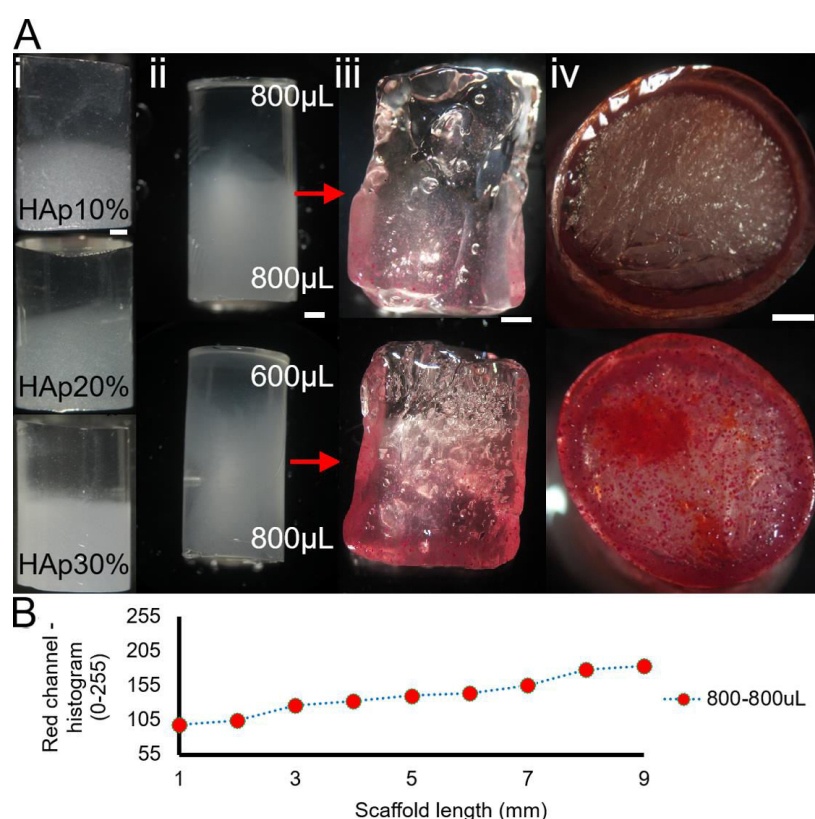


Figure S4.1 | Gradient of HAp variations in GG hydrogels enabled by HAp concentration and injected volumes. (A) Microscopic images of (i) hydrogels obtained at 60°C with 10, 20 and 30% (w/w) HAp. (ii) Layer size varied by injected volumes. (iii) Alizarin red staining showed the HAp distribution after freeze-drying process. (iv) Top (residual HAp) and bottom (rich HAp) layers. Scale bar = 1 mm. **(B)** Histogram profile of HAp red dots quantified by image J along the structure length.

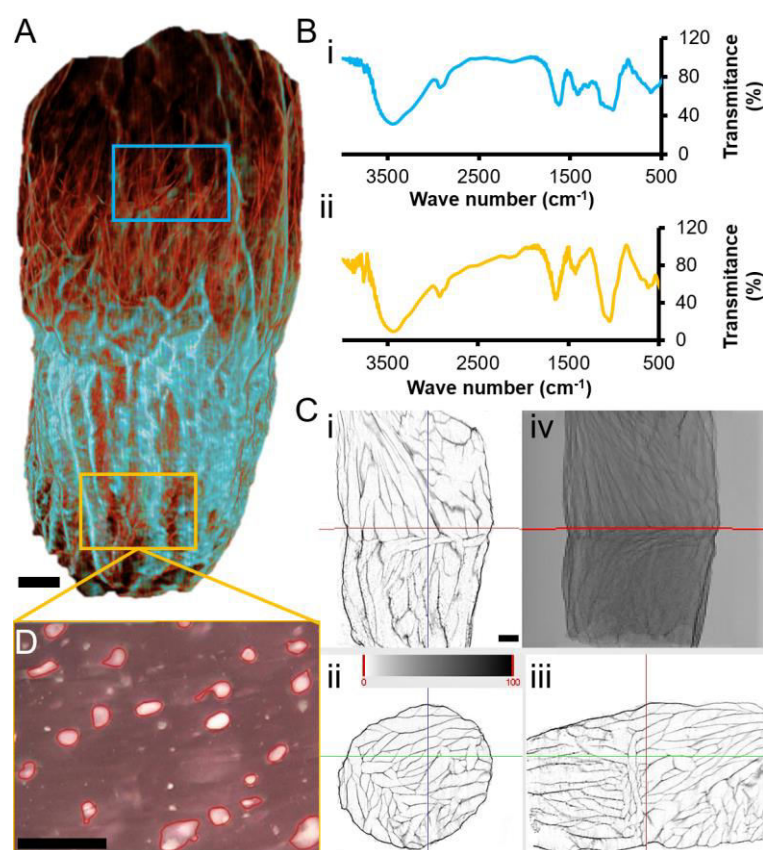


Figure S4.2 | Phasic composition and structural characterization of 3-D graded structures.

(A) A 3-D reconstruction of the gradient structures is shown. Hard-material and soft-material were identified by X-ray diffraction and were represented by blue and red coloration according to the histogram from 0 to 255; 0-40: soft material; 40-255: hard material. (B) The composition of top and bottom phases of the structures were analyzed by infrared diffraction. Pure GG was identified in the top phase and the incorporation of HA was observed in the bottom phase, when considering the orientation of the structures represented in A. (C) Cross-sectional projections were shown from (i) sagittal, (ii) transversal and (iii) coronal views from the X-ray images (iv). (D) Microscopic images were obtained from HAp distributed in the hydrogels. Scale size = 1 mm.

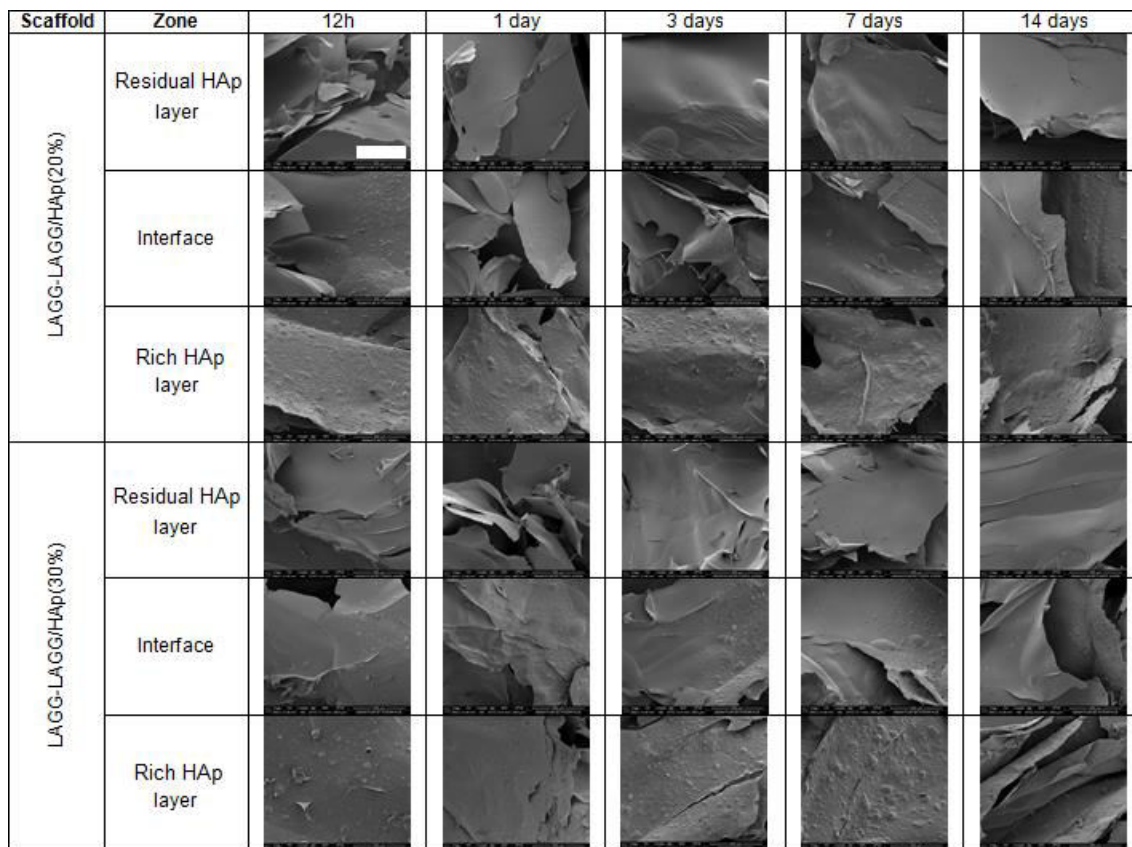


Figure S4.3 | Bioactive to non-bioactive gradient transition mediated by microparticles.

The apatite layer formed by the incorporation of HAp at 20 and 30% (w/w) was shown. The structures presented a bioactive behavior in the regions containing HAp, which disappears with the decreasing gradient, as shown by the SEM images (scale bar = 500 μ m).

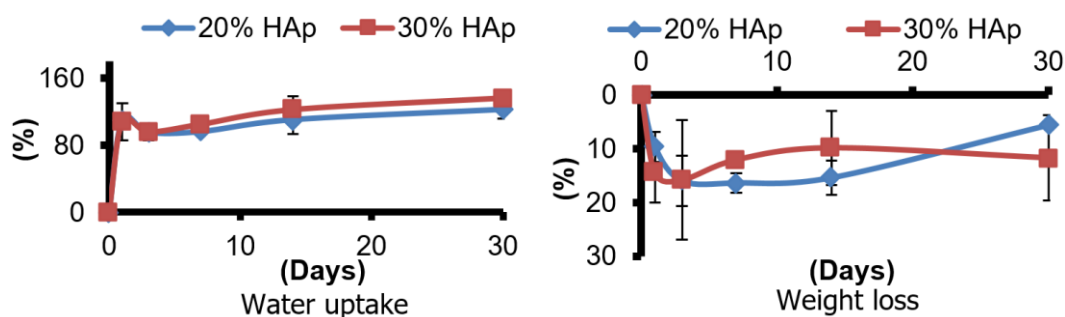


Figure S4.4 | Water uptake and weight loss. Water uptake (left) and weight (right) were quantified for structures with two different concentrations of HAp, 20 and 30% (w/w). Data expressed as Mean $\pm$ SD (n=3, *p<0.05).

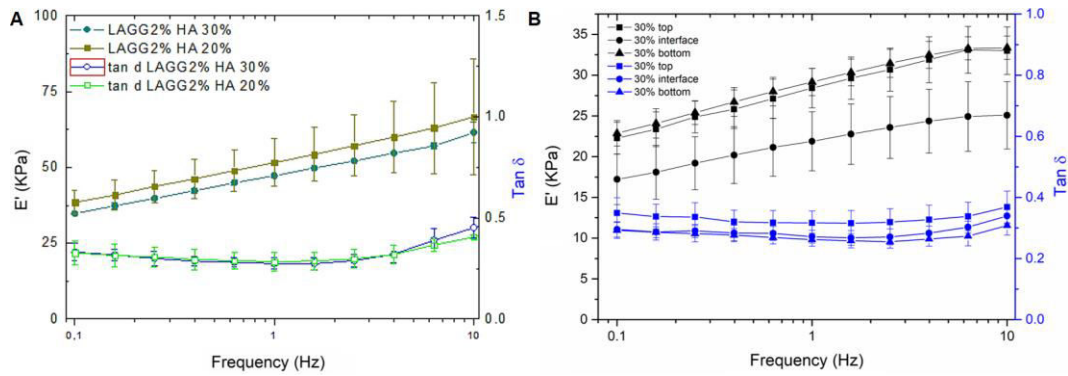


Figure S4.5 | Dynamic mechanical analysis was performed to characterize the whole structure and each layer. (A) Structures with incorporation of HAp at 20 and 30% (w/w) were characterized, showing a slight increase in E' with the increasing concentration of HAp ($n = 5$). **(B)** Each structure of 30% HAp was sliced in three similar regions of 5 mm ($n = 4$). Storage modulus (G') and energy dissipation were characterized per layer. Data expressed as Mean $\pm$ SD (* $p < 0.05$).

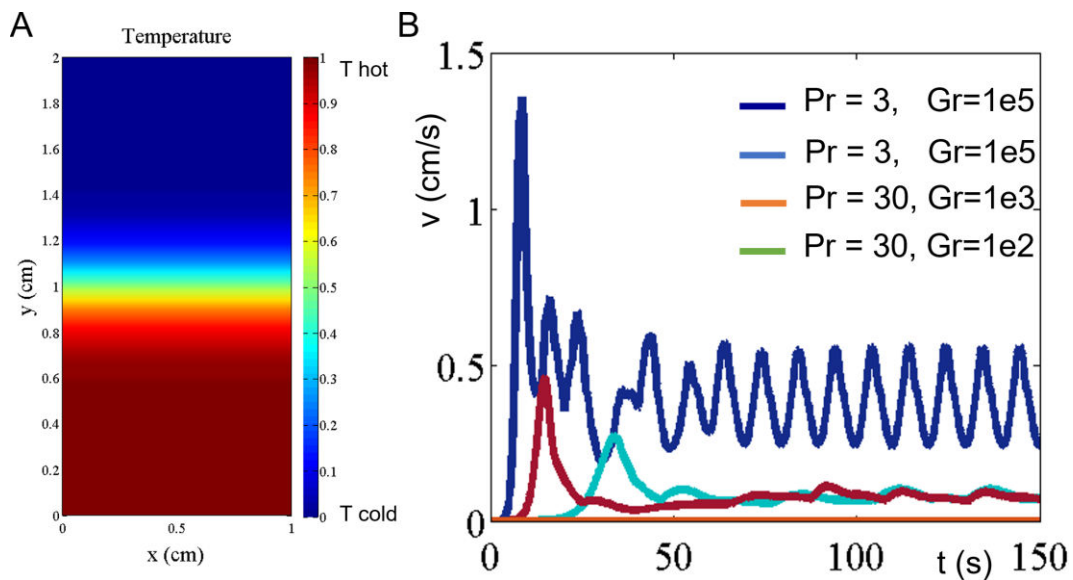


Figure S4.6 | Initial temperature gradient and screening of Grashof numbers. (A) An initial condition was set to observe the formed gradient of temperatures when a T_{hot} is applied on the base and T_{cold} is applied on the top of the system, being the sides periodic conditions. **(B)** A screening of Grashof numbers was performed for Prandtl number = 3 and 30. The transition from non-convection to convection was observed with a stationary solution, later becoming a periodic solution until it reached an average of frequencies and speeds, creating then a regime.

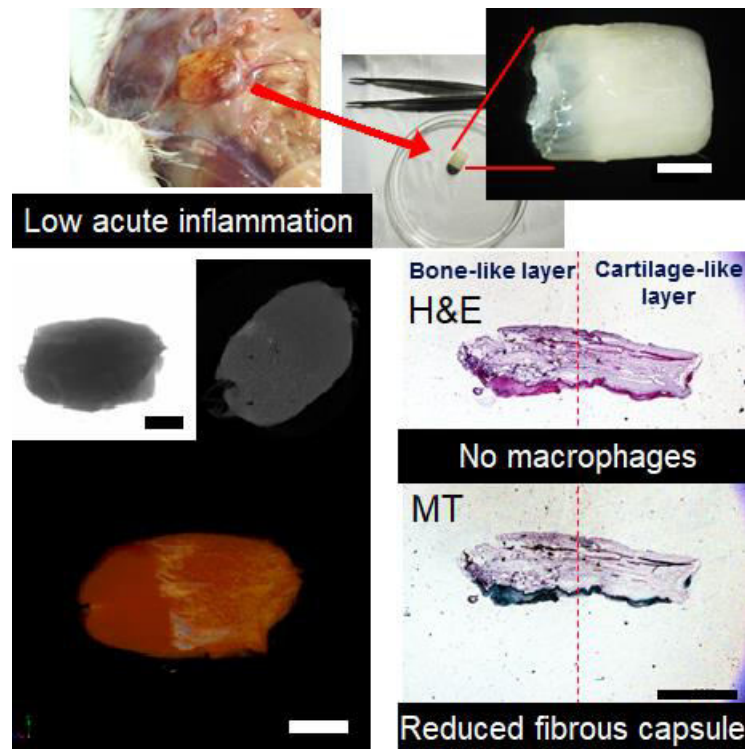


Figure S4.7 | Subcutaneous assay performed in mice to study inflammatory response and to assess the maintenance of a HAp gradient during the *in vivo* implantation. The results show low inflammatory inflammation, reduced or no macrophages invasion and reduced fibrous capsule at 30 days.

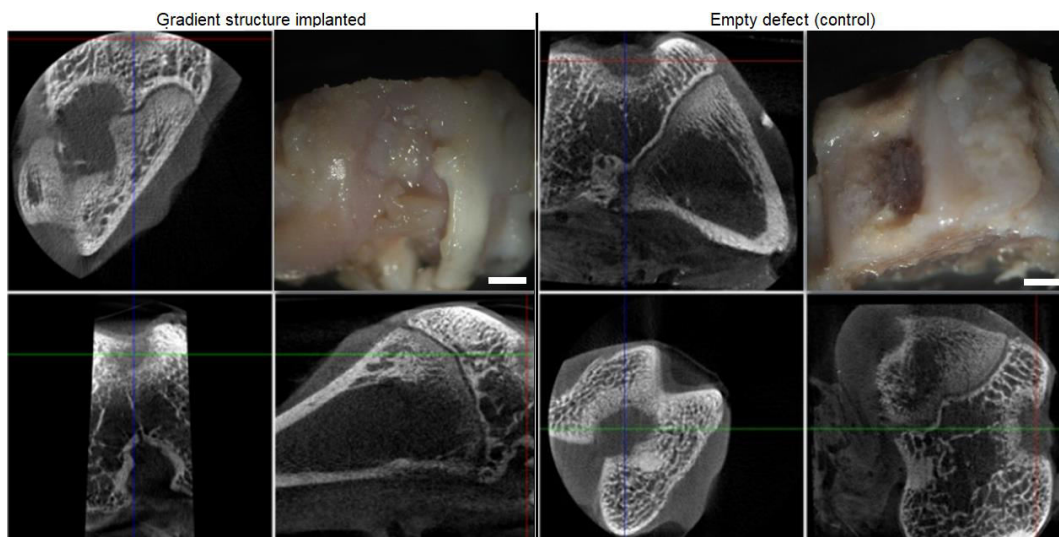


Figure S4.8 | X-rays cross-sectional and macroscopic views of explanted rabbit knees. Gradient structures (left) and empty defects (right) were assessed after 30 days. X-rays imaging show coronal, transaxial and sagittal sections. Macroscopic images show the top view of the explanted knee.

CHAPTER 5

CONTROL OVER 3-D POROSITY

Based on the following publication:

RF Canadas, T Ren, A Tocchio, AP Marques, JM Oliveira, RL Reis, U Demirci. Tunable anisotropic networks for 3-D oriented neural tissue models. (Submitted)

5. TUNABLE ANISOTROPIC NETWORKS FOR 3-D ORIENTED NEURAL TISSUE MODELS

5.1 Abstract

Organized networks are common in nature showing specific tissue micro-architecture, where cells can be found isotropically or anisotropically distributed. Since native tissues present characteristic stiffness and cell arrangements, when addressing an *in vitro* tissue model, it is important to grant control over mechanical properties while achieving anisotropic porosity of polymeric networks, especially in three-dimensions (3-D). While progress was achieved organizing cells in two-dimension (2-D), reported fabrication techniques for aligned networks in 3-D are limited. Here, we describe the use of a biomimetic extra-cellular matrix system allowing programming anisotropic structures into precise pore diameters in 3-D. Using control over polymeric composition, crosslinking directionality and freezing gradient dynamics, we revealed a mechanism to top-down biofabricate 3-D structures with tunable micro-porosity capable of directing cellular responses at millimeter scale such as axonal anisotropic outgrowth that is a unique characteristic of the brain cortex. Further, we showed the combination of this approach with a specially designed microfluidic system forming a neural-endothelial interface to be applied in a microfluidic organ-on-a-chip configuration which can potentially be broadly applied to multiple organ systems.

5.2 Introduction

In nature, microarchitecture and mechanical features of tissues and organs are directly linked to their function^{1,2}. Diseases come along with alterations in tissue architecture together with mechanical changes³⁻⁶. Among these changes, the most altered features are tissue elasticity, extra-cellular matrix (ECM) composition, and porosity architecture.

Biomimetic 3-D platforms have been created based on different approaches such as cell spheroids⁷, organoids⁸ and engineered constructs based on scaffolds⁹. Cell spheroids and organoids present advantages based on cell self-assembly, enabling spatial self-organization. However, fine control over cellular arrangement, phenotype, and reproducibility has been reported to be challenging^{10,11}. Scaffold and hydrogel-based engineering methods attempt to support cell adhesion and organization recreating the native tissue 3-D architecture *in vitro* by ideally controlling network geometry and mechanical properties. Although reported success in controlling hydrogels assembly¹², mobility¹³ and arrangement¹⁴

e.g. using magnetic forces ¹⁵ or acoustic waves ¹⁶, the current techniques to engineer constructs offer limited control over the 3-D micro-architecture, not gathering simultaneously efficient mechanical and biological properties, usually due to fabrication limitations and lacking recapitulation of the constituents of natural ECM and its specific porosity geometry ¹⁷⁻¹⁹. Among these diverse techniques to bioengineer scaffold networks, stretched fibers ²⁰, electrospinning ²¹ and 3-D printing ²² allow control over linear deposition of fibers achieving anisotropic patterns. However, stretched fibers and electrospinning produce compact fibrous matrices blocking cell penetration in a 3-D environment and usually present poor mechanical properties. 3-D printing systems (e.g. extrusion, droplet based, stereolithography) present limitations such as fibers fusing, collapsing or lack of layer thickness and porosity resolution ranging at the microscale ²³.

Moreover, multiple tissue types show specific organization associated with their function, such as linear hollow tubular arrangements of cells present in vessels as vascular and lymphatic networks ^{24,25}, or anisotropic bulk matrices as in neocortex, peripheral nervous, and cardiac systems, or even tendons and muscles. These tissues exhibit anisotropic cellular patterns, and their unique ECM varies in elasticity and stiffness, so these mechanical and geometrical properties have to be correlated and effectively mimicked for successful biofabrication of *in vitro* functional tissue models ²⁶⁻²⁹. In particular, the cerebral cortex presents an anisotropic outgrowth of neurites, which was recapitulated by Kim et al ²⁰ in a 3-D system at the microscale, but this 3-D architecture was never engineered at a robust millimeter scale with control over neural columns width resembling the human neocortex as needed for a larger spectrum of applications in drug discovery, TE and regenerative applications.

Here, we report an innovative 3-D system able to organize neurons and direct neurites outgrowth in a ECM-like network made of a photo-crosslinkable blend of a fibrous protein with a polysaccharide (figure 5.1a). The degradation rate and mechanical properties can be controlled by adjusting the ratio of the polymeric blend, while the 3-D anisotropic structure and its pore size can be tuned by varying the temperature of the freezing gradients and guiding the crosslinking (figure 5.1b). The top-down fabricated oriented porous structures in a single step process resulted in pore sizes ranging from tens to hundreds of micrometers overcoming the resolution of similar techniques and ranging the size of human neocortex columns (figure 5.1c) ³⁰⁻³². As a model system, to mimic the brain cortex where neural cells are anisotropically organized, primary neurons were injected in the 3-D oriented porous systems creating 3-D neural anisotropic constructs at a large millimeter scale, which has not been achieved before (figure 5.1d). Further, we also aligned primary neurons interfaced with endothelial cells under dynamic conditions controlled by a microfluidic platform designed for co-cultures as an organ-on-chip type model,

demonstrating the versatility of the reported system and its relevance for interfaced tissues mimicry (figure 5.1e).

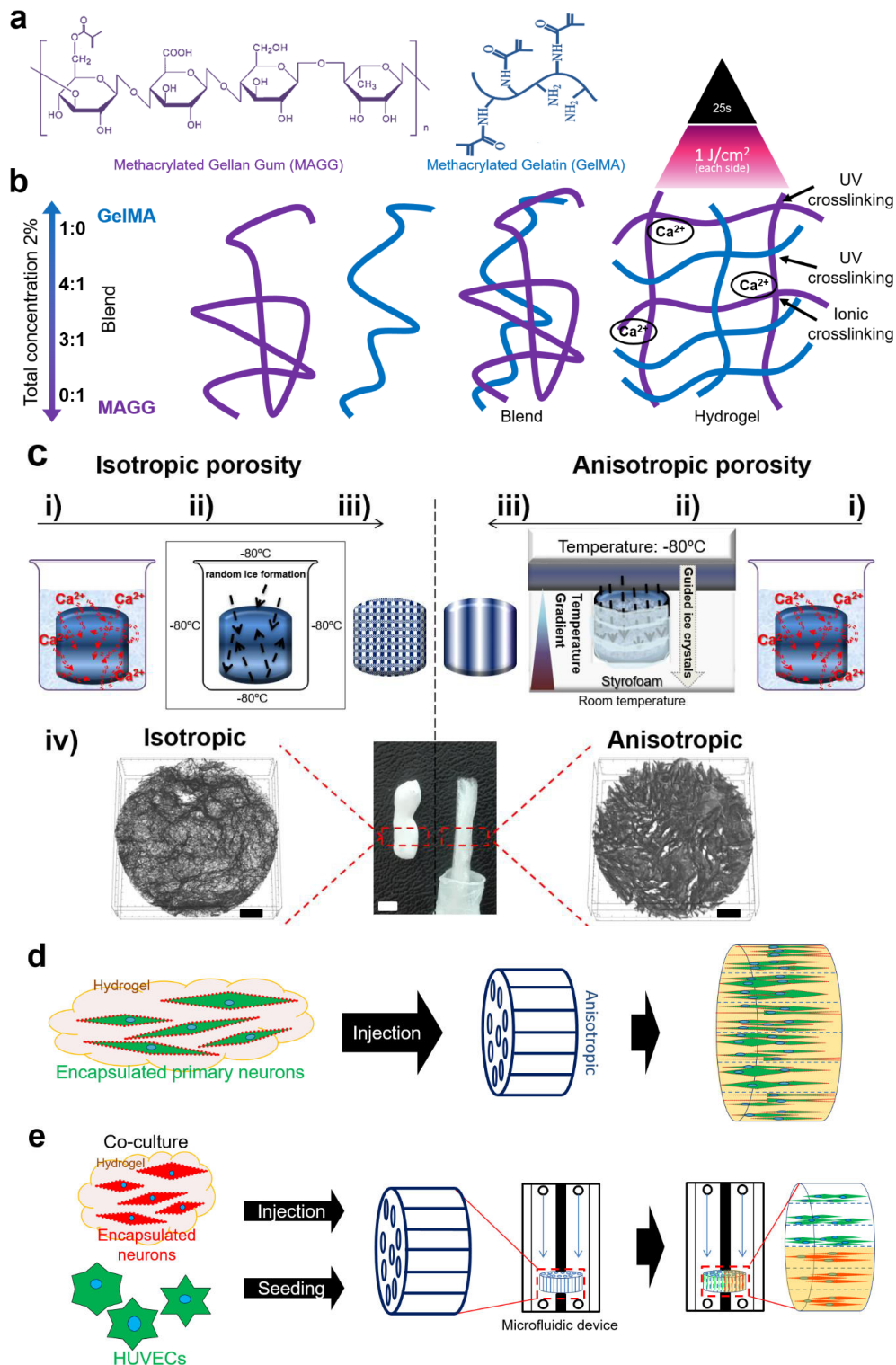


Figure 5.1 | Fabrication and manipulation of the properties of 3-D oriented porous systems for *in vitro* modeling. (a) Hydrogels formulations were produced combining GelMA and MAGG. (b) The tested blends were produced at 3:1 and 4:1 ratios of GelMA:MAGG keeping a total polymer concentration of 2% (w/v). Photo-crosslinking was used to fabricate the hydrogels. (c i) After

photo-crosslinking, ionic crosslinking was used to stabilize the blend and (c ii) the porosity orientation was controlled by freezing strategies (c iii) before lyophilization, (c iv) obtaining isotropic and anisotropic 3-D porous structures, as represented by the micro-CT reconstructed cross-sections (scale bar = 500 μ m) and macroscopic photography (scale bar = 2 mm). (d) Primary mouse neurons were embedded in hydrogel before injection in the 3-D oriented porous structures and cultured over 7 and 14 days (e) Interfaced 3-D neural and endothelial heterotypic constructs were produced as an interfaced tissues approach culturing HUVECs and mice neurons in a custom microfluidic device.

5.3 Results

5.3.1 Physicochemical control and characterization of hydrogel-based systems

To evaluate the optical properties of each hydrogel blend, microscopic imaging was performed to assess light distortion through the hydrogels by laying the hydrogels on top of paper written sheets, which showed translucent networks (figure 5.2a-i). To quantify this translucency, absorbance spectroscopy measurement was performed at 495nm. GelMA and GelMA-MAGG 4:1 were characterized by $\sim$ 25% light transmission while GelMA-MAGG 3:1 and MAGG reached about 10% transmittance (figure 5.2a-ii).

To evaluate the network remodeling ability of each hydrogel, water absorption and enzymatic degradation by collagenase type I over 7 days were performed (figures 5.2b-i and 5.2b-ii). By decreasing the concentration of GelMA or increasing the ratio of MAGG, the swelling was increased, and degradation was slowed down showing control over network remodeling properties. Water absorption reached the maximum of around 37-fold increase of the hydrogel initial weight at 12h and the lowest degradation of about 19% of hydrogel's mass, for the formulation of pure MAGG. The lowest water absorption of about 23 fold increase was observed for GelMA and 3:1 over 12h and 7 days, respectively, and the fastest degradation of around 56% was found for the formulation 4:1 GelMA:MAGG at 7 days.

To evaluate the G' and G'' of the different formulations (figure 5.2b-iii), rheological assays were performed showing a control over G' ranging from 61 to 898 Pa and G'' from 42 to 216 Pa by tuning the blend ratio. The highest G' was found for GelMA-MAGG 3:1 (898.1 ± 178.3). The ratio between storage and G'' was higher for formulations composed of the largest proportion of MAGG, namely GelMA-MAGG 3:1 and MAGG 2%, indicating a greatest elasticity and shape recovery ability. The control over physicochemical properties was demonstrated and was dependent on the different crosslinking profiles occurring which were represented by a schematic suggested in figure 5.2c.

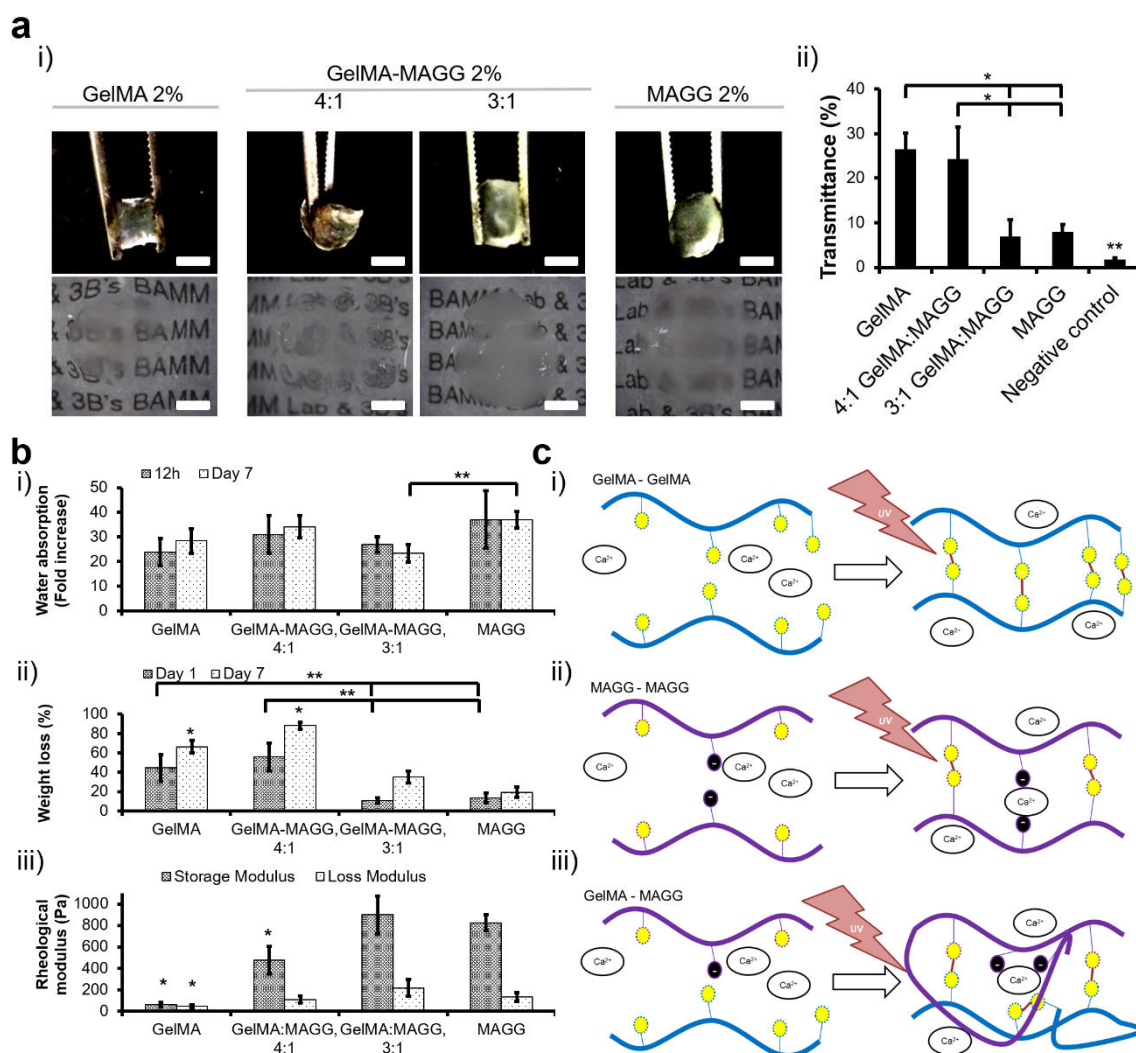


Figure 5.2 | Physicochemical characterization of GelMA-MAGG based hydrogels. (a i) Translucency of hydrogel formulations was assessed macroscopically using dark field (scale bar = 1.5 mm) and white paper background with written text (scale bar = 0.5 mm). (a ii) Transmittance was quantified by spectrophotometry at 495 nm and normalized to PBS; wet paper in PBS was used as a negative control since it is not translucent. (b i) Water absorption at 12h and 7 days and (b ii) degradation by collagenase type I at day 1 and 7 were quantified for each formulation. (b iii) Mechanical properties were evaluated by rheology for each formulation. (c i) GelMA-GelMA, (c ii) MAGG-MAGG and (c iii) GelMA-MAGG crosslinking mechanisms by UV and calcium ions were schematically represented. Data expressed as (mean $\pm$ SEM).

To characterize the variation of 3-D structure porosity, pore size, wall thickness and interconnectivity with the GelMA-MAGG blend composition, a 3-D reconstruction by micro-CT was performed (figure 5.3a) and quantitatively analyzed as observed in figure 5.3b. The dependence of pore size on the blend ratio was

observed. Porosity ranged from 88 to 95% in all the formulations being the minimum and maximum correspondent to GelMA-MAGG 3:1 and 4:1, respectively (figure 5.3b-i). The introduction of MAGG in the system created significantly larger pores at the ratio 4:1, which significantly decreased as the proportion of MAGG increased, reaching the smaller pore size of all formulations at pure MAGG (33 μm) as represented at figure 5.3b-ii. Wall thickness showed no significant differences ranging from 7 to 10 μm in all the formulations. Interconnectivity ranging from 57% to 99% showed a similar profile as observed for pore size, increasing with the introduction of MAGG (figure 5.3b).

To assess cell proliferation, NIH-3T3 fibroblasts were cultured in the several formulations instead of neurons, since neurons do not proliferate. Metabolic activity was measured (figure 5.3c-i) over 7 days and correlated to the cell number. Increasing GelMA proportion in the blend resulted in significantly increased cell metabolic activity over 7 days in all conditions indicating that there was cell proliferation in all the formulations.

To screen the cell viability in the GelMA-MAGG formulations, both NIH-3T3 fibroblasts and SH-SY5Y neuroblastoma cell lines were cultured. The formulation GelMA-MAGG 3:1 was selected based on its favorable balance between mechanical properties, degradation rate, and 3T3 metabolic activity as previously reported. Cells were cultured over 7 days and characterized by live/dead assay (figure 5.3c-ii and 5.3-d-i). High cell viability (>80%) was observed for both NIH-3T3 and SH-SY5Y (figure 5.3-D-ii).

To assess the ability of primary neurons to adhere and extend neurites in the formulation of 3:1 GelMA-MAGG, primary neurons from mouse were cultured over 7 days. Neurons adhered, and neurite outgrowth was observed as shown in figure 5.3-d-iii.

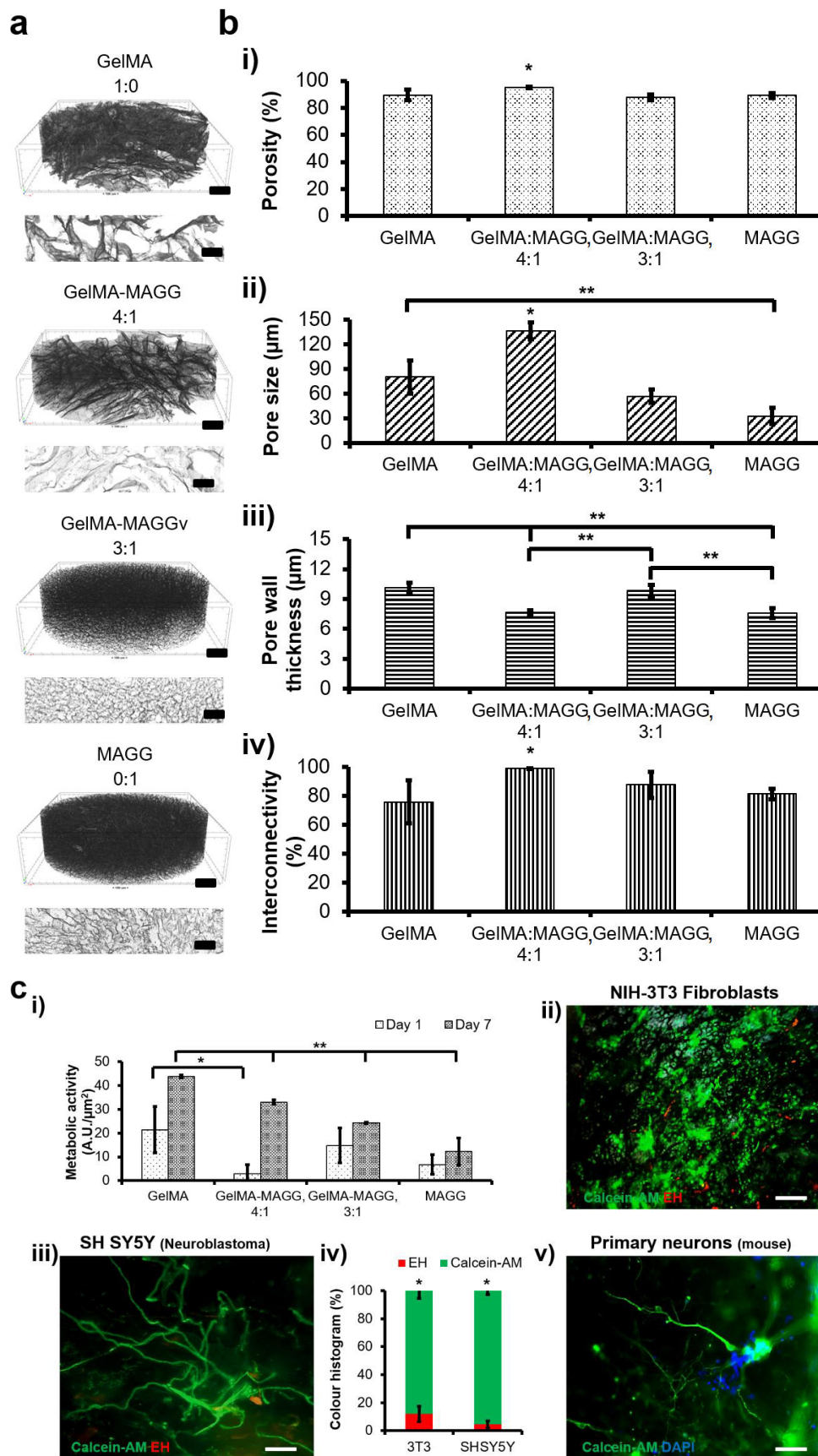


Figure 5.3 | 3-D porous structures characterization and cell response assessment in GelMA-MAGG formulations. (a) 3-D reconstruction of the porous structures was made by X-ray

micro-CT (scale bar = 500 μm). Cross-sections were reproduced showing the pore size and shape for each formulation (scale bar = 250 μm). (b) The micro-architecture was characterized by quantifying the (b i) porosity (%), (b ii) mean pore size (μm), (b iii) mean wall thickness (μm) and (b iv) interconnectivity (%). (c i) NIH-3T3 fibroblasts were cultured over 7 days and their metabolic activity assessed by Alamar blue® assay at day 1 and day 7 for correlation with proliferation. (c ii) Live-dead assay was performed to NIH-3T3 at day 7 of cell culture to evaluate the viability. Calcein-AM was used to stain live cells as green and ethidium homodimer the dead cells as red (scale bar = 50 μm). (c iii) SH-SY5Y neuroblastoma cells were seeded in the GelMA-MAGG 3:1 formulation (scale bar = 50 μm) to evaluate the adhesion, neurite extension and viability by live/dead staining. (c iv) Live/dead staining's were quantified for each cell type at day 7. (c v) Primary neurons from mouse were cultured over 7 days and stained with DAPI and calcein-AM to evaluate the ability of primary neurons to adhere, extend and keep viable over 7 days (scale bar = 50 μm). Data expressed as mean $\pm$ SEM.

The summary of tunable properties characterized for this blend formulations and evidenced on figures 5.2 and 5.3 is listed and summarized in table 5.1, with grey bars representing the feature tendency by changing the polymeric ratio.

5.3.2 Guiding porosity of 3-D oriented porous systems to obtain isotropic or anisotropic structures

To control the pore orientation of 3-D structures, freezing gradients were used after crosslinking and evaluated by microscopy and micro-CT. Porous isotropic (figure 5.4-i) and anisotropic structures (figure 5.4-ii) were obtained. When applied a gradient freezing step, the anisotropic degree significantly increased from 0.19 to 0.52 (figure 5.4a-iii), and the interconnectivity increased from 83% to 90% (figure 5.4b-iv).

To control the pore size of the oriented porous structures the freezing temperature was varied. The control over the pore size of the anisotropic structures was observed (figure 5.4a ii). The crosslinking was guided before the freezing step to reach the smallest pore size as represented (figure 5.4b-ii).

To quantify the pore size of oriented porous structures, microscopic images were acquired and analyzed (figure 5.4c). When starting this process with different temperatures, as -80 °C or -196 °C, the pore diameter significantly decreased from 376 μm to 64 μm . To further decrease the pore diameter obtained at -196 °C, the UV-crosslinking was preceded by a guided ionic-crosslinking with Ca^{2+} from top to the bottom of the hydrogel resulting in a pore size significantly decreased from 64 μm to 18 μm .

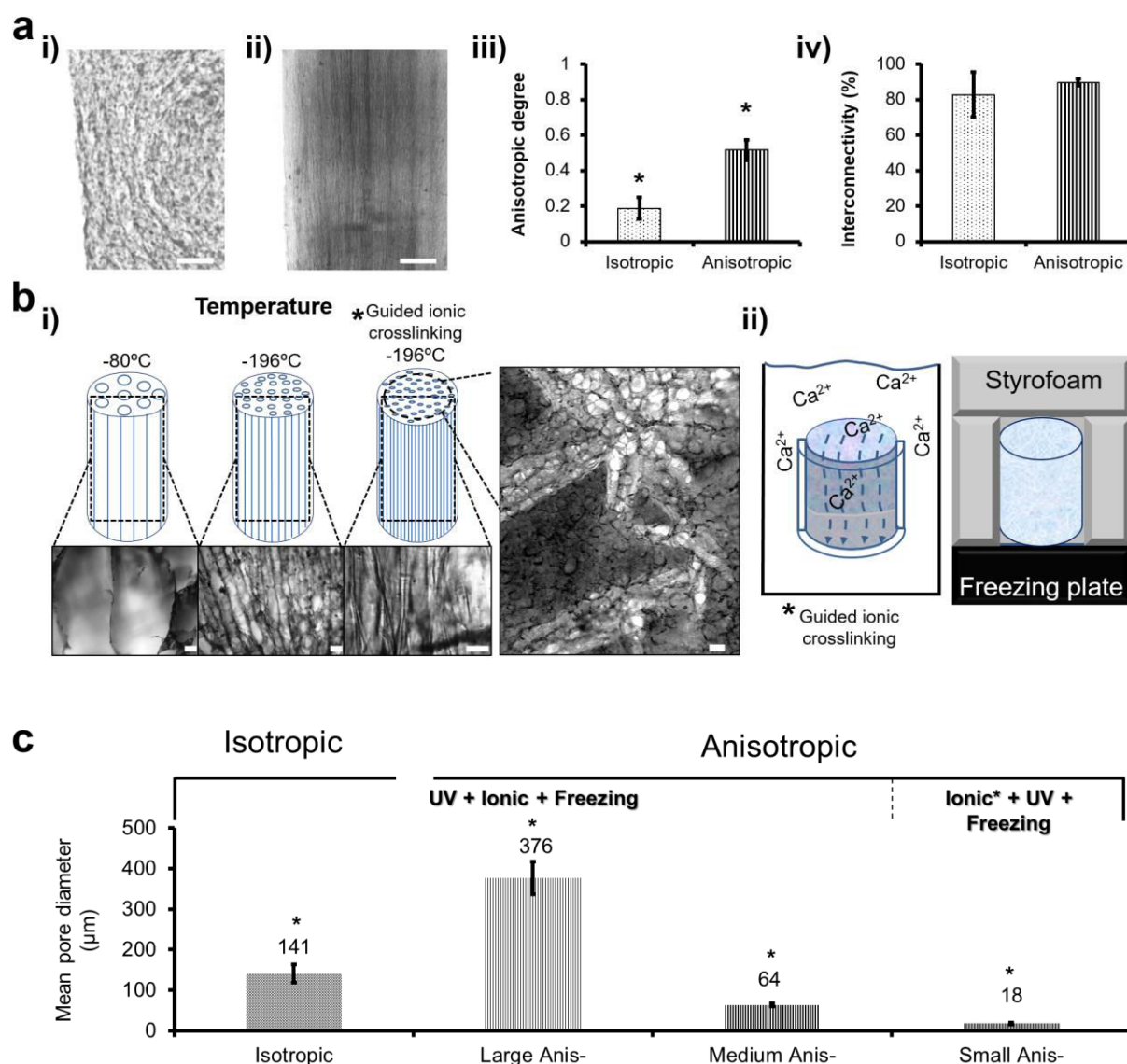


Figure 5.4 | Control over porosity architecture as isotropic and anisotropic porous geometry. (a i) Isotropic and (a ii) anisotropic porous structures were qualitatively characterized by micro-CT and representative transversal cross-section were shown (scale bar = 1 mm). (a iii) The degree of anisotropy of the 3-D porous structures was quantified by micro-CT (0 = totally random porosity; 1 = perfectly aligned porosity). (a iv) Structures interconnectivity was also quantified by micro-CT. (b i) The control over the pore size of anisotropic structures was shown by microscopic images (scale bar = 100 μm). (c) Pore diameter was quantified according to each crosslinking and freezing gradient applied condition. The quantification was performed for isotropic and anisotropic structures showing random and aligned pore orientation of different diameter ranging from 18 to 376 μm. Data expressed as mean ± SEM.

5.3.3 Controlling diameter of linear porosity obtained on anisotropic structures for neurite guidance

To assess the effect of pore size and orientation on neurites outgrowth, mouse primary neurons were cultured in aligned porosity varying the pore diameters from around 20 to 400 μm . Neurites outgrowth were randomly oriented in the structures with isotropic porosity (figure 5.5a-i and ii). The aligned and layered orientation of neurites was observed in the oriented porous structures. Different pore diameters led to an arrangement of oriented neurites outgrowth with layers of different diameters according to the size of the porosity. The frequency of orientation between 90 and -90° was quantified (figure 5.5a-iii). The absence of a peak means a random orientation angle of neurite outgrowing creating an isotropic neural tissue, while the presence of a peak in the orientation angle frequency represented a tendency of the neurites to extend in a specific direction, resulting in an oriented neural tissue. The frequency of orientation was affected by the pore diameter, since decreasing the diameter increased the frequency of an orientation angle. These neurite layers interconnect from one to a second pore allowing for interlayer communication at several points of the porous structures.

To have indication of the neural activity in the constructs, the presence of postsynaptic vesicles was assessed by cytochemistry (figure 5.5b-i). To qualitatively observe the distribution of postsynaptic junctions, postsynaptic density protein 95 (PSD95) was selected as a marker of excitatory sites³³ and functional matured synapsis, as previously demonstrated in other studies^{34–36}. Synaptic vesicles were observed distributed in the oriented arrange of neurons (figure 5.5b-ii) and this event was quantified by counting the PSD95 puncta in horizontal and vertical sections which is related to neuronal activity and anatomy (figure 5.5b-iii)^{37,38}. Considering the study by Kim *et al*/³⁹ where an average of 10 of PSD95 puncta was expressed per 10 μm of neurites in mouse primary neurons, the expression of PSD95 in the present study (7.9 puncta per 10 μm of neurites) was on the expected level when counted in the orientation of the pore direction and half of this amount was observed in the vertical orientation, meaning perpendicular to the pore direction. The number of puncta significantly increased in horizontal when decreasing the pore size, while this number increased in vertical by increasing the pore size. 3-D sections of acquired confocal images (Figure 5.5b-iv) showed the co-localization of Tuj-1 and PSD95 as expected.

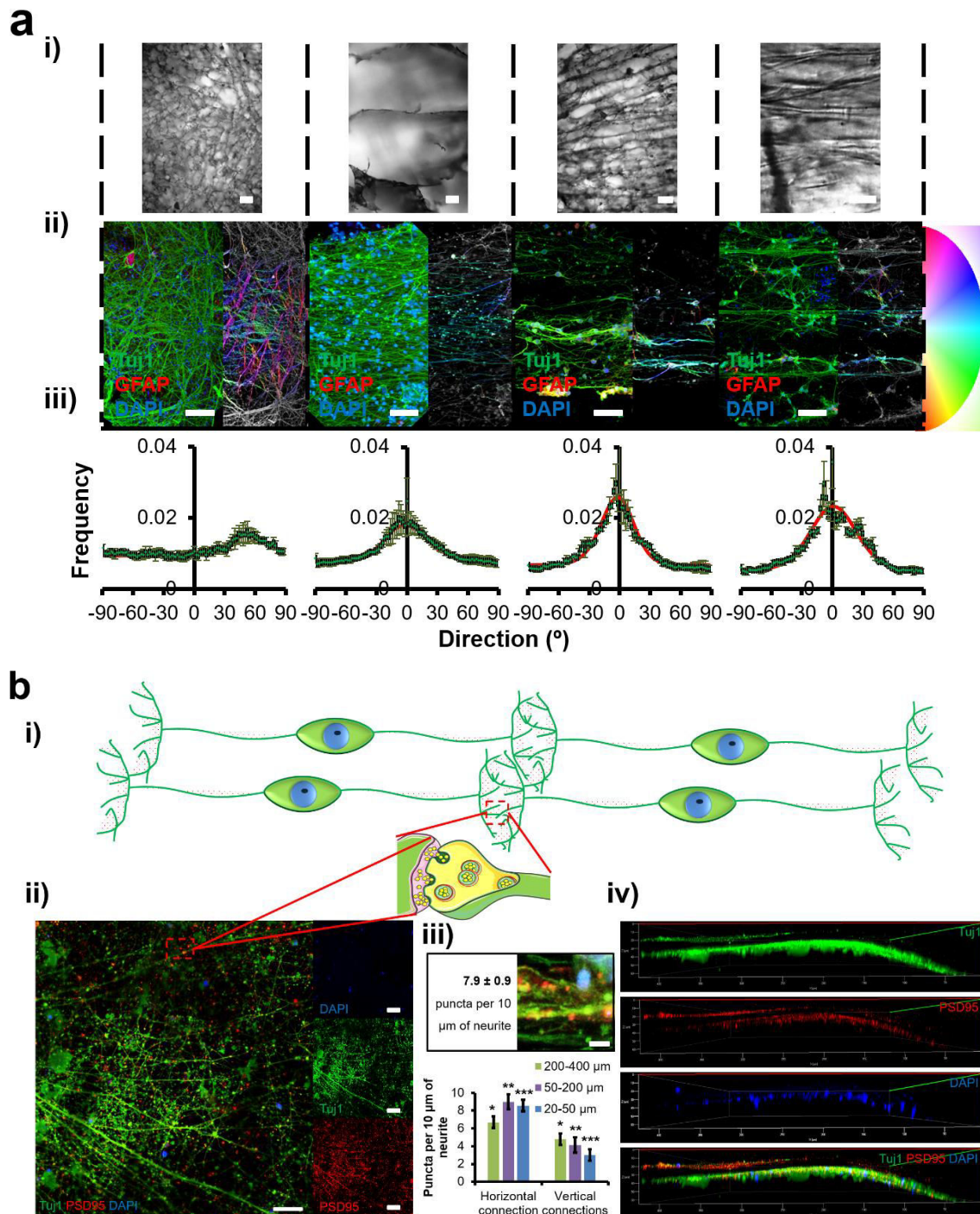


Figure 5.5 | Anisotropic porosity guiding neurite outgrowth from mice primary neurons. (a i) Representative bright-field images were obtained according to porosity architecture and pore size (scale bar = 50 μm). (a ii) Primary neurons were cultured in the 3-D oriented porous structures to evaluate the neurite outgrowth guidance ability. Tuj-1 and anti-glial fibrillary acidic protein (GFAP) antibodies, and the fluorescent dye DAPI were used to stain the neuron-specific class III β -tubulin (green) of primary neurons, GFAP of glial cells (red) and cells nuclei (blue), respectively, and images were acquired by confocal microscopy (scale bar = 100 μm). A color map was created showing the neurites orientation angle represented by colors (images shown side by side with confocal images) (a iii) Representative images of

neural cell culture were acquired for cell distribution and orientation analysis and quantification. A fitting curve was traced based on a Gaussian function of the frequency of orientation angle events. (b i) A schematic of synaptic vesicles distribution and connection between neurons was designed to represent the functionality of these vesicles in a neural tissue. (b ii) To show the presence and distribution of the synaptic vesicles representative images of PSD-95 immunostaining were acquired by confocal microscopy. RGB channels of PSD95 (red) and Tuj-1 (green) colocalization showing the distribution of the postsynaptic proteins (red) in the oriented porous structures, were represented; cell nuclei (blue) were stained with DAPI (scale bar = 100 μm). (c iii) Quantification of the postsynaptic proteins was performed and an overall average of 7.9 puncta was counted and represented by microscopic image (scale bar = 2 μm). Each anisotropic construct was classified by pore size ranges creating 3 sets, and vertical or horizontal sections of PSD-95 puncta were counted to analyse their distribution according to the orientation per pore size group. (c iv) A vertical 3-D projection of PSD95 immunostaining was obtained for cross-sections of 3-D fractions of constructs. The 3-D reconstructions showed the distribution and colocalization of PSD95 (red), Tuj-1 (green) and DAPI (blue) (scale bar = 100 μm). Data expressed as mean $\pm$ SEM.

5.3.4 Microfluidic custom design for tissue interfaces: heterotypic culture of HUVECS and primary neurons

To create a 3-D neural-endothelial interfaced tissue, an organ-on-a-chip platform was designed for perfusion of oriented porous structures by two different culture media conditions (figure 5.6a-i). To verify the chemical gradient formed at this interface, two different dyes were flowed through a bulk GelMA hydrogel (figure 5.6a-ii) and the staining profile was traced (figure 5.6a-iii). The control over the interfaced environments was observed and a gradient obtained at the middle region of the hydrogel.

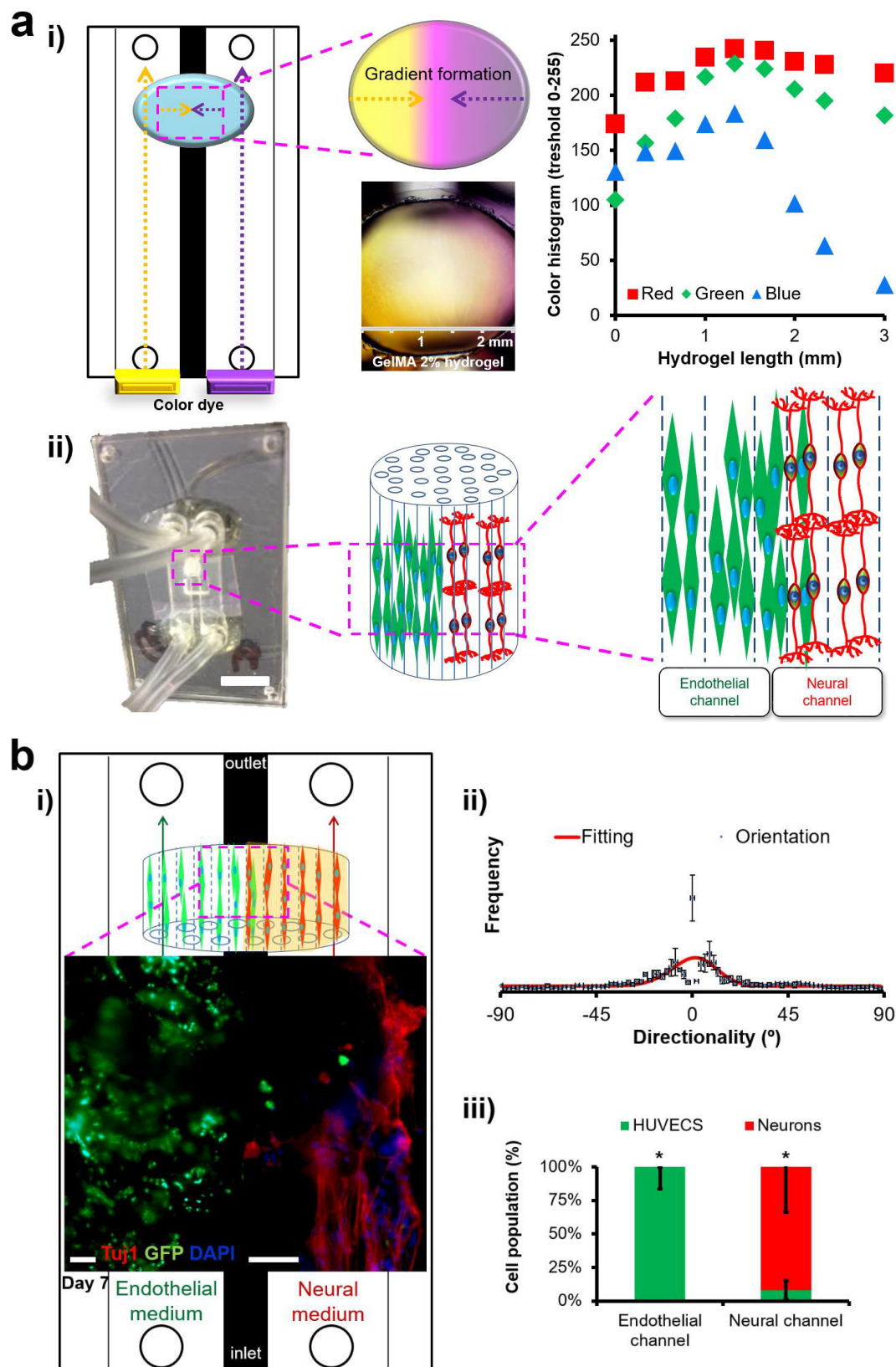


Figure 5.6 | Organ-on-a-chip microfluidic system adapted to create interfaces and perfused co-cultures. (a i) Schematic of the microfluidic device specially designed for perfusion of 3-D tissue interfaces and heterotypic cultures. Yellow and purple dyes were flowed to perfuse a 3-D hydrogel made of GelMA (2% w/v) to show the interface created by two connected channels within the 3-D hydrogel. A

magnification of the perfused GelMA hydrogel colored by dyes was represented (scale bar = 0.5 mm). RGD histogram was quantified at several points of the colored hydrogel. The color dye profile was quantified and traced as a color histogram. (a ii) A picture of the custom-made microfluidic device is shown (scale bar = 3 mm). To create an endothelial-neural interface, HUVECs and primary neurons were cultured under perfusion of endothelial and neural culture media flowed in each channel as a heterotypic culture and represented by the schematic. (b i) Mouse primary neurons isolated from cerebral cortex were suspended in Matrigel pre-gel solution (10M cells/mL) and injected in half of the 3-D oriented porous structure. HUVECs were directly seeded in the structure. Neural and endothelial media were perfused populating the construct over 7 days. (b ii) Neurite outgrowth alignment was quantified and a fitting curve traced based on a Gaussian function of the frequency of orientation angle events. (b iii) To quantify the neurons and HUVECs migrating to each other channel an immunostaining was performed to Tuj1 and HUVECs were expressing green fluorescent protein (GFP, green).

To proof the microfluidic tissue interface concept, heterotypic cultures of primary neurons and endothelial cells was performed as these are the main cells of the blood-brain-barrier. Primary neurons embedded in the hydrogel were injected in one half of the 3-D oriented porous structure and HUVECs expressing GFP were directly seeded in the second half (figure 5.6b-i). The primary neurons showed ability to align in the orientation of the porosity (figure 5.6b-ii) and kept their position in the microchannel perfused by the neural culture medium up to 7 days of culture (figure 5.6b-iii). HUVECs crossed at low number the interface towards the neural side, keeping in higher cell number in the side of the structure perfused by the endothelial culture medium (figure 5.6b iii). The control over 3-D cell stratification and orientation together with a culture media gradient was observed.

5.4 Discussion

In this study, we fabricated anisotropic 3-D structures with tunable mechanical and structural properties by controlling the ratio of methacrylated GG and gelatin for modulation of neurite extension and orientation. These polymers were selected to represent a fibrous protein and a polysaccharide resembling the natural extra-cellular matrix (ECM), respectively. By controlling the ratio of GelMA and MAGG in the photo-crosslinkable mixture, hybrid hydrogels with tunable degradation rate, elastic modulus, and pore size, were obtained. These tunable properties are connected with the blend molecular interactions. GelMA is photo-crosslinked to other GelMA or MAGG molecular chains. While the methacrylation occurs on the

primary alcohol of GG, gelatin was modified on the amine groups, increasing the space between molecular chains when crosslinked. So, GelMA-GelMA covalent crosslinking bonds produce longer space in between chains than GelMA-MAGG or even longer than MAGG-MAGG. MAGG also reacts to other MAGG molecules, creating the shorter bonds of this system by ionic strength, since these were not spaced by a methacrylate group. The same trend was previously shown by studying the effect of crosslinking density on mechanical properties of gelatin-based structures⁴⁰. This is the mechanism that allows having control over the 3-D structure properties and subsequent engineered construct. This control is crucial for applications in drug delivery and TE, where it can be tuned to release drugs in a specific timeframe controlling the degradation rate, or to fabricate an engineered graft to match the host tissue regeneration speed. Moreover, this mechanism allows mimicking structural and mechanical features of a large spectrum of human tissues *in vitro* as it is the case of different anisotropic tissues such as cardiac muscle, peripheral nerve or cerebral cortex. The set of properties tunable by this mechanism is summarized in table 5.1.

Having the control over remodeling properties, such as elasticity, degradation and swelling, as described above, a strategy to control 3-D pore orientation at a single step was developed. Here we showed that, decreasing the freezing temperature led to smaller ice crystals formation and resulted in smaller pore diameter. Since the lowest possible temperature was achieved at -196 °C using liquid nitrogen, a guided ionic crosslinking was applied before the freezing step to further decrease the porosity. The crosslinking reaction starting from the top of the hydrogel was forced to occur linearly through the polymer solution leading to a vertical bonding of GG chains, as described before^{32,41}. While the freezing temperature controlled the pore diameter by the nucleation of different sized ice crystals achieving bigger ice crystals at -80 °C and smaller at -196 °C, the vertical ionic crosslinking induced the contraction of polymeric chains caused by ionic forces. This combination of processes, described for the first time in this study, led to the production of the smallest oriented pore diameter with an average of 18 µm pore size, decreasing the diameter obtained only by freezing gradients, and increasing the range of pore diameters in 3-D oriented porous structures at the microscale applying a simple fabrication technique. The largest pore size of the anisotropic structures was obtained at the lowest freezing temperature, reaching 376 µm at -80 °C. So, the developed bioengineering method provided the ability to control the size of neurons columns at a relevant size scale, since the fabricated scaffolds were as tall as the 2 mm of neocortex, and neurons columns were varied in diameter from ~20 µm up to ~400 µm. Furthermore, the 6-layers of the native cortex vary in cell density, which we could modulate by varying the pore size.

Freeze-drying process presents as main limitation in the production of 3-D structures the formation of a polymer-lean phase and difficulty in precisely tuning with a high resolution the pore size since the

fabrication is susceptible to the thermal quenching kinetics, leading to low structural stability and generally weak mechanical properties of the fabricated materials. However, in our study we showed control over this limitation by modelling the ratio of GelMA and MAGG, reinforcing the mechanical properties with MAGG and double crosslinking forms creating this way a 3-D stability improved by a dual-crosslinking system. Moreover, the mechanical properties of the networks were improved keeping optimized features capable of supporting high performance for several cell types, meaning good cell adhesion, viability, proliferation and extension together with an adequate degradation rate that allows keeping the scaffold architecture during the *in vitro* cell culture. By reinforcing the structures, this limitation was transformed in an advantage since interconnectivity is increased by this event.

In nature, neurons extend their neurites oriented through several layers making the brain cortex and hippocampus anisotropic, which is fundamental for a meaningful function of these tissues ^{1,42-44}. Having this in mind, we cultured primary neurons in the 3-D oriented architectures having the neurite outgrowth been successfully guided in an organized 3-D millimeter scale as never reported before. Moreover, we observed different cell arrangements of neurites in each anisotropic architecture, and the release of PSD-95 post-synaptic marker followed the neurite orientation in the 3-D neural arrangements, which is more similar to what happens *in vivo* where synaptic vesicles have been shown to regulate neural activity ⁴⁵. Being the brain and its tissues difficult to non-invasively monitor *in vivo* to study healthy and disease physiology, and drug response, *in vitro* models with a realistic functional 3-D arrangement are essential for further progresses in the field of neurosciences. The system developed in this study allows arranging neural geometry more closely to the observed in the natural environment, being this way a potential solution for increasing *in vitro* reliability and mimicry, as well as reproducibility when compared to animal models.

Currently, constructs at a millimeter scale end-up with a necrotic core due to poor diffusion of culture medium, which is a problem to be assessed in the TE field. In the last approach of the present study a microfluidic device was designed to fit a construct. Moreover, this device was designed for interfaces, which are globally present in nature. Tissue barriers play a main role when testing efficacy of a bioactive molecule during the drug discovery process ^{46,47}. To create such an interface, endothelial and neural culture media were cultured in the microfluidic approach and perfused by a chemical gradient of culture media supporting both cell types viable over a week. At day 7, neurons were aligned adjacent to endothelial cells creating a heterotypic cell culture with control over biological gradients and orientation of cells and GFs in a 3-D space.

The presented system can potentially be used to model several tissues *in vitro*, especially anisotropic neural tissues characterized by aligned neurites outgrowing from primary neurons. Here we combined

the ability of tuning degradation and mechanical properties by material engineering techniques with a meaningful neuronal organization. Degenerative conditions in the brain result in degradation, regional stiffness changes, altered neuron networks morphology and ultimately neuronal function loss^{48,49,50} as observed during aging⁵¹, and Alzheimer's or Parkinson's diseases⁵². Moreover, the brain vasculature morphology is also affected⁵³, which can be targeted by the developed 3-D system combined with the microfluidic approach designed for interfaces and heterotypic cell cultures.

5.5 Materials and methods

Methacrylation of gelatin and gellan gum. GG (Gelzan™, molecular weight: 1,000,000), gelatin (from porcine skin, gel strength 300, Type A), and methacrylic anhydride were purchased from Sigma-Aldrich. MAGG and GelMA were synthesized by reacting GG and gelatin with methacrylic anhydride. Briefly, 4 g of GG was dissolved in 400 mL of distilled water at 70°C for 1 h, and 8 mL of methacrylic anhydride was added at 50°C. The reaction was continued for 6 h at 50°C controlling the pH at 8 by adding 5N NaOH solution (flow rate was continuously adjusted to keep the pH over 6h). After 6 h the solution transferred to dialysis tubing (molecular weight cutoff: 12–14 kDa, Spectrum Labs, Inc.) and dialyzed in distilled water for 4 days, changing water twice a day. The solution was lyophilized to obtain pure MAGG, and it was stored at –80°C until further use. Similarly, 2 g of gelatin was dissolved in 20ml of phosphate buffered saline (PBS, 1X, Invitrogen) at 50°C, and 1.6 mL of methacrylic anhydride was added at a flow rate of 0.1 mL/min for 16 min. The reaction was continued for 2 h at 45°C, and then dialyzed against distilled water at 40°C for 5 days, changing water twice a day. The solution was lyophilized to obtain pure GelMA, and it was stored at –80°C until further use.

The degree of methacrylation (DM) of MAGG was measured by ¹H NMR (Varian Inova 500). MAGG was dissolved in D₂O at 10 mg/ml at 50°C to obtain the spectrum was obtained. The DM of MAGG, defined as the number of methacrylate groups attached to GG divided by the number of hydroxyl groups of unreacted GG, was calculated by integrating the 1.5 ppm peak from the methyl group of the rhamnose unit, and the 2.0 ppm peak from the methyl group of the methacrylate group.

The reagent TNBSA was used to determine the free amino groups in GelMA. Absorbance and concentration present a linear relationship up to 1 mg/mL. The modification of gelatin by methacrylation is inversely proportionally to the absorbance of the solutions at 335 nm and was quantified using a standard curve of concentrations for calibration.

Preparation of GelMA and MAGG based hydrogels. 0.5% (w/v) 2-hydroxy-1-[4-(2-hydroxyethoxy)phenyl]-2-methyl-1-propanone (Irgacure 2959, Ciba Specialty Chemicals) was added to the polymeric solution at 50 °C for 1 h to fabricate the hydrogels. The lyophilized MAGG and GelMA were dissolved (by this order) at 2% (w/v). In the blends of 3:1 and 4:1 a total concentration of 2% (w/v) was kept in deionized water under constant stirring. First, MAGG was dissolved at 80°C for 1 h and then GelMA was added and dissolved at 50 °C. After dissolution, photo-crosslinked hydrogels were obtained by exposure to UV light (320-500 nm, 7.14 mW/cm², EXFO OmniCure S2000) for 60 sec. After photo-crosslinking, the hydrogels were immersed in CaCl₂ solution at 0.5 M concentration for 24 h for total ionic-crosslinking of MAGG molecular chains. For all the isotropic structures, silicon molds (10 mm height, 7 mm diameter) were used. The obtained structures were punched and sliced in cylinders of 3x3 mm. For the fabrication of anisotropic structures with smaller pore diameter, a guided ionic crosslinking was performed before the photo-crosslinking. To guide the ionic crosslinking, cylindrical molds are covered on the bottom part with Parafilm™ and the top part covered with paper sheet. The paper sheet is used to adsorb a CaCl₂ solution of 0.5 M feeding the ionic crosslinking of the polymeric solution by capillarity (consult figure 1 in supplementary data to see the described set up). The structures were submitted to an oxygen plasma treatment for 3 cycles of 5 min for sterilization and surface hydrophilicity cure before cell seeding.

Hydrogels translucency assessment. The hydrogels' optical translucency was qualitatively and quantitatively evaluated. The qualitative evaluation was performed optically and microscopically against white paper sheet with the text “BAMM Lab & 3B's” to evaluate the light distortion occurred through the hydrogel. The quantification was determined by visible light spectrophotometry at 495 nm wavelength and normalized to PBS; wet paper in PBS was used as a negative control. Five measurements per condition were performed.

Measurement of rheological modulus. Rheological measurements were performed using an ARES-G2 rheometer (TA Instruments). All measurements were run with 8 mm serrated plate geometry on swollen samples immersed in PBS during the test using a special solvent dish and conditioned at 37 °C. The swollen sample diameter was always 8 mm, and sample thickness was 1 mm. The rheometer gap was set at 800 μm and a dynamic time sweep was performed with 1 Pa stress, 1% strain, and 1 rad/s. 5 measurements per condition were performed.

Assessment of water absorption and weight loss. To study the swelling kinetics of the developed hydrogels, PBS (Gibco, with monovalent ions) solution was used. Hydrogel samples at 1% (w/v) were immersed in 1 mL of each solution at 37 °C. At different time points, the hydrogels (n=4) were removed from the solutions and quickly blotted on a filter paper. Their wet weight was measured (wt) and compared to the initial wet weight (w0). The swelling ratio (Sk) was defined according to eq 5.1.

equation 5.1.
$$Sk (\%) = (wt - w0) / w0 \times 100$$

The degradation profile of the GelMA-MAGG polymeric blend formulations was studied according to eq. 5.2. Weighed hydrogels (n=4) were incubated in a 13 ng/mL collagenase type I solution at 37 °C for 7 days. The samples were removed, freeze-dried and weighed again to determine mass loss.

equation 5.2.
$$\text{weight loss } (\%) = (m0 - mf) / m0 \times 100\%$$

Fabrication of anisotropic 3-D oriented porous structures. The anisotropic fabrication is preceded by the production of hydrogels as previously described. The anisotropic structures were fabricated using the hydrogels and applying freezing gradient temperatures, reaching the -80 °C or -196 °C. These temperatures were reached by using dry ice or liquid nitrogen, respectively. To create the temperature gradient, a custom made styrofoam mold involving silicon tube (100 mm height × 6 mm diameter) was used covering the polymeric solution all around except the top, where a frozen plate at -80 °C or -196 °C was directly placed for 2 h. As a third strategy the UV-crosslinking was preceded by a guided ionic crosslinking. After freezing for 2 h and before the lyophilization, the frozen structures were kept in a freezer at -80 °C. The obtained structures were formed by porosity differences in both ends of the cylindrical shaped scaffolds. The portion of the scaffold used for each trial was sliced from the second half of the formed cylinder. In every condition the porosity decreases through the scaffold from the top to the bottom and to avoid that variation the first half of each scaffold was discarded (meaning the half in contact with the frozen metal plate).

Characterization of 3-D architecture of 3-D porous structures formulations. Freeze-dried structures were analyzed after lyophilization using high-resolution micro-CT. The structure of the sample was acquired by X-ray, reconstructed and then qualitatively and quantitatively analyzed. Several morphometric parameters, as porosity, mean pore size, pore wall thickness, interconnectivity and anisotropic degree were calculated. 3-D perspectives of each formulation were built. Samples were acquired with a SkyScan 1272 scanner (v1.1.3, Bruker, Boston, USA), with a pixel size of 8 µm. The X-ray source was set at 50 kV and 200 µA. Approximately 600 projections were acquired over a rotation

range of 180°, with a rotation step of 0.6°. Data sets were reconstructed using standardized cone-beam reconstruction software (NRecon 1.6.10.2, Bruker). The output format for each sample was bitmap images. Representative data set of the slices was segmented into binary images with a dynamic threshold of 30–255 for soft polymer analysis (grey scale values). Then, the binary images were used for morphometric analysis (CT Analyser, v1.13, Bruker) and to build the 3-D models. Measurement of pore diameter was assessed using FIJI® software to measure the pore diameter of bright field images collected by optical microscopy (n = 5). More than 20 pores were measured per sample for each formulation.

Live/dead cell viability assay. The effect of each formulation on cell viability was assessed after 7 days culturing NIH-3T3 fibroblasts (ATCC) and SH SY5Y (ATCC). Cells were seeded within the 30 min after sterilization in a 24-well suspension cell culture plate (VWR) and kept in the CO₂ incubator (1M cells/scaffold in 50 µL culture medium per seeding). A cell suspension of NIH-3T3 was prepared by trypsinization (trypsin/EDTA solution, Invitrogen) and seeded onto the several freeze-dried hydrogels formulations prepared as previously described. NIH-3T3 constructs were cultured in α-MEM (Life Technologies) supplemented with 10% of FBS (Thermo-Fisher Scientific) and 1% penicillin-streptomycin (Life Technologies) at 37 °C, in a humidified atmosphere with 5% of CO₂. SH SY5Y were seeded onto the 3:1 freeze-dried formulation. SH SY5Y constructs were cultured for 3 days with DMEM-F12 and 5% FBS (Thermo-Fisher Scientific), 1% penicillin-streptomycin (Life Technologies) and supplemented with 10 µM retinoic acid for more 4 days at 37 °C, in a humidified atmosphere with 5% of CO₂. Cell viability of cells seeded in the 3-D structures was characterized after 7 days of cell culture staining with Live/Dead calcein AM and ethidium homodimer-1 in PBS (Life Technologies) for 20 min. All formulations were studied performing triplicates and cultures on tissue culture polystyrene well plates were used as a positive control for cell viability.

Alamar blue assay. NIH-3T3 fibroblasts were seeded and cultured as previously described. Three hours later, the constructs were moved to a new 24-well suspension culture plate and 900 µL of α-MEM was added for each construct supplemented with 10% FBS (Thermo-Fisher Scientific), 1% penicillin-streptomycin (Life Technologies). The same culture medium was used as described before, being changed every 2 or 3 days. *In vitro* metabolic activity was assessed at day 1 and 7 of culture using AlamarBlue® CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI, USA) (n = 9), at 37°C in a humidified 5% CO₂, in a concentration of 10% in culture medium. Then, the medium was removed by aspiration and the constructs were washed with fresh medium three times and kept in culture

until the end of the assay. Scaffolds without cells were used as a negative control for fluorescent intensity correction.

Isolation and culture of primary cerebral cortex neurons. All experiments were carried out according to animal care standards set forth by the National Institute of Health and were approved by the Institutional Animal Care and Use Committee (IACUC) at Stanford University. Primary cortical neurons were isolated from embryonic day 18 (E18) C57bl/6 mice provided by Jackson Laboratory. Freshly micro-dissected whole mouse cortices were dissociated by trituration after 30 min of enzymatic digestion using Neuronal Isolation Enzyme (Thermo-Fisher Scientific) in HBSS (Life Technologies). The cells were then suspended in neurobasal medium with B27 supplement (Life Technologies) and 500 μ M glutamine (Life Technologies) until seeding/injection. Culture conditions were maintained at 37 °C with a 95% relative humidity, and 5% CO₂. Primary neurons isolated from cerebral cortex of mouse embryos at week 18 were embedded in Matrigel® followed by injection of the cells in the isotropic or anisotropic porous structures (10 M cells/scaffold embedded in 50 μ L matrigel®). The constructs were cultured in 24-well tissue culture plates for 7 and 14 days in neurobasal medium with B27 supplementation and 500 μ M glutamine at 37°C in a humidified 5% CO₂.

Immunofluorescence staining. Cells were fixed with 4% paraformaldehyde for 20 min at RT and washed with PBS. Constructs were blocked in 1% BSA (Sigma) and cells were permeabilized with 0.3% Triton-X 100 (Sigma) for 2 h at RT. Cells were incubated with rabbit anti-tuj1 (A7811, Abcam) and mouse anti-GFAP (ab5245, Cellsignal) overnight at 4 °C. Samples were washed and incubated for secondary goat anti-mouse Alexa Fluor 568 and goat anti-mouse Alexa Fluor 488 antibodies (Life Technologies) for 2 h at RT. Mouse anti-PSD95 was visualized with goat anti-mouse Alexa Fluor 488 (Life Technologies). DAPI was used as nuclear counter staining in all the cases. After washing, 3-D structures were visualized with Zeiss LSM710 inverted confocal microscope.

Neurite shape, extent and orientation analysis. Neurite orientation angle was determined using FIJI® software. Orientation analysis was performed with the FIJI® software applying the Directionality tool (n=5). A color map was traced indicating the neurite orientation by color correspondence.

Statistical analysis. The investigated parameters were expressed as mean $\pm$ SD or confidence interval. Student's t-test was used to compare group means, and a p-level < 0.05 was considered statistically

significant. Statistical analyses were carried out with Microsoft Excel 2010 and GraphPad Prism 5 for Windows. In all cases, not-paired two-tailed Student's t-test and nonparametric Mann-Whitney test were used to determine significance of the results.

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5.8 Supplementary Information

5.8.1 Supplementary Materials and Methods

Gelatin and GG were modified by methacrylation to create photo-crosslinkable and stable hydrogels. To test the photo-crosslinking speed and ability, a photo-mask patterned with squares was used. The gels were exposed 25s to UV at low intensity (figure S5.1).

Different cell types were seeded at 1M cell/scaffold density over 7 days *in vitro* to build constructs with 3x3mm made of 3T3 fibroblasts (figure 5.5) or SH-SY5Y neuroblastoma cells in the GelMA-MAGG 3:1 formulation. SH-SY5Y cell line was supplemented with retinoic acid at day 3 for neurite extension evaluation (figure 5.5).

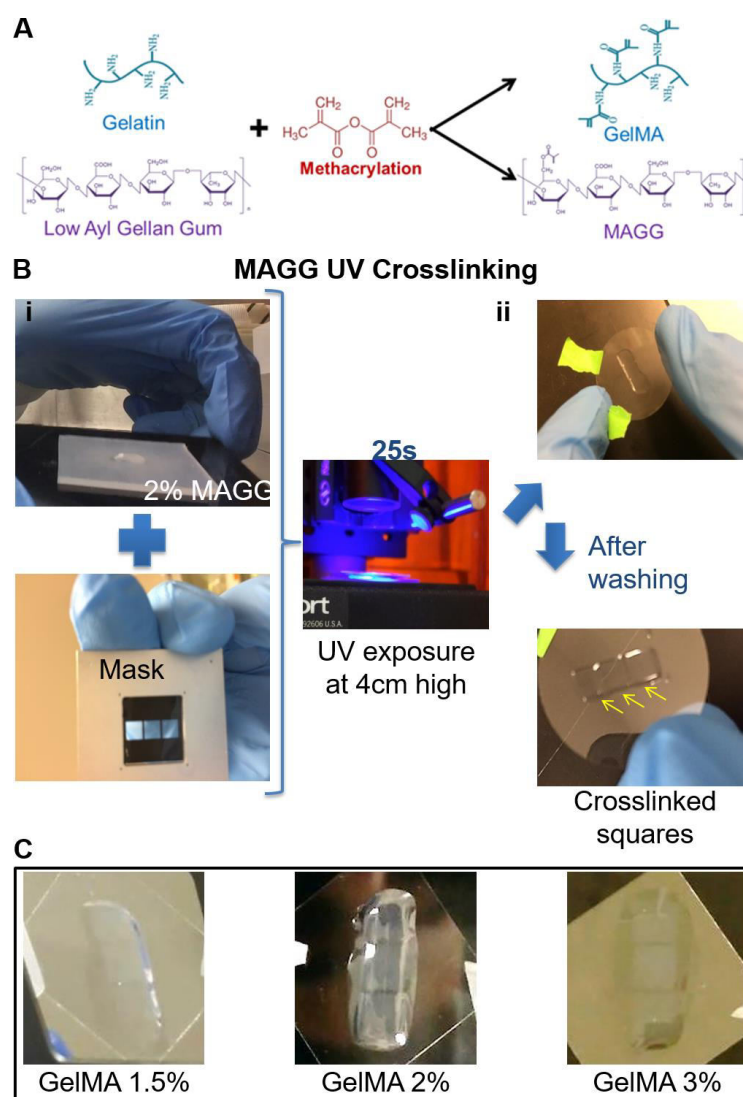


Figure S5.1 | Methacrylation of gelatin and GG and hydrogel fabrication. (A) Gelatin and low acyl GG were modified by methacrylation (b) The crosslinking ability by UV light of MAGG was assessed

using photomasks and the formation of patterned squares was observed (c) GelMA was assessed for photo-crosslinking using the same principle at different concentrations.

5.8.2 Supplementary Results

Since the methacrylation occurs by replacing these groups, the reaction is inversely proportional to the substitution. A modification of 69.3% by methacrylation is calculated for gelatin (figure S5.2A i). To calculate the percentage of LAGG modified in MAGG, NMR is performed showing 71.0% of methacrylation degree (figure S5.2A ii).

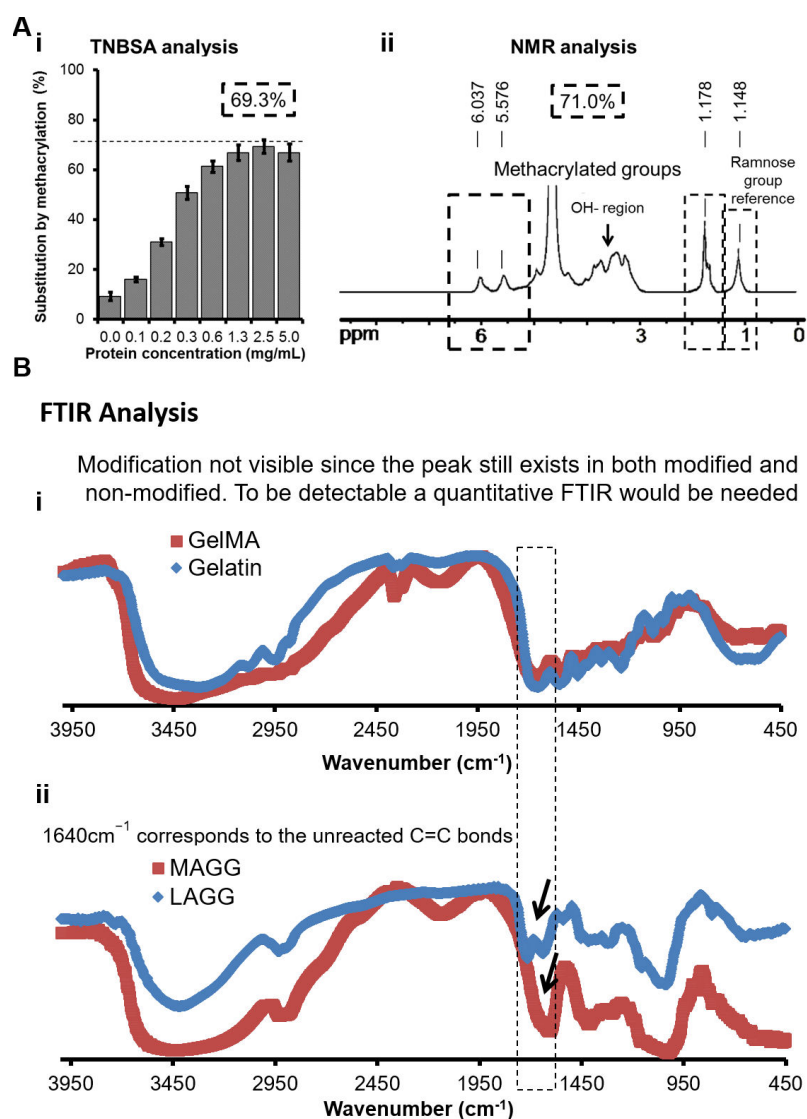


Figure S5.2 | Characterization gelatin and GG methacrylation. (A) Gelatin and GG were modified by methacrylation at 69.3 and 71.0%, respectively. (B) FTIR to gelatin (i) was not conclusive, but to GG (ii) showed the change in the peak corresponding to the modified molecular group.

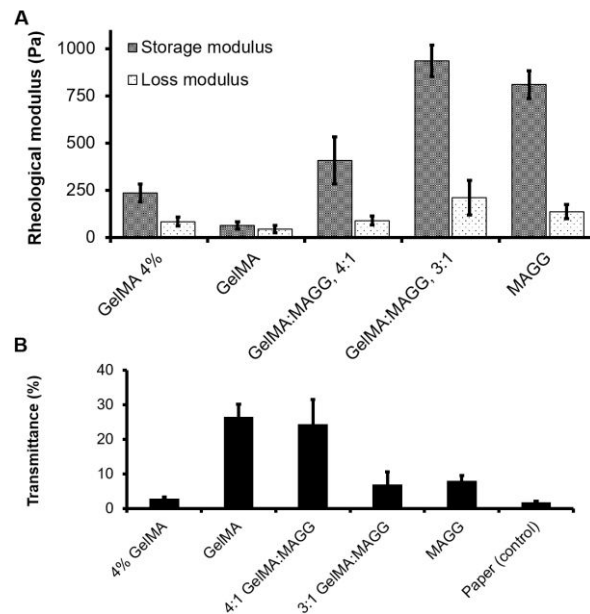


Figure S5.3 | Physicochemical characterization of GelMA and MAGG. (A) Rheological properties of several formulation including GelMA 4% were assessed. (B) Light transmittance was also assessed at GelMA 4%.

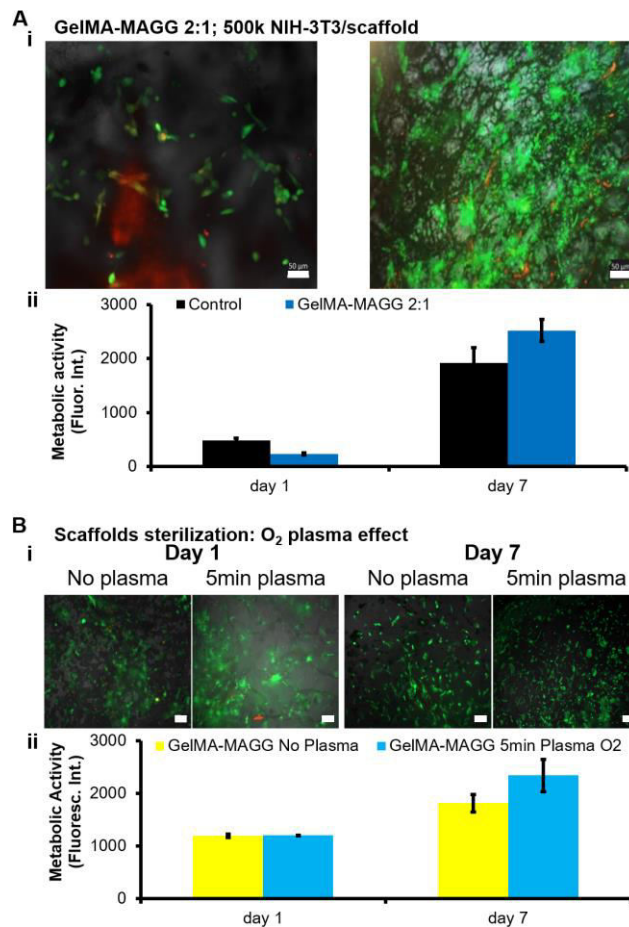


Figure S5.4 | Biological assessment for cytotoxicity and sterilization efficiency. (A) Viability was tested for GelMA:MAGG at day 1 and 7. (ii) Live/dead staining was performed showing good cell

adhesion and proliferation. (ii) Metabolic activity was also quantified. (B) The efficiency of plasma sterilization method was assessed.

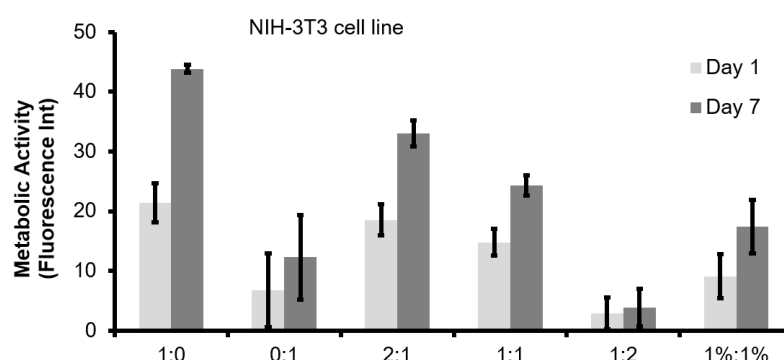


Figure S5.5 | Biological assessment for cytotoxicity and sterilization efficiency. Different concentrations of GelMA:MAGG hydrogels were fabricated and tested for metabolic activity over 7 days.

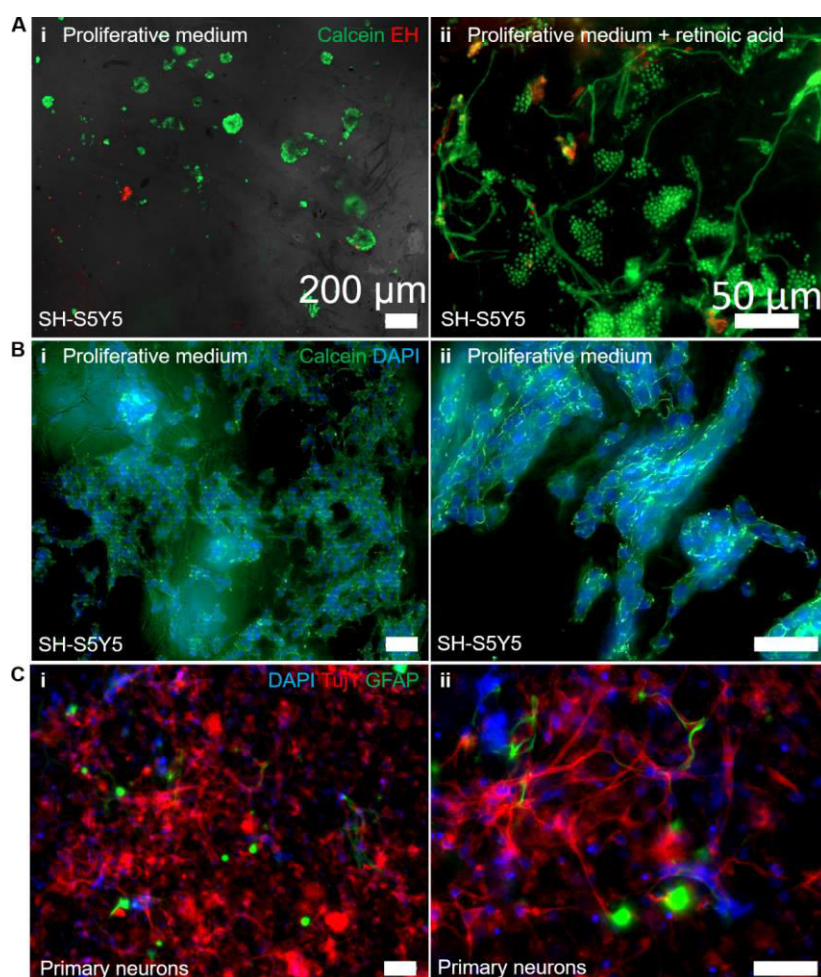


Figure S5.6 | Neurites extension assessment and neural viability. SH SY5Y were used for viability tests (A) under neurite extension medium or (B) proliferative medium. Since the current approach

was designed for primary neuron constructs preparation, (C) primary neurons were cultured in the 3-D structures to assess neurite extension.

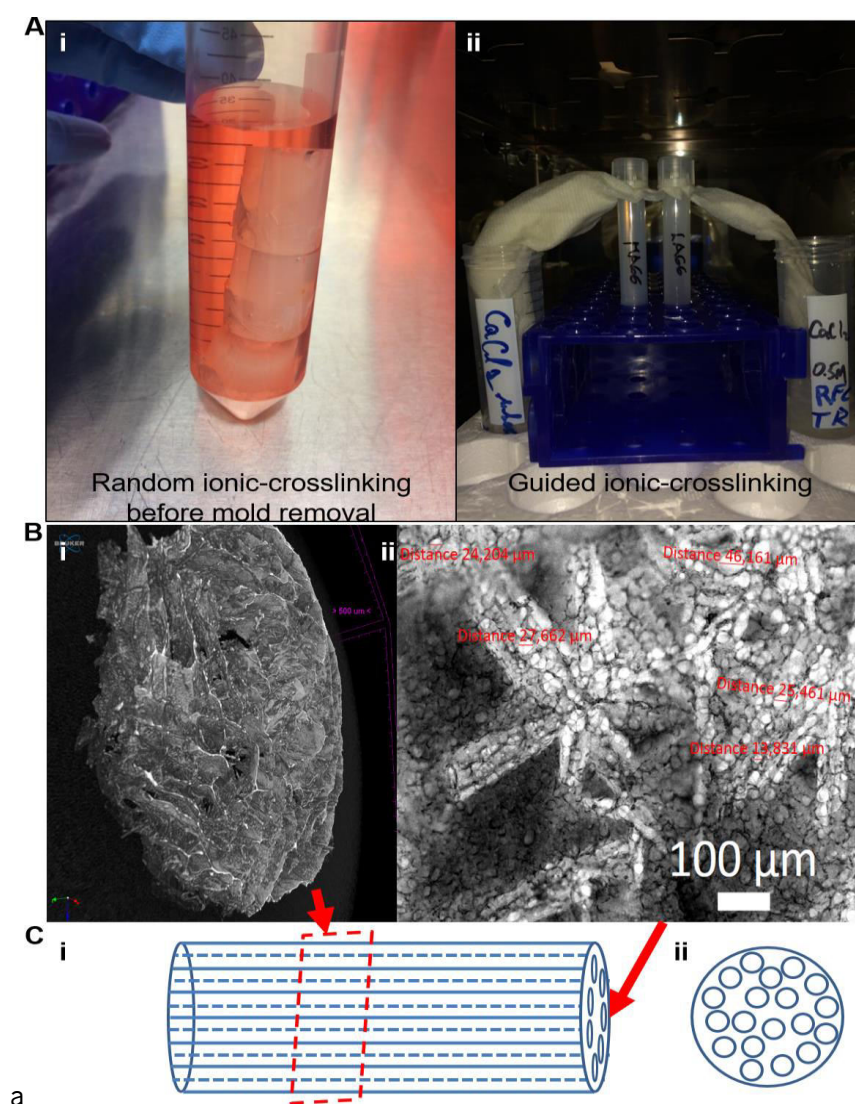


Figure S5.7 | Guided crosslinking set-up and oriented porosity reconstruction. (A) Ionic crosslinking guidance strategies were tested as (i) randomly performed, or (ii) guided. (B) Crosslinking and freezing guided methods led to anisotropic 3-D structures formation, as (C) represented by the scheme.

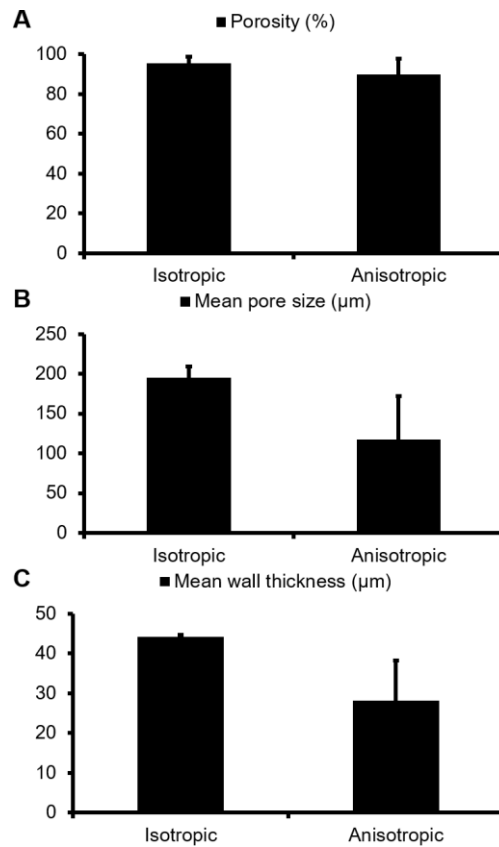


Figure S5.8 | Guided crosslinking set-up and oriented porosity reconstruction. (A) Porosity was slightly affected by the structure porous orientation. (B) The mean pore size decreased when comparing to isotropic structures performed at -80°C freezing temperature. (C) Mean wall thickness decreased with the pore size.

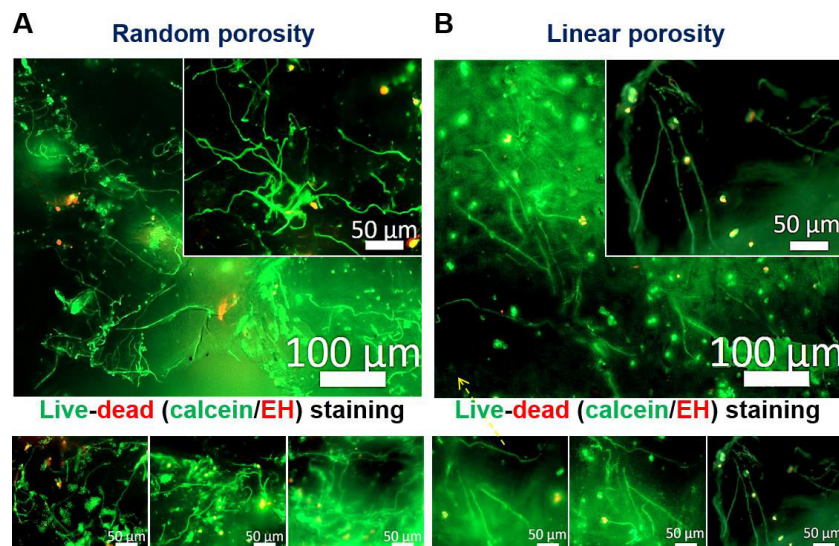


Figure S5.9 | SH-SY5Y neurite guidance assessment. SH-SY5Y were cultured in isotropic and anisotropic structures showing, respectively, (A) neurite random extension, or (B) neurite linear extension.

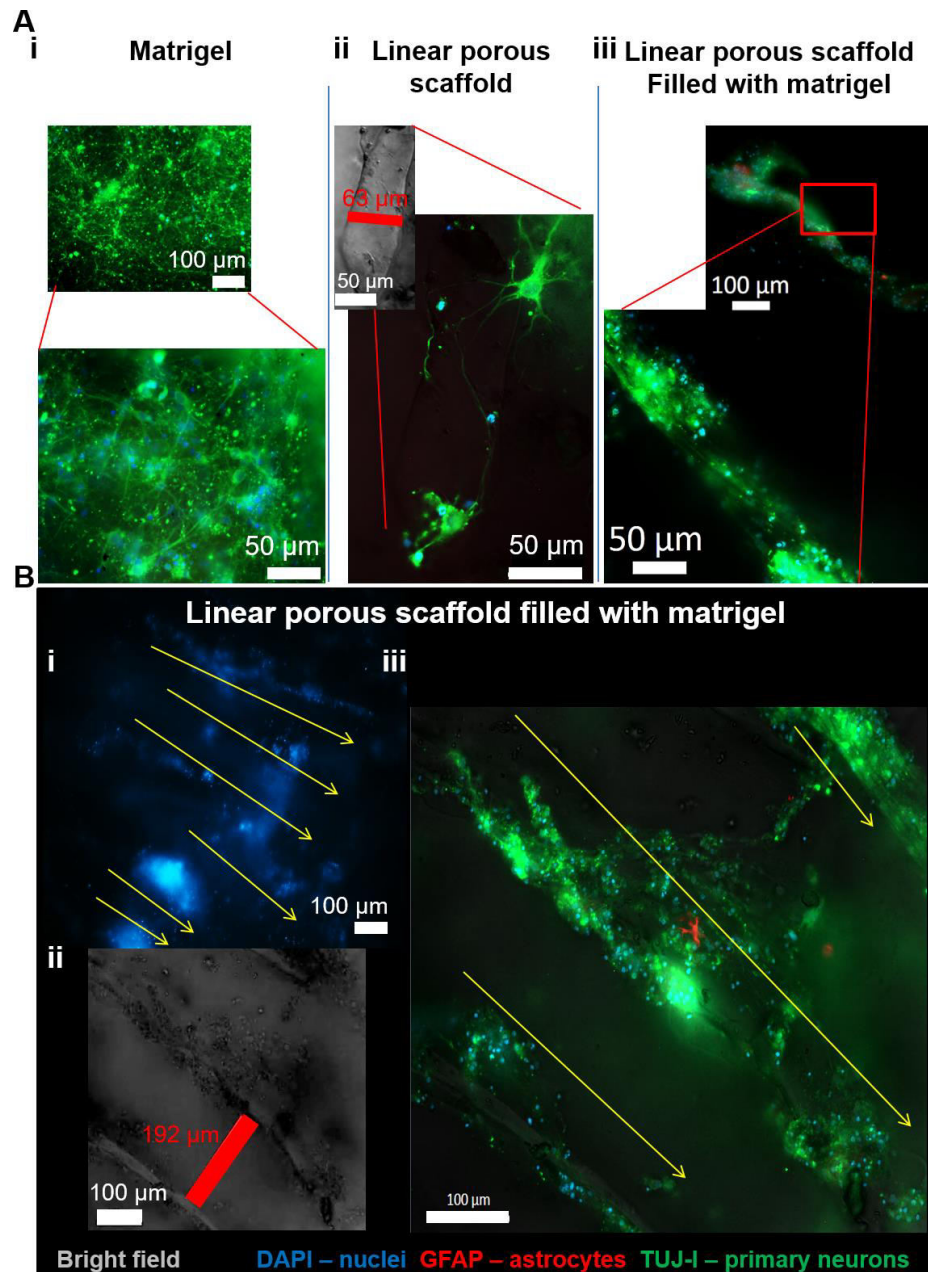


Figure S5.10 | Primary neuron neurite guidance and columns formation assessment. (A) To assess guidance of neurite extension, (i) primary neurons were cultured in Matrigel, or (ii) seeded in porous oriented structures. (iii) To fill the and keep the cells in the pores, primary neurons were encapsulated and injected in the structures. (B) Several layers of aligned neurons were observed, (ii) guided by the material architecture, and (iii) having contact across layers.

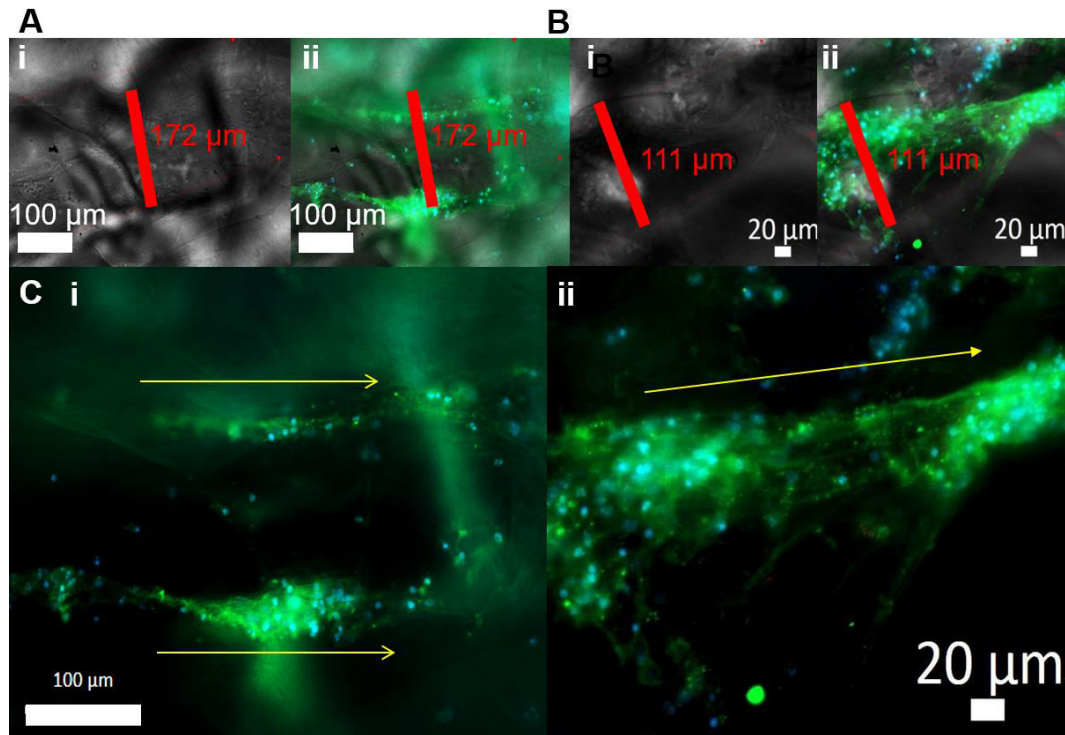


Figure S5.11 | Porosity guiding neurite extension. (A - i) Structure channels (ii) were filled with neurons. (B i) Pore diameter changed (ii) and the size of neurons column formation is controlled by the pore diameter. (C) Pore diameter can also change the way neurons distribute, (i) organizing in two layers attached to each wall, or (ii) creating a single column through the pore.

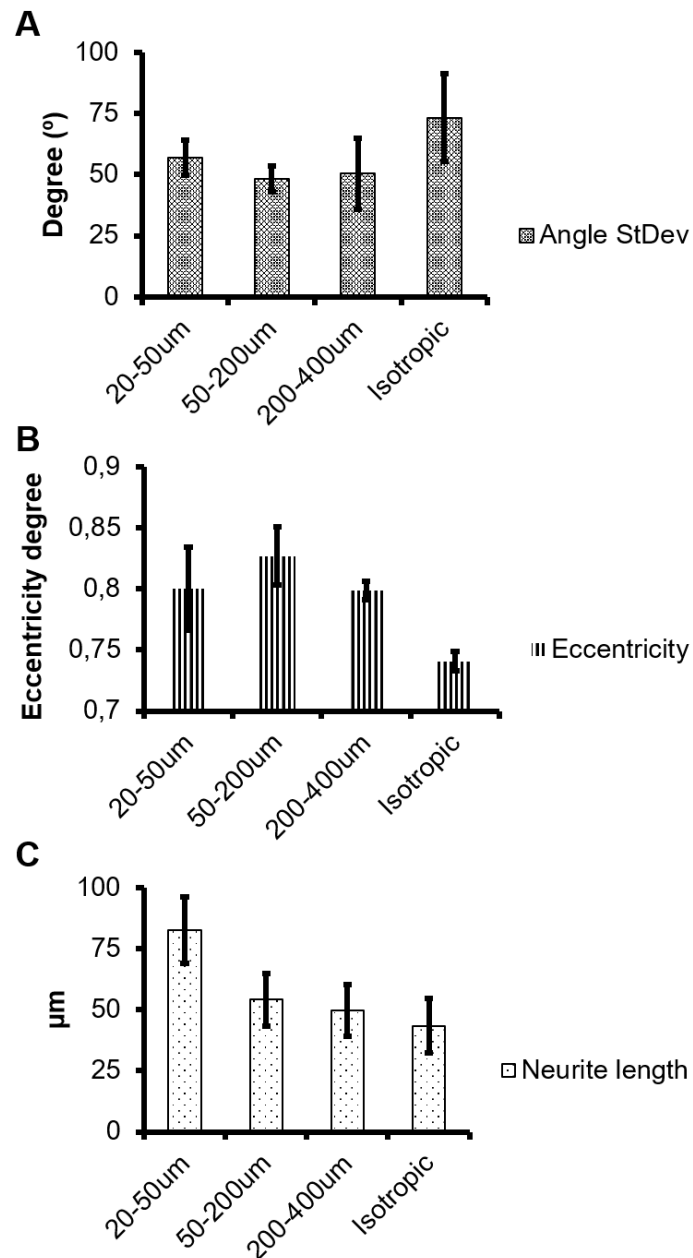


Figure S5.12 | Neurite quantitative characterization. (A) The angle SD was plotted to observe how disperse is the orientation of each anisotropic formation. The dispersion increases in the isotropic pattern. (B) The cell shape eccentricity was assessed, meaning the values closer to 1 refer to more extended cell shapes. (C) The neurite length was assessed showing that decreasing the pore diameter increases the neurite extension length.

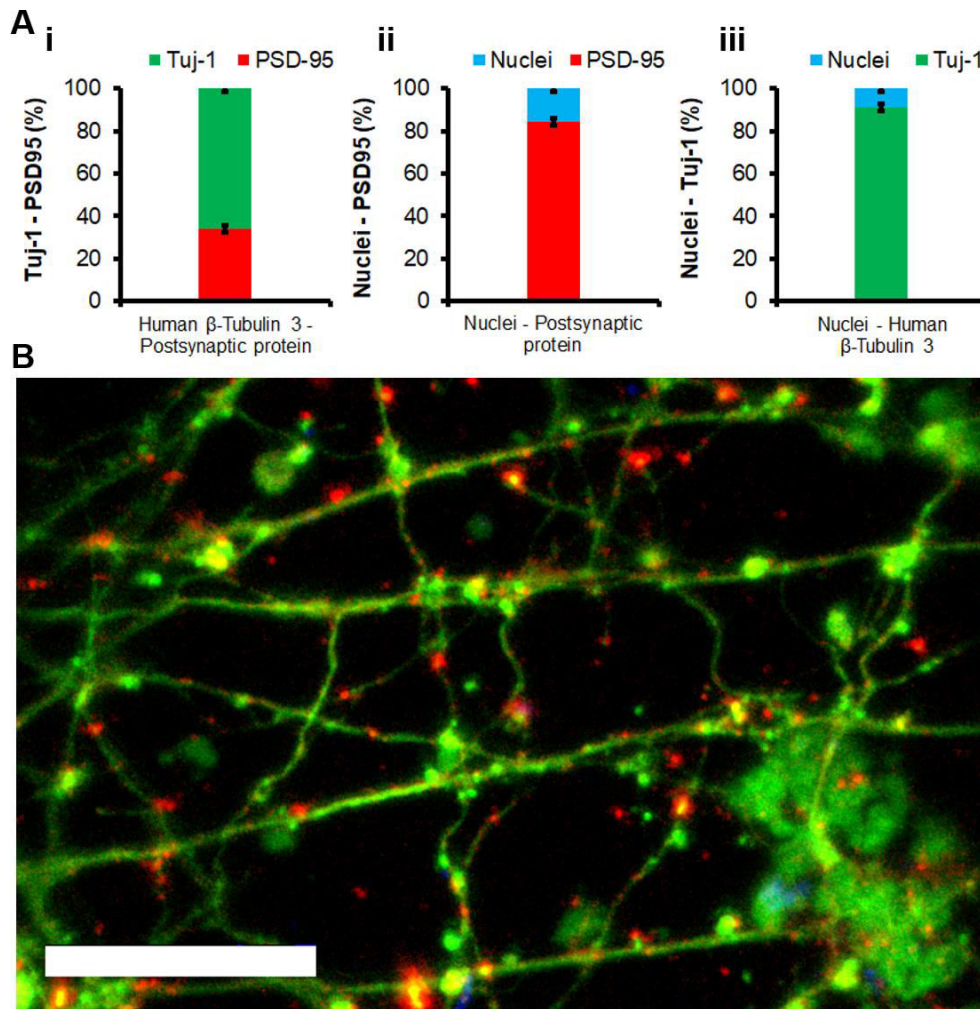


Figure S5.13 | Post-synaptic protein expression. PSD-95 was stained to assess the localization of post-synaptic proteins. (A) Ratios of immunostainings were performed as a method to understand the amount of PSD-95 expression relative to other cellular number indicators, such as (i) Tuj-1/PSD-95 (ii) Nuclei/PSD-95, and, as a comparison (iii) Nuclei-Tuj-1. (B) A representative of the PSD-95 puncta localization is presented, showing Tuj-1 as green, PSD-95 as red and nuclei as blue.

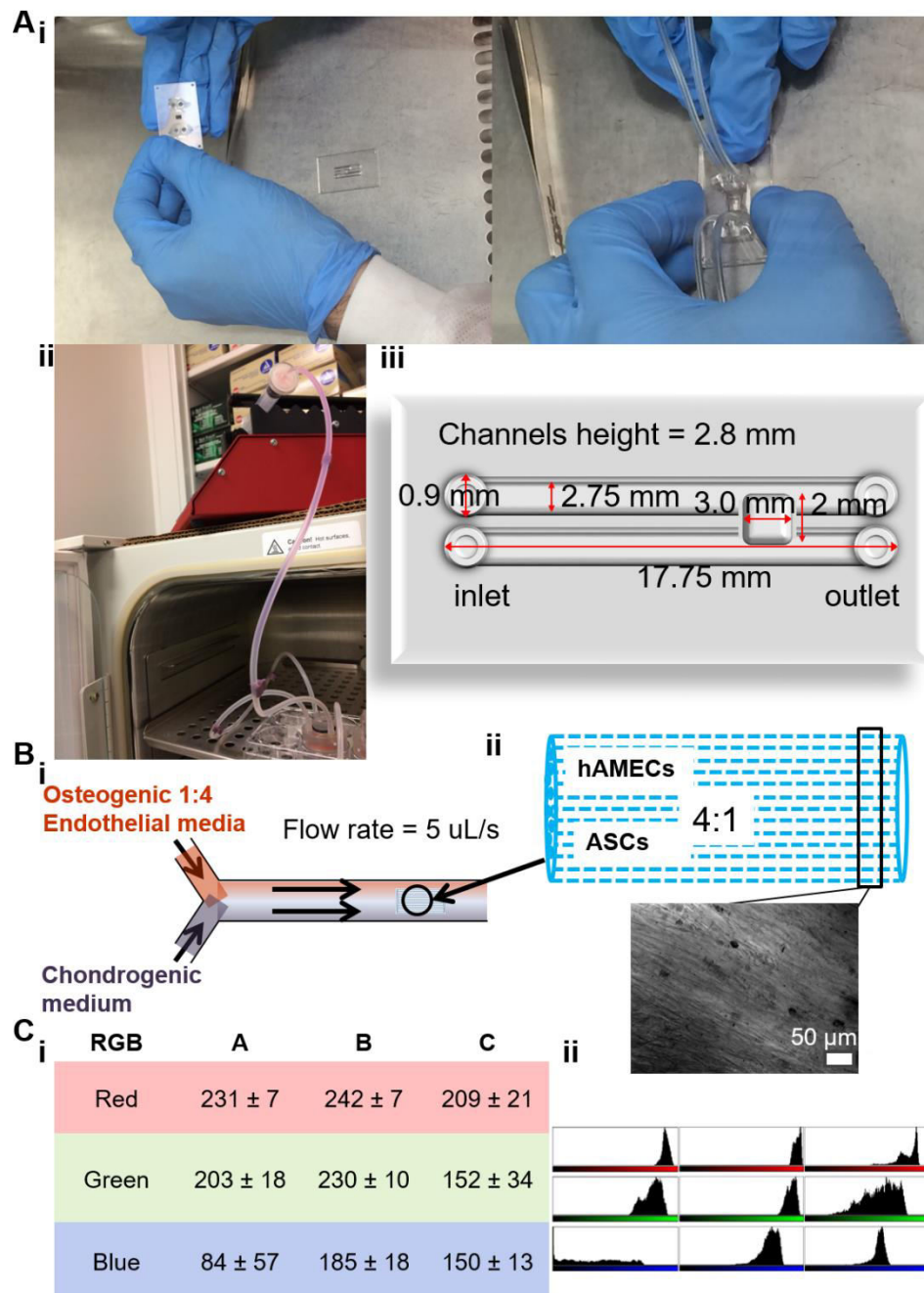


Figure S5.14 | Microfluidic device assembling and gradient formation quantification. (A) Microfluidic device (i) parts were assembled with a (ii) flowing pump for cell culture perfusion and incubation. (B) The strategy comprises a dual-flow of culture medium to create a gradient of biochemical signals. (ii) Anisotropic structures were used to be perfused under dynamic conditions, being the support for a heterotypic culture of endothelial cells and neurons. (C) Red and blue dyes were flowed on top and bottom channels to observe the gradient formation. (i) RGB colors were quantified and (ii) plotted showing the change of RGB colors from top – A, interface – B, and bottom – C regions, where blue is increasing and red decreasing.

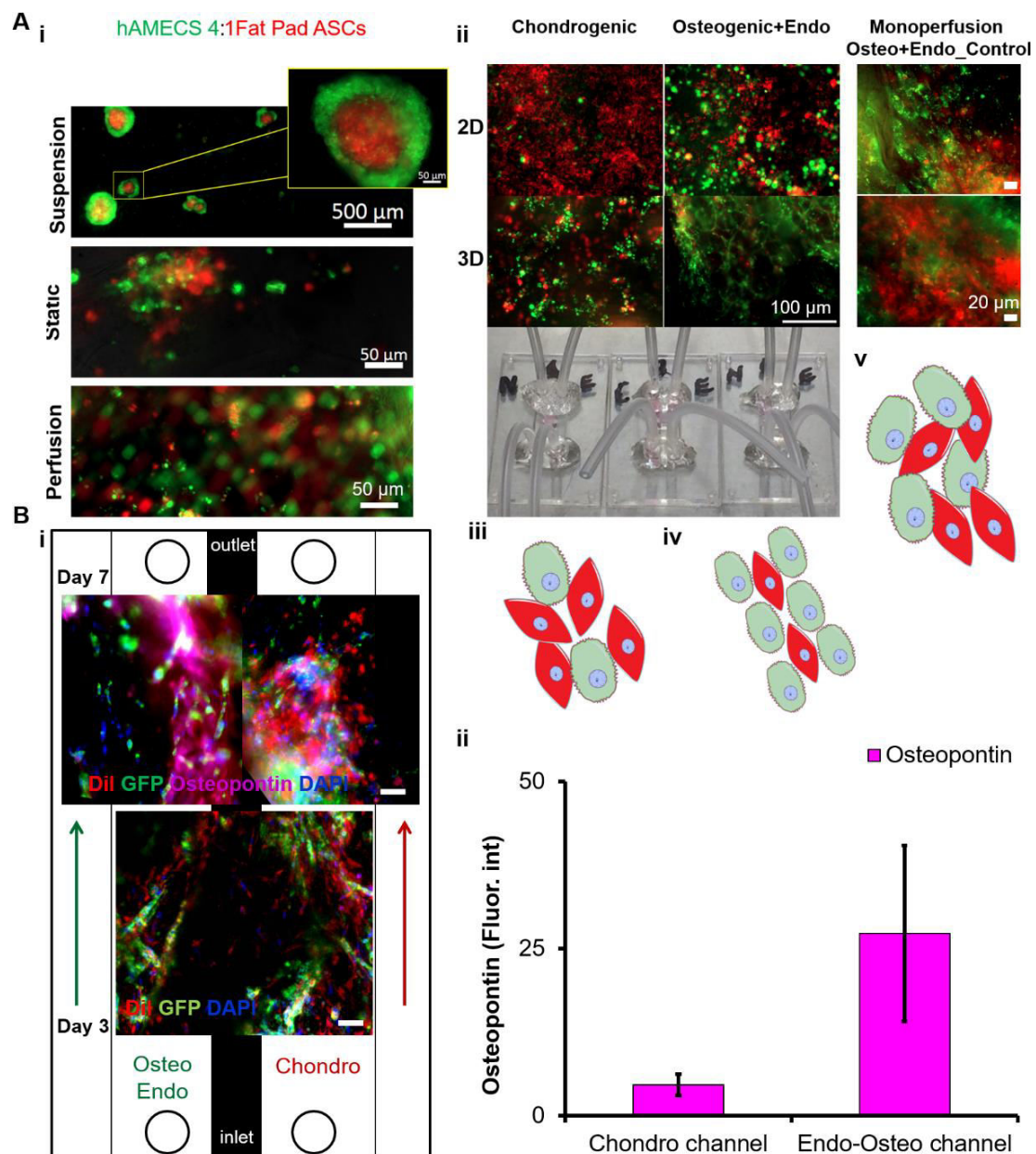


Figure S5.15 | Interface microfluidic approach on OC differentiation. hAMECs were co-cultured with fat pad-derived ASCs. (A) Cells were either cultured in suspension, in static or perfused conditions. (ii) Cells populated differently when cultured under perfusion of chondrogenic or osteo-endothelial conditions. The prevalence of hAMECs increased in the osteogenic conditions in both 2-D and 3-D environments. Cell tubular-like arrangements were observed when in 3-D structures. When perfusing both channels with the osteo-endothelial medium, no prevalence of one cell type over the other was observed. (B) Osteopontin was quantified in the condition of 3-D co-culture under interfaced chondrogenic and osteo-endothelial media. An increase of osteopontin localization was observed in the osteo-endothelial side.

CHAPTER 6

BIOREACTOR FOR 3-D HIERARCHICAL CELL CULTURES

Based on the following publication:

Canadas RF, Oliveira JM, Marques AP, Reis RL, Multi-chamber bioreactor, methods and uses, WO2016042533A1, 2016. <https://patents.google.com/patent/WO2016042533A1/en>

6. MULTI-CHAMBERS BIOREACTOR, METHODS AND USES

6.1 OBJECT OF INVENTION

The present disclosure relates to a multi-chamber bioreactor, preferably in a polymeric material with a 3-D structure, adapted for cell-mono and co-culture, with at least two entries and outputs of culture medium adaptable to be used as a static or dynamic culture system, depending if culture media is perfused or manually replaced.

The disclosure subject matter also relates to a technique based on a bioreactor device that allows the creation of two or more different tissues integrated with the natural phenotype, using an integrated and continuous 3-D support structure.

6.2 DESCRIPTION

6.2.1 Background Art

There are several different systems for 2-D and 3-D cell culturing, which can be categorized into static (e.g. tissue culture polystyrene) and dynamic systems (e.g., bioreactors).

In the case of static culture systems, 2-D, or flat culture plates, well culture plates, Petri dishes and T-flasks are the most common used technologies. There is also the possibility to combine both 2-D systems, using a well culture plate with trans-well inserted to create a flat membrane over (and in the middle) of the well, allowing to have two different surfaces, interconnected, in the same culture well.

Recently, new culture well plates have been created to be 3-D. A mesh with a 3-D architecture is designed in the bottom part of the well, allowing the cells to be cultured in a more biomimetic arrange, comparing to human body environment. A disposable chamber adapted to accommodate 3-D structures was also recently created.

However, in TE and RM there is a lack of physical structures to support multilayered and complex tissues (Keeney and Pandit, 2009). The state of the art is characterized by several bilayered structures with sharp interfaces (Nukavarapu and Dorcemus, 2012). It is extremely challenging to make a continuously gradient structure that allows a smooth interface formation of two tissues with different phenotypes, and one of the biggest problems is not related with the structure itself, but greatly dependent on the cell culturing conditions in a 3-D environment. In this sense, there is a need for devices or bioreactors enabling to

induce the homogenization of the cells inside of the 3-D structure or scaffolds, and at the same time, allowing having the optimal conditions for the production of multilayered tissue mimicking the native ones. These facts are unveiled in order to illustrate the technical problem addressed by the present disclosure.

6.2.2 General description of the invention

In order to address the above mentioned technical problems and despite deficiencies related to the above solutions, it is necessary to develop a reactor and a method to obtain synthetic tissues grafts with fused cells multilayers that mimic the native ones. Also has a further advantage that avoids the use of a binder between the synthetic tissues layers and improves the cell proliferation that can be evaluated by metabolic activity measurements.

To enable induction of multilayered phenotypes in a single tissue mimicking the native ones, appropriate nutrition/chemical cues (e.g., supply of culture medium with or without bioactive agents) shall be optimized for each region of the multilayered construct.

One of the aspects of the present subject matter is a multi-chamber bioreactor with or without 3-D structure, adapted for cell mono- and co-culture, with at least two entries and outputs of culture medium, adaptable to be used as a static or dynamic culture system.

An aspect of the present disclosure relates to a polymeric multi-chamber bioreactor, preferably transparent, comprising:

- at least a first and a second fluid-tight chamber for receiving, respectively, a first fluid culture medium and a second fluid culture medium comprising, respectively, a first and a second cell culture, and an opening between the first and second chamber for interchanges between the chambers;
- each chamber comprising at least an inlet and an outlet of fluid, preferably a fluid culture medium;
- the first and second chamber comprising, respectively, a first and a second cell culture scaffold, wherein the first and second scaffold are in contact, wherein the chamber and the scaffolds are arranged such that when the bioreactor is in use, an interchange of cells of the first, or of the second, or of both the first and the second cell culture occurs between the chambers through one or both scaffolds.

In another embodiment, the multi-chamber bioreactor disclosed can comprise 2, 3, 4, 510, 20 n chambers.

In another embodiment, of the multi-chamber bioreactor, the first and second scaffolds may be each one of the two layers of a bilayer scaffold.

In another embodiment, of the multi-chamber bioreactor, the pore size of the scaffolds is such to allow the interchange between the chambers of cells of the first cell culture, or of the second cell culture or of both the first and second cell culture.

In another embodiment, of the multi-chamber bioreactor, the pore size of the scaffolds is such to allow the interchange between the chambers of cells of both first or second cell culture. The size of a 3-D cell culture - scaffolds - can be influenced by the ability to diffuse fresh culture medium, because cells in the inner part of the 3-D structure die if the fresh medium cannot diffuse inside the structure, replacing at the same time the metabolic waste of the cells, which has an acidic nature.

Furthermore, the range of pore size is also dependent on the ability of the medium to get into and exit from the interior of the 3-D structure. Introducing the ability to have a stirring movement, the diffusion potential will increase, allowing an increased size of the 3-D culture (resulting in the ability to produce larger tissues *in vitro*) and pore size range, allowing to have a structure with smaller pore size, larger porosity and surface area.

In another embodiment, of the multi-chamber bioreactor, the pore size of the scaffold can range between 5 μm -2 mm; preferably between 10 μm -500 μm , more preferably 10 μm -49 μm . The pore size of the scaffold can be achieved by X-ray micro-CT, which perform an acquisition of the structure by X-ray in 180° or 360°, layer by layer, also reconstructing the structure three dimensionally and has the ability to characterize several parameters as surface area, pore size, porosity, trabecular size and interconnectivity using several software analysis.

In another embodiment, of multi-chamber bioreactor, the porosity of the scaffolds can range between 10-98 %, preferably between 70-90 % which can be characterized also by micro CT.

In another embodiment, of multi-chamber bioreactor, one or both scaffolds are composed of a polymeric, ceramic, hybrid or composite material, or combinations thereof; preferably in a transparent polymer.

In another embodiment, of multi-chamber bioreactor, wherein one or both scaffolds are biodegradable, or chemically degradable, or photodegradable, or combinations thereof.

In another embodiment, of multi-chamber bioreactor, wherein one or both scaffolds may be degradable such that, when the bioreactor is in use, the interchange of cells occurs gradually.

In another embodiment, of multi-chamber bioreactor, the cultures are mono-cell cultures or cells co-cultures.

In another embodiment, of multi-chamber bioreactor, the first and second cultures are mono-cell cultures and/or cell co-cultures.

In another embodiment, of the multi-chamber bioreactor, one or more chambers can comprise a detachable cap.

In another embodiment, of the multi-chamber bioreactor wherein the chamber cap can be suitable for compressing the respective cell culture and scaffold.

In another embodiment, of the multi-chamber bioreactor wherein either the first or the second culture medium can be air.

In another embodiment, of the multi-chamber bioreactor can further comprise pumping means wherein the chambers and the pumping means are arranged such that, when the bioreactor is in use, the flow of the fluids between each chamber inlet and the respective chamber outlet is laminar.

In another embodiment, the multi-chamber bioreactor may further comprise a conductive metal, as a coating or filling the cell scaffold, in contact (direct or indirect) with the cell culture, able to induce an electric pulsatile stimulus over the cell culture. This pulsatile pulse can be used as a stimulus to certain cell cultures but also to promote transfection of the cells by microporation.

In another embodiment, the multi-chamber bioreactor may further comprise glucose, urea, pH, temperature, O₂ pressure and CO₂ pressure sensor, or combinations thereof.

In another embodiment, the multi-chamber bioreactor may further comprise in the bottom part a magnet. The magnet may be used to attract magnetic particles into the culture for transfection.

In another embodiment, of the disclosed subject matter, the diffusion of culture medium of the multi-chamber bioreactor, may be improved, submitting the said bioreactor to a stirring movement (rotational movement). Namely the rotation can be promoted by magnetic stirring, having magnets performing the attraction between the bottom part of the bioreactor and the stirring position in a plate.

Another aspect of the present subject matter relates to the use of the multi-chamber bioreactor to obtain synthetic tissue grafts and/or *in vitro* tissue models for drug screening.

Another aspect of the present subject matter relates to a synthetic tissue graft obtainable by the use of the multi-chamber bioreactor, comprising a multilayered tissue containing at least two different fused cell layers.

In another embodiment, the synthetic tissue graft obtainable can be for: OC interface tissue; a skin interface tissue; an intestine epithelial barrier; a blood-brain barrier; a lung epithelial barrier, among others.

The synthetic tissue graft of the present disclosure for use in human or veterinary medicine.

Another aspect of the present subject matter relates to the use of the disclosed synthetic tissue grafts to use in RM and TE, in particular in the treatment of diseases that involve the regeneration, replacement or treatment of biologic tissues.

In another embodiment, the multi-compartmentalized chambers, allow flowing at least two different culture media.

In an embodiment, the dual culture chambers can have both, a porous central layer – a 2-D or a 3-D structure. This kind of chamber is used to culture cells with different conditions in each chamber. The design of the multi culture chambers allows avoiding the mixture of the culture media, having connection between the two compartments between the porosity of the central 3-D porous structure. This 3-D porous structure is designed to be easily discarded from the chamber, allowing an independent analysis.

Throughout the description and claims the word "comprise" and variations of the word, are not intended to exclude other technical features, additives, components, or steps. Additional objects, advantages and features of the present subject-matter will become apparent to those skilled in the art, upon examination of the description or may be learned by practice of the invention. The following examples and drawings are provided by way of illustration, and they are not intended to limit the present subject-matter. Furthermore, the present subject-matter covers all possible combinations of particular and preferred embodiments described herein.

6.2.3 Brief description of the drawings, pictures and proof of concept

The following figures provide preferred embodiments for illustrating the description and to provide proofs of concept, and should not be seen as limiting the scope of present subject-matter.



Figure 6.1 | Dual-chambers. (A) Top view of a dual-chamber with opened top and top cap on the right side, turned down (scale bar = 15 cm). (B) Dual-chambers placed in a commercial 6-well plate. (C) Dual-chamber bioreactor system assembled for flow perfusion.

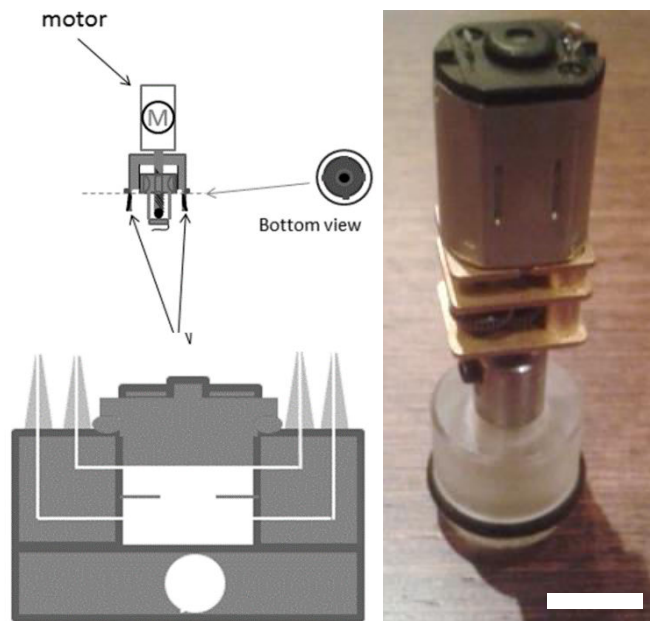


Figure 6.2 | Cross section of the dual chamber, with a detail of the screw adapted to fit top hole of the dual chamber bioreactor, motor, and bottom view detail. On the left: a schematic of the compressive drive to attach on the top of the dual-chamber. On the right: a picture of the first prototype fabricated as a compressive drive (scale bar = 10 mm).

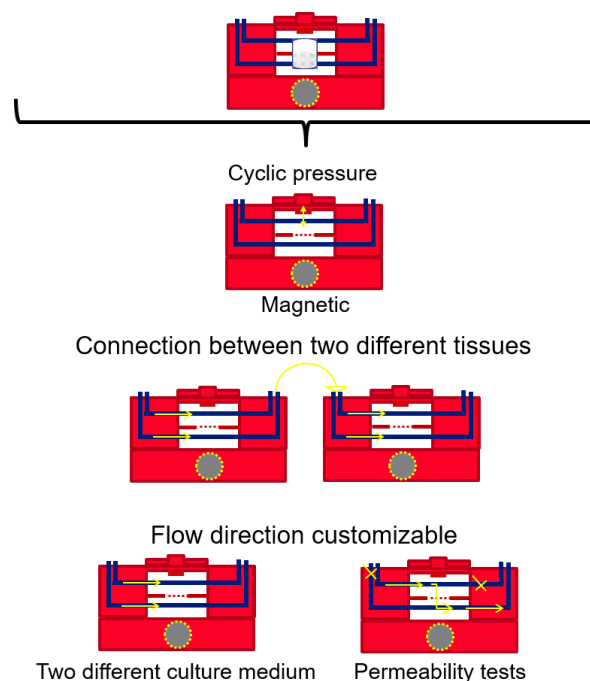


Figure 6.3 | Schematic representation of the versatile dynamics of the dual-chambers.

Cyclic pressure can be applied if an optional compressive drive replaces the top cap. Two dual-chambers can be connected from the outlet of the first to the inlet of the second chamber. By interrupting one of

the inlets and outlets, the direction of the flow can be controlled to be parallel or perpendicular to the interface.

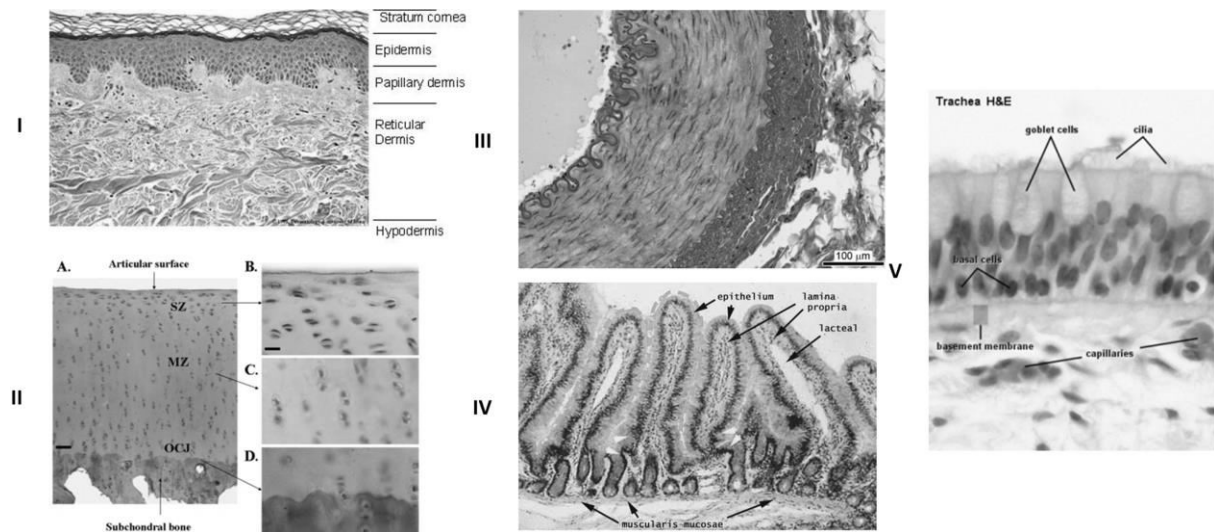


Figure 6.4 | Represents examples of multilayered tissues and barriers in human body that could be mitigated by the present subject-matter; wherein: I - is skin layers; II - is OC interface; III - is blood-brain barrier; IV - is Intestine epithelial barrier; V - is Lung epithelial barrier.

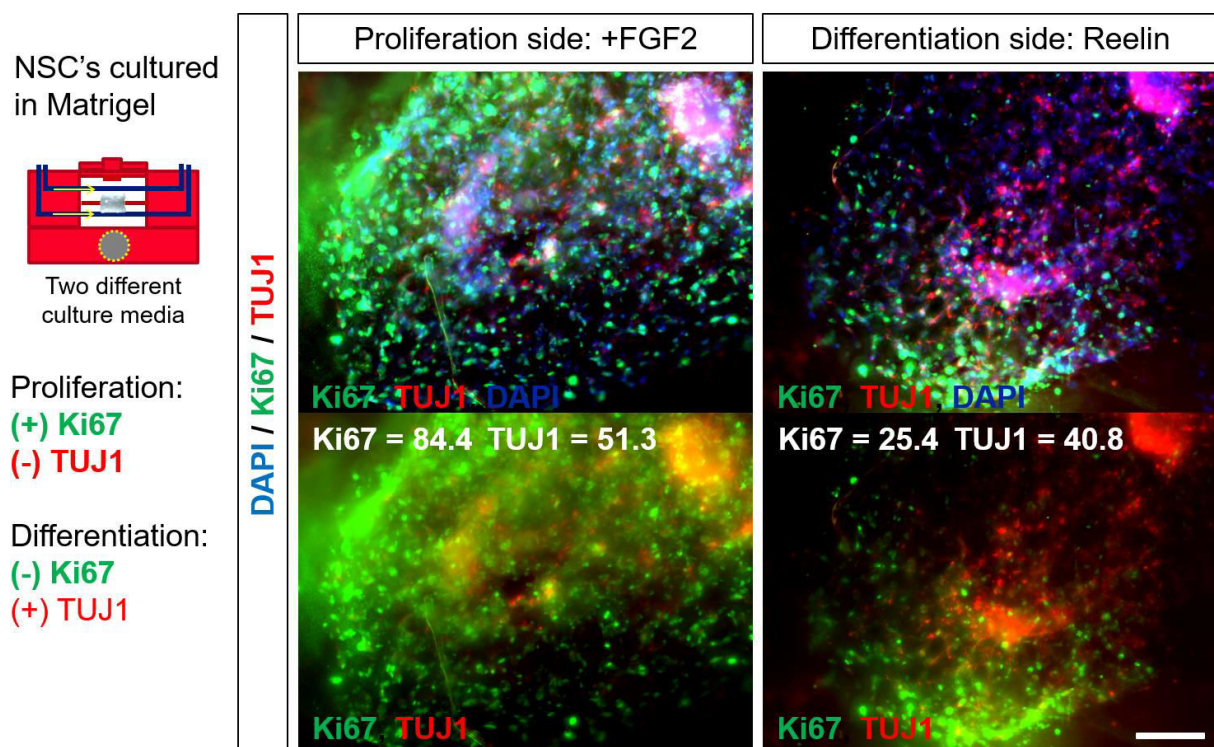


Figure 6.4 | Proliferative NSCs region interfaced with differentiated neurons. NSCs were encapsulated in Matrigel® and placed in the interface of the dual-chamber bioreactor. A proliferative culture medium supplemented with FGF was perfused interfaced with a neuronal differentiation medium

supplemented with Reelin. Two regions in a single hydrogel were characterized, a proliferative region of NSCs interfaced with neuron differentiated from NSCs. Ki67 was used to stain the proliferative cells and TUJ1 the neurons (scale bar = 500 μ m).

Each chamber may have detachable caps for the top and the bottom compartments. The top cap may present the possibility to adapt a compression drive, allowing to test a compressive stimulus over the cell culture.

In another embodiment, the plurality of chambers of the bioreactor may have dimensions to adapt to commercial 6-well tissue culture plates (38.4 mm diameter, 17.5 mm height). This way and being the top and bottom of the chambers detachable, the culture can be observed by microscopy. Microsensors can be added to the system to monitor several biochemical parameters, such as oxygen tension, pH, temperature, or glucose and urea concentration and physical parameters like pressure.

Different tissues such as bone and cartilage (OC) and dermis and epidermis (skin), or barriers as, blood-brain, intestinal epithelium and lung epithelium, are examples of interfaces that should be integrated, presenting two or more different phenotypes in each side and are extremely difficult to be reproduced in the laboratory bench without an adapted system.

There is a need for devices or bioreactors allowing to have optimal conditions for the production of multilayered tissues mimicking the native ones. To achieve this goal physical stimuli and appropriate nutrition/chemical cues (e.g., supply of culture medium with or without bioactive agents) shall be optimized for each region of the multilayered tissue.

The present disclosure concerns a technique, based on a bioreactor device, to enhance the creation of continuous smooth gradient interfaces, able to create two or more different tissues integrated, with the natural phenotype, using an integrated and continuous 3-D support structure.

6.2.4 Description of preferred embodiments

An embodiment, of the present disclosure refers to a dual chamber bioreactor that is composed by a set of dual culture chambers interconnected, and flow pump(s).

The dual culture chambers present two independent culture medium entries and outputs, which permit induction of independent and different shear flows, and a central separator with a hole for insertion of the scaffold, whereby there is integration of the two chambers. The dual chambers have a magnetic bar attached to the bottom part.

In another embodiment, the dual chamber bioreactor may incorporate connected dual culture chambers from the outlet of a first dual-chamber to the inlet of a second dual-chamber. The dual culture chambers may have a central barrier with a hole to insert the bilayer scaffold, with a porosity no more than 50 μm , preferably 10-49 μm , more preferably 20-30 μm . This kind of well attends to culture cells with different conditions in each chamber. The design of the dual culture chambers allows to avoid the mixture of the culture media. In the bottom part of the dual culture chamber is attached a magnetic bar, which can be attract by a stir plate or to perform transfection by magnetic attraction. Each chamber has detachable caps for the top and the bottom compartments. The top cap presents the possibility to compress the scaffold, allowing to test a compressive stimulus. The dual chambers may have dimensions to adapt to commercial 6-well tissue culture plates, namely 11 to 39 mm diameter; in particular, 38.4 mm diameter, 15-20 mm height, in particular 17.5 mm. All of the pieces that compose the dual chambers are autoclavable.

6.2.4.1 OC tissue graft development using the dual chamber bioreactor

To develop an OC analogue, which can be further use in as an *in vitro* 3-D tissue model, undifferentiated ASCs isolated from fat pad were cultured in a bilayered scaffold, aiming an *in situ* cell differentiation into chondrocyte and osteoblast-like cells. The bilayered scaffolds comprise the cartilage- and bone-like layers, which are composed of pure GG and GG with dispersed HAp, respectively.

In vitro mature and homogeneous OC tissue formation is achieved by culturing ASCs within the GG-HAp/GG bilayered scaffold by means of using the dual chamber bioreactor. An optimized chemical mediation is provided at each compartment of the dual chamber, i.e. in one compartment is provided the osteogenic medium and in the second compartment is provided the chondrogenic medium. Chondrogenic differentiation culturing cocktail is performed based on well-established protocols. Although osteogenic differentiation of ASCs can require additional GFs, due to the presence of HA in the bone-like part of the scaffold, the conditions to maintain both cell types in co-culture needed to be optimized. The optimization, taking advantage of the culture system, accounts for the presence and the influence of osteogenic/chondrogenic mediators such as dexamethasone, L-ascorbic acid-2-phosphate, β -glycerophosphate, and bone morphogenic proteins (BMPs), fibroblast growth factor (FGF), platelet-derived GF and TGF- β , respectively.

The mixture of the different culture medium is prevented due to the independent flow through the two compartments of the chamber. This maximizes the differentiation potential of the ASCs towards each lineage, in the respective scaffold layers. The system characterized by the dual chamber bioreactor and

the produced living tissue aims to be used as a 3-D *in vitro* OC model. These 3-D tissue models enable a continuous analysis of the GFs production and allow culture conditions optimization, thus holding a great promise for application in TE and RM, and screening of bioactive molecules or drugs.

6.2.4.2 Development of an OC human tissue interface *in vitro* model

In an embodiment, to develop the OC analogue (bone and cartilage interface), undifferentiated hASCs will be cultured in a bilayered system, aiming at *in situ* cell differentiation into chondrocyte and osteoblast-like cells. These hASCs can be derived from different tissues as the abdominal fat or from fat pad (also known as Hoffa's body) behind the knee. The osteogenic differentiation can be achieved by a specific chemical composition of the culture medium or be the physical stimulus from the structure in the bony part induced by the HÁ or nano-CaP particles dispersed within a silk-based structure. An optimized chemical mediation that will be responsible for the chondrogenic differentiation of the hASCs encapsulated in MAGG incorporated in a silk backbone structure.

The goal is to integrate a bony part with good vascularization and enervation with an avascular cartilage layer. To promote this vascularization hAMECs can be co-cultured in the bone-like layer.

Also primary cells as osteoblasts and chondrocytes isolated from humans, for example with osteoarthritic phenotype, can be used to culture and create an *in vitro* disease model. This way would be possible to study the disease phenotype in a more realistic 3-D structure.

To make possible the existence of the optimal environmental conditions to promote the production of these two layers continuously integrated, an adapted bioreactor is needed. The bioreactor will allow the culture of both layers integrated in two different chemical mediums. Moreover, the cartilage-like layer will be cultured in a lower flow perfusion medium and hypoxic conditions.

6.2.4.3 Development of skin tissue *in vitro* model with dermis and epidermis

In addition, a skin model can be developed; an epidermal analogue will be created by culturing hASCs-derived epidermals in a GG-keratin membrane, while the dermal analogue aiming at vascularized neodermis formation will be achieved by co-culturing hASCs and hAMECs in a GG-Hyaluronic acid matrix. The goal is to achieve a continuous epidermis supported by hASCs, and dermis vascularization promoted by hAMECs.

In order to create an environment that more realistically represent an *in vivo* situation, the bioreactor will be used once again using the flow pump to induce the culture medium flow. The use of the bioreactor may improve tissue interface standardization, with a continuous and controlled medium composition. To

improve skin tissue maturation, an air-liquid interface can be created using the dual-chamber bioreactor, allowing the flow of an optimized cell culture medium in the dermis layer and having an air phase in the epidermis layer, supported by the dual-chamber.

This bioreactor system will also make possible to continuously analyse GFs production and allow the optimization of cell culture conditions.

The proposed engineered tissues can be used as *in vitro* models and will be validated after performance comparing with parallel *in vivo* models. Ultimately these models are of great interest to predict *in vitro* TE constructs outcome, avoiding superfluous *in vivo* trials and minimizing their variability by providing controlled testing conditions. Furthermore, these engineered tissues will be the first step to produce human tissues in lab with the final goal of transplantation.

6.2.4.4 Validation of the developed engineered skin as a graft

In order to validate the developed models, *in vivo* tests will be performed in rat OC and full-thickness excisional skin models. Similar defects will be produced in the *in vitro* (our developed models), *in vivo* models and clinically available TE products will be tested. OrCel™ Bilayered Cellular Matrix, a bilayered cellular matrix, in which normal human allogeneic skin cells (epidermal keratinocytes and dermal fibroblasts) are cultured in two separate layers, will be tested for skin regeneration. Chondro-Gide®, consisting in an acellular membrane with a multilayer structure, formed by collagen types I and III, with one compact and one porous side, will be tested in the OC model. The performance of the products for tissue regeneration will be compared between *in vitro* and *in vivo* models based on type/composition and organization of deposited ECM, cellular organization within the different layers, vascularization of the bone and dermal/hypodermal layers. These results will allow us to ensure that the proposed models could be used to test *in vitro* skin and OC TE constructs, thus minimizing animal experimentation.

The disclosure is of course not in any way restricted to the embodiments described and a person with ordinary skill in the art will foresee many possibilities to modifications thereof.

The above described embodiments are obviously combinable.

The following claims further set out particular embodiments of the disclosure.

6.3 CLAIMS

1. A polymeric multi-chamber bioreactor comprising:
 - at least a first and a second fluid-tight chamber for receiving respectively a first fluid culture medium and a second fluid culture medium comprising respectively a first and a second cell culture, and an opening between the first and second chamber for interchanges between the chambers,
 - each chamber comprising at least an inlet and an outlet of fluid,
 - the first and second chamber comprising respectively a first and a second cell culture scaffold,
 - wherein the first and second scaffold are in contact,
 - wherein the chamber and the scaffolds are arranged such that when the bioreactor is in use, an interchange of cells of the first, or of the second, or of both the first and the second cell culture occurs between the chambers through one or both scaffolds;
 - wherein the pore size of the scaffolds is such to allow the interchange between the chambers of cells of the first cell culture, or of the second cell culture or of both the first and second cell culture.
2. The multi-chamber bioreactor according to any of the previous claim further comprising pumping means wherein the chambers and the pumping means are arranged such that, when the bioreactor is in use, the flow of the fluids between each chamber inlet and the respective chamber outlet is laminar.
3. The multi-chamber bioreactor according to the previous claims wherein the first and second scaffolds are each one of the two layers of a bilayer scaffold.
4. The multi-chamber bioreactor, according to any of the previous claims, wherein the pore size of the first scaffold, or of the second scaffold, or of both the scaffolds ranges between 5µm-2 mm; preferably between 10µm-500µm, more preferably 10 µm-49 µm.
5. The multi-chamber bioreactor, according to any of the previous claims, wherein the porosity of the scaffolds is between 10-98 %, preferably between 70-90 %.
6. The multi-chamber bioreactor, according to any of the previous claims, wherein one or both scaffolds are of a polymeric, ceramic, hybrid, composite material or combinations thereof.
7. The multi-chamber bioreactor, according to any of the previous claims, wherein one or both scaffolds are biodegradable, or chemically degradable, or photodegradable, or combinations thereof.
8. The multi-chamber bioreactor according to the previous claims wherein one or both scaffolds are degradable such that, when the bioreactor is in use, the interchange of cells occurs gradually.

9. The multi-chamber bioreactor, according to any of the previous claims, wherein the first and second cultures are mono-cell cultures.
10. The multi-chamber bioreactor, according to any of the previous claims, wherein the first and second cultures are cell co-cultures.
11. The multi-chamber bioreactor, according to any of the previous claims, wherein one or more of the chambers comprises a detachable cap.
12. The multi-chamber bioreactor, according to any of the previous claims, wherein the chamber cap is suitable for compressing the respective cell culture and scaffold.
13. The multi-chamber bioreactor, according to any of the previous claims, wherein either the first or the second culture medium is air.
14. The multi-chamber bioreactor, according to any of the previous claims, further comprising a conductive metal in contact with the cell culture, able to induce an electric pulsatile stimulus.
15. The multi-chamber bioreactor, according to any of the previous claims, further comprising glucose, urea, pH, temperature, O₂ pressure CO₂ pressure sensors, or combinations thereof.
16. The multi-chamber bioreactor, according to any of the previous claims, further comprising in the bottom part, of said bioreactor, a magnet.
17. Use of the multi-chamber bioreactor for drug scanning or screening.
18. A synthetic tissue graft obtainable by the use of the multi-chamber bioreactor described in claims 1-17, comprising a multilayered tissue containing at least two different cell layers fused.
19. The synthetic tissue graft, according to claim 18, wherein the synthetic graft is synthetic OC interface tissue, or synthetic skin interface tissue, or synthetic intestine epithelial barrier, or synthetic blood-brain barrier, or synthetic lung epithelial barrier.
20. The synthetic tissue graft, according to claims 19, for human or veterinary medicine application.
21. The synthetic tissue grafts, according to the previous claim, for RM and TE purposes.
22. The synthetic tissue grafts, according to the claims 21-22, to be used in the treatment of diseases that involve the regeneration or treatment of tissues.

CHAPTER 7

DYNAMIC SYSTEM FOR AUTOMATED 3-D CELL CULTURE

Based on the following publication:

Canadas RF, Oliveira JM, Marques AP, Reis RL, Rotational dual chamber bioreactor: methods and uses thereof, WO2014141136A1, 2014. <https://patents.google.com/patent/US20160024453A1/en>

7. DYNAMIC PLATFORM FOR A ROTATIONAL DUAL CHAMBER BIOREACTOR: METHODS AND USES THEREOF

7.1 OBJECT OF INVENTION

This disclosure refers to the development of a rotational dual chamber bioreactor for cell culture in bi- and multi-layered scaffolds, in the context of TE and RM strategies. The rotational dual chamber bioreactor comprises at least two dual culture chambers, a multiposition magnetic stirrer plate, and at least two flow pumps which can be coupled to a monitor device to register biochemical parameters such as pH, pO_2 , glucose and urea sensors and physical parameters like pressure.

The dual culture chambers have a central barrier with a hole to insert the bilayer scaffold. This kind of chamber is used to culture cells with different conditions in each compartment of the chamber, avoiding medium exchange. Each chamber can rotate 180° (horizontal), being the rotations per minute controlled independently. The rotation is promoted by magnetic stirrers, having magnets performing the attraction between the bottom part of the dual chamber and the stirring position of the plate. The multiposition stirrer plate is adapted dimensionally to the requirements of the culture plate dimensions used, and also allows 180° vertical rotation. Each dual chamber has detachable caps for the top and the bottom chambers. The top cap has the possibility to compress the scaffold and thus providing a compressive stimulus with torsion simultaneously. This compression is applied by attaching a top plate adapted to fit the top caps of each dual chamber.

7.2 DESCRIPTION

7.2.2 Background Art

A bioreactor may refer to a device or system meant to seed cells onto scaffolds and grow cells or tissues, under specific biochemical and/or mechanical conditions, in the context of cell culture. Therefore, tissue development is facilitated by bioreactors. In this context, bioreactors are devices engineered to deliver appropriate spatial and temporal nutrient transport that may also incorporate mechanical or other physical stimulation, in a well-defined and controlled environment. Bioreactors that provide well-defined

and controllable environments are useful for fundamental studies to optimize tissue growth, and it can act as production units for large-scale tissue fabrication and bioprocesses. Bioreactor systems can provide reliable model systems for fundamental studies of cell biology and play a key role in improving the quality of engineered tissues.

Different bioreactors have been reported in the literature, including mixed flasks [Vunjak-Novakovic et al 1996], rotating vessels [Freed et al 1998; Freed et al, 2000], perfused cartridges [Carrier et al, 2002], and bioreactors with different mechanical stimulation [Altman et al, 2001; Altman et al, 2002].

Bichamber bioreactors and oscillating bioreactors have also been described for application in OC TE [Wendt et al, 2003; Chang et al, 2004; Valonen et al, 2010].

The main limitation of the current bioreactors for OC TE is that the newly formed tissue(s) is not homogeneously distributed within the bilayered scaffolds [Chen et al, 2013]. Furthermore, there are no bioreactors adapted for bilayered scaffolds that support different culture medium for each layer of the bilayered constructs, allowing inducing rotatory stimulus, compression and vertical movement to avoid cell sedimentation and undesired OC tissue malformation, at the same time.

Several patents have been granted based in the use of bioreactors for different applications. The following examples should be taken into account by their relevance in the area of this invention:

US2006141623 (A1) Patent of 23rd June 2006 describes systems, modules, bioreactor and methods for the automated culture, proliferation, differentiation, production and maintenance of tissue engineered products in a general way. However, this system description doesn't refer to double chamber (a chamber with two compartments), to rotating movements of each chambers independently nor to all system turning movement.

WO 2007/012071 of 25th January 2007 refers to a bioreactor device, and a method and system for fabricating tissues and growing cells and tissues in the bioreactor device. The bioreactor device includes a bioreactor chamber for containing a sample, where sample growth in response to mechanical, electrical, and bio-factors stimulation is monitored through one or more optical ports. Embedded sensors are provided for measuring fluid pressure, pH, temperature, and oxygen tension. The bioreactor device can receive different types of mechanical loadings, including fluid shear, hydrostatic pressure, matrix compression, and rotation. The system does not present a double chamber for bilayered structures.

WO Patent 2008/098165 of 14th August 2008 refers to oscillating cell culture bioreactor. The bioreactor has a gas permeable, closed-loop chamber for cell or tissue culture, and an oscillating means for moving the gas permeable, closed-loop chamber bi-directionally along an axis horizontal to an axis normal to the closed-loop chamber to force convection of cells and fluid in the gas permeable, closed-loop chamber. The bioreactor optionally includes a TE scaffold, an inlet means, an outlet means, and integrated sensors. Another aspect provides a bioreactor having a plurality of gas permeable, closed-loop chambers for cell or tissue culture.

EP Patent 1990402 of 12th November 2008 describes a bioreactor to apply mechanical forces as an anabolic stimulus. The invention describes a bioreactor designed to be used in the field of cytomechanics. The system comprises an electro-active polymer actuator, also known as artificial muscle, which is bonded to a deformable, water impermeable biocompatible substrate/matrix. This layered structure is the flexible membrane of the bioreactor.

US Patent 7604987 of 20th October 2009 relates to a bioreactor presenting a chamber for containing cells or tissue cultures within a culture medium. The bioreactor also comprises a detector capable of detecting a change in one or more metabolites associated with growth of the cell or tissue cultures within the chamber and a chamber drive capable of rotating the chamber at a first speed about a first axis and a second speed about a second axis, the second axis being disposed at an angle relative to the first axis. In use, the magnitude of the first speed and the second speed are independently variable of each other. However, this bioreactor is composed just by one chamber for all structures samples, preventing co-culturing cells with two different media separately in each structure and each structure have no independent chamber container.

WO Patent 2010/040699 of 15th April 2010 relates to multi-culture bioreactor system that can maintain stem cells and differentiated cell types in physically isolated environments but can allow biochemical communication between these cells. Despite the system allows the co-culture and biochemical interaction between several chambers, these different chambers are physically isolated, and consequently does not allow obtaining integrated bi- or multi-layered cultured scaffolds on individual optimized environments.

WO Patent 2010/064943 of 11th June 2010 describes a bi-directional continuous perfusion bioreactor for tri-dimensional culture of mammal tissue substitutes. The bioreactor induces mechanical cellular stimuli by creating shear forces caused by the flow perfusion using different pressure gradients, controlled by two pumps, a vacuum and a peristaltic one, positioned within a circular flow system designed for the bioreactor and also through rotational engines positioned in specific locations. This bioreactor is able to grow tissues of large dimensions, through the control of the perfusion and flow gradient within the scaffolds, thereby obtaining both access of the nutrients to the interior and the removal of the metabolic products of the interior of the material.

EP Patent 2236597 A1 of 6th October 2010 presents a high-throughput sensorized bioreactor for applying hydrodynamic pressure and shear stress stimuli on cell cultures. This hydrodynamic pressure is generated inside at least one culture chamber by means of variation of the mean distance, with a controlled speed, of a surface relative to the surface on which the culture is positioned and between which the culture medium is free to flow.

CN 101899393 (A) Patent of 1st December 2010 belongs to the field of bone TE and cell mechanics relating to a dynamic load and recirculating perfusion bioreactor, which comprises a main body, a sliding sleeve module and a three-dimensional culture cabin module. The invention comprises a piezoelectric ceramic that penetrates into the three-dimensional culture cabin through the hole. The moving end of the piezoelectric ceramic is connected with a loading rod and the culture cabin main body is also provided with a liquid inlet and a liquid outlet which are externally connected with a peristaltic pump. The bioreactor does not perform rotational movements nor is adapted for dual chamber.

EP Patent 2151491 A3 of 23rd November 2011 discloses a multichamber bioreactor, however this system has not rotational movement, which is a limitation regarding homogenization of culture, because cell sedimentation can occur by gravity.

CN102796664 Patent of 28th November 2011 is related with a human body ligament TE bioreactor that can perform tension/compression and two-way torsional multidimensional stress loading. Moreover, it can automatically detect the loosening condition of the bracket and perform bracket loosening on-line compensation; and has the characteristics of simple and compact structure and high use convenience.

This bioreactor does not perform inversion of the culture chamber and is not applied to bilayered structures.

US2012035742 (A1) Patent of 9th February 2012 describes methods, devices and systems for bone TE using a bioreactor to culture human bone grafts. This bioreactor is composed by a unique chamber without rotating movements.

WO 2013103306 A1 Patent of 11th July 2013 refers to a bioreactor composed of watertight chamber and internal matrix for the generation of cellularized medical implants. The bioreactor's internal matrices and external chamber are simultaneously manufactured, preferably through a rapid prototyping process. The system is also characterized by an external chamber possessing a shape adapted to the shape of the implant to be cellularized (internal matrix) enabling a greater efficiency in the cellular colonization and culture of the implant. This bioreactor is not adapted to perform mechanical stimulus nor real time monitoring.

CN203269948 Patent of 6th November 2013 describes a device for tendon and tendon sheath *in vivo* co-culture construction. The device comprises a TE tendon and tendon sheath co-culture bioreactor, a control unit and a measuring unit, wherein the TE tendon and tendon sheath co-culture bioreactor comprises TE tendon and tendon sheath culture units and a pneumatic tendon sliding mechanism. The system comprises also monitoring sensors for pressure and flow. The system does not present different speed rotations for the several chambers.

US2014030762 (A1) of 30th January 2014 relates to a bioreactor for cell culture on a three-dimensional structure, comprising one culture chamber, the inner walls of which form a vertical duct, with a diameter that widens regularly from the duct inlet to the duct outlet, means enabling the culture medium to flow in said vertical duct. The invention also relates to the advantageous use of these bioreactors in TE, for the production of tissue grafts, notably a bone or cartilage graft. This patent does not describe dual-chambers, rotational movements or bilayered structures culture.

7.2.3 Summary

The present disclosure provides a rotational dual chamber bioreactor adapted for co-culture of cells in bi- or multi- layered scaffolds. For the first time allows culturing in dual chamber developing homogeneous

tissues with bi- or multi layered by applying rotational movement in horizontal and vertical axis. The horizontal movement with two or more different programmable speeds, ranging from zero to 0.12sec/degrees at no load, can be applied independently for each dual chamber, which can be used as a mechanical stimulation over the cells cultured onto a scaffold placed in that chamber, while the vertical movement with a programmable delay of time is applied for all the dual chambers together to avoid cell sedimentation by gravity. This bioreactor, which can also induce different shear flows of two different culture mediums independently circulating in each chamber, coupling two or more different flow pumps to the bioreactor and it is useful for simultaneously developing different mature tissues *in vitro*, i.e. OC tissues. Furthermore, a top plate can be attached, fitting the top caps of each dual chamber, providing automatic mechanical compression and torsion according with the rotational movement performed by each chamber. The programmed rotational movement will directly set the compression speed also.

Thus the present disclosure describes a bioreactor comprising a multiposition magnetic stirred plate on which are placed at least two dual chambers, a top plate fitting the top cap of each dual chamber and at least two independent flow pumps.

A preferred embodiment, of the present invention the dual chambers are fixed to the stirred plate by magnetic attraction and the referred stirred plate has a rotational movement, which is horizontal and/or vertical rotational movement.

In another embodiment, of the present invention, the horizontal rotational movement is applied independently for each dual chamber, ranging from 0° to 180°.

A preferred embodiment, of the present invention, the rotational movement comprises two or more programmable speed scales ranging from zero to 0.12sec/degrees.

In another embodiment, of the present invention, the vertical rotational movement is applied to all the chambers together, ranging from 0° to 180°.

A preferred embodiment, of the present invention, the dual chamber comprises a central barrier with a hole in which is inserted the multilayered scaffolds; independent culture medium entries and outputs; a magnet in the bottom of the chamber; compressible and/or detachable top caps; and microsensors to register biochemical and physical parameters.

In another embodiment, of the present invention, the dual chamber is a dual culture chamber whose dimension is adapted to the culture plate dimension, preferably 38.4mm in diameter and 17.5mm in height.

It is also an objective of the present invention to describe the use of the above described bioreactor in medicine or RM, in particular in the biotechnology, pharmaceutical industry, biology or TE fields.

It is a further object of this invention to describe the use of the above described bioreactor for the development of *in vitro* tissue models and TE of different organs, drug screening and/or to mimic the articular joint movement and other body mechanical stimuli.

7.2.4 General description of the invention

The technology represents a more realistic bioreactor mimicking different physical stimuli. The system is adapted for multilayered scaffolds cultured with two different culture media. Furthermore, the bioreactor overcomes the static culture disadvantages since, further than perfusion, it is able to perform 180° turnings avoiding cell sedimentation, promoted by a servomotor.

In respect to the bioreactor dynamics, this system is projected to the dual chambers perform compression and torsion of the top cap over the scaffolds and consequently over the cells by adding the top plate to the system. These chambers are bi compartmentalized for bi- or multilayered scaffolds. Each chamber position can perform 180° horizontal rotations to mimic for example the articular joint movement and improving culture medium diffusion.

Moreover, the dual culture chambers present two independent culture medium entries and outputs, which permit induction of independent and different shear flows.

All of the pieces that compose the dual chambers are autoclavable. The stirrer plate is controlled by a keyboard linked to an arduino (Atmel®) and a LCD display. The arduino is also linked to a servo control module. The stirrer plate can also incorporate a wi-fi system to control the stirring at distance, using computer software.

The bioreactor presents the possibility of having a determined number of samples in an equal number of independent culture positions or chambers. These bi-compartmentalized chambers allow flowing of two different culture media, rotating independently with two or more different programmable speeds. Furthermore, the entire system has 180° turning movement to avoid cell sedimentation promoted by the servomotor.

Regarding the data collection and user-friendly features, the dual chamber's size is adapted to meet the requirements of the user regarding the sizes of existing culture plates. Furthermore, the chambers are placed by magnetic attraction, feature that facilitate the removal and replacement of the dual chambers, as well as the analysis of individual chambers without affecting the remaining operating chambers.

The described technology can be used to develop different *in vitro* tissue models platforms that allows replacing or decreasing the animal models for scientific research and in the pharmaceutical field for drug

screening and evaluation, decreasing the time consuming, the animal unnecessary use, and the involved costs (drug discovery and testing and TE products development).

In the biotechnology, biology and TE fields, nowadays, the culture medium diffusion into 3-D porous scaffolds is a challenge to be overcome in order to develop 3-D tissue able to be used in the clinic. Applying such a technological solution, as the Rotational Dual Chamber Bioreactor, this problem will be solved. Furthermore, the technology under invention could mimic native stimuli, as for example the articular joint movement, which contributes as a platform to develop research on articular joints opening avenues to study disorders as OA.

Innovations, advantages and some applications:

- Multiple and independent positions that can be stimulated by rotational movement up to 10 programmable speeds scales.
 - o This feature can be useful for two different goals: first the improvement of culture medium diffusion into the 3D culture scaffolds; secondly, it can simulate, for example, an articular movement performed by our joints, since the rotation is of 180° horizontally.
- All system can automatically turn 180° up and down in a specified delay of time.
 - o Cells sedimentation can occur in 3-D cell culture. With this turning movement this issue will be avoided. Furthermore, if the turning movement is not desirable in a specific study, this feature can be disabled.
- Dual Chambers adapted for 3-D bilayered structures that can support the growth of two different cell lineages (or stem cells fate)
 - o Nowadays, in TE and RM field, the complexity of the developed structures to be implanted is increasing. There is also the need to mimic native tissues, such as the articular joint movement, for the appropriate development of *in vitro* tissue models for drugs screening. Thus, the goal is to develop more complex structures that can include two types of different tissue (for example, in the cartilage and bone in OC unit). To address this goal, different cell lines or primary cultures (e.g., adult stem cells from different origin) need to be used and cultured together.
- Each one of the dual chambers can have different conditions and can be checked without interfering with the other chambers in culture.
 - o Cells co-culture could be improved, studying both cell lines secretome over time (under the effect of a specific drug).
- Allows the flow of two different culture media in the two parts of each dual chamber.

- o Interfaces of our body could be tested in a dynamic system, as for example the blood-brain barrier.
- o Two different drugs could be applied flowing in the same dual-chamber, or to test the diffusion from one to the other compartment of each dual-chamber through a cell sheet/membrane.
- o Using the Rotational Dual-Chamber Bioreactor, the optimization of two different culture media used in co-cultures is definitely possible for two different tissues or cell lines growing simultaneously. The existing bioreactors only allow 3-D cell cultures under a single specific culture medium.
- The bioreactor will be adapted to perform cyclic compression movements
 - o In our body, a lot of physical stimuli are interfering with our health. For example, OA is generated by chemical mediators but also by physical loads. Applying such cyclic pressure, models for this and similar disorders can be created, allowing developing a more realistic platform for drug testing, avoiding the ethical and technical problems associated with animal models.

These are just few examples of some interesting applications and solutions possible with this bioreactor. Overviewing, we are presenting a new concept of bioreactor with a lot of variables that can be enabled and disabled independently, but all of them can contribute to have a more realistic, controlled, fast and accessible environment. The broader features of the presented bioreactor make it a valuable tool for broad studies ranging from fundamental science to the applied pharmaceutical and medical/surgical fields.

7.2.5 Brief description of the drawings, pictures and proof of concept

Without intent to limit the disclosure herein, this patent presents attached drawings of illustrated embodiments for an easier understanding.

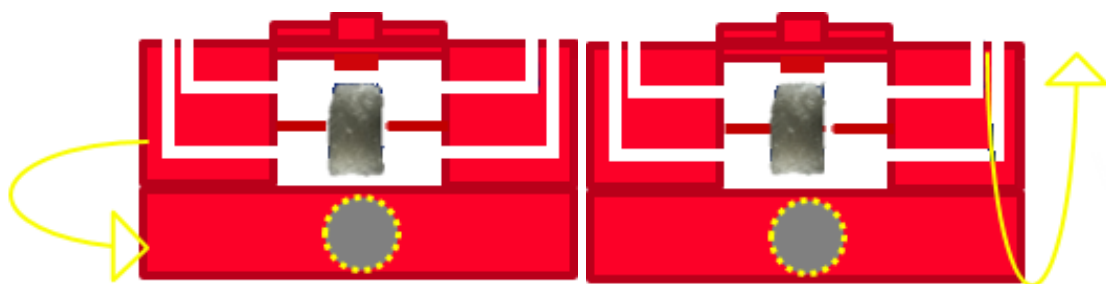


Figure 7.1 | Perspective view of the dual chamber (with open view for the inside); wherein is present a top cap of each dual chamber, a central part of the chamber where is comprised the bi-

compartmentalization with the central hole for structure insertion, and the bottom part adapted for insertion of a magnetic bar to fix the dual chamber to the stirred plate by magnetic attraction. When attached to the dynamic platform, the dual-chamber can perform horizontal and vertical movements, as indicated by the yellow arrows.



Figure 7.2 | Perspective picture of the dual-chambers. On the left, the dual-chamber is attached to the bottom part designed to attach in the newest version of the dynamic platform. On the right the dual chamber is attached to the bottom part which fits a commercial 6-well plate (scale bar = 10 mm).

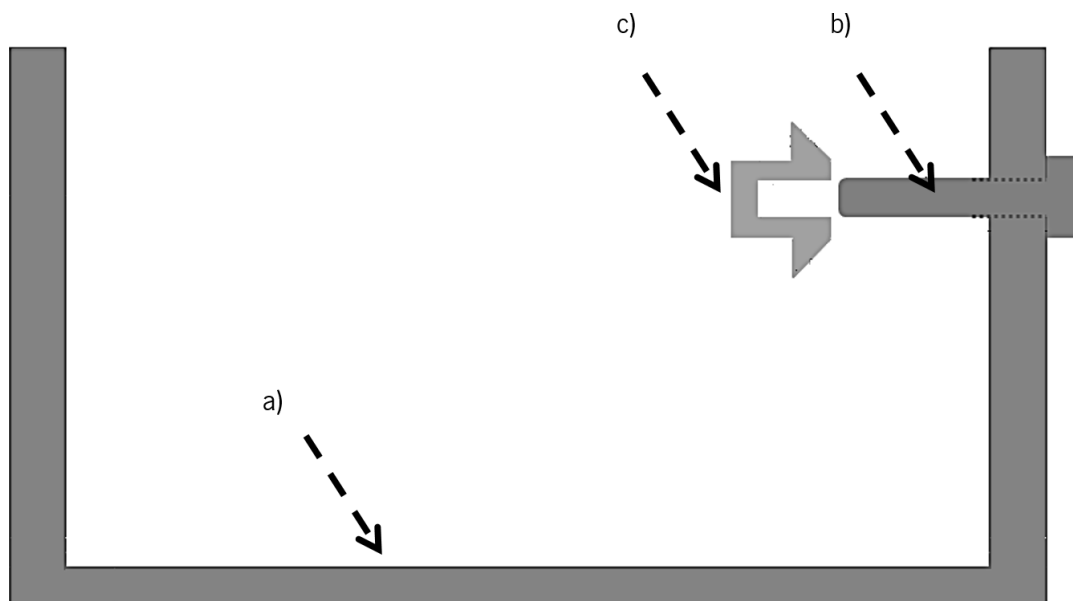


Figure 7.3 | Lateral view of the holder (2nd generation) for the dynamic platform, constituted by a a) basal plate, b) a screw to entry in the c) second piece that is part of the dynamic platform.

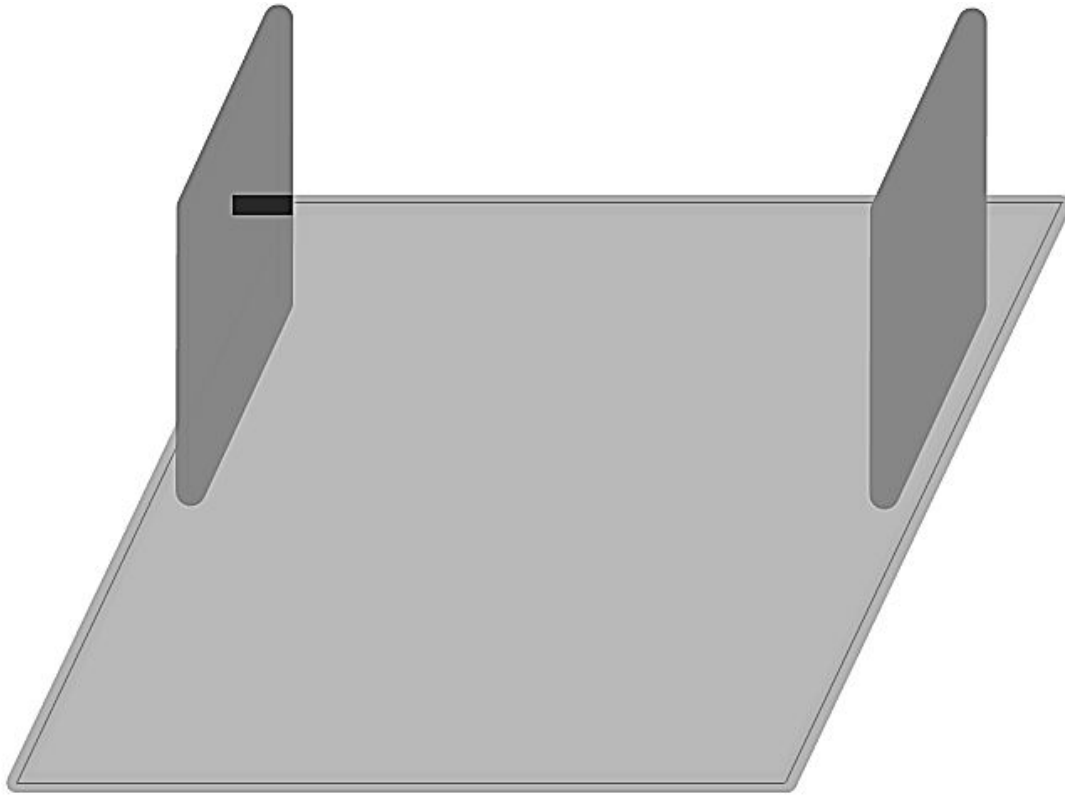


Figure 7.4 | Perspective view of the holder (2nd generation) for the dynamic platform.

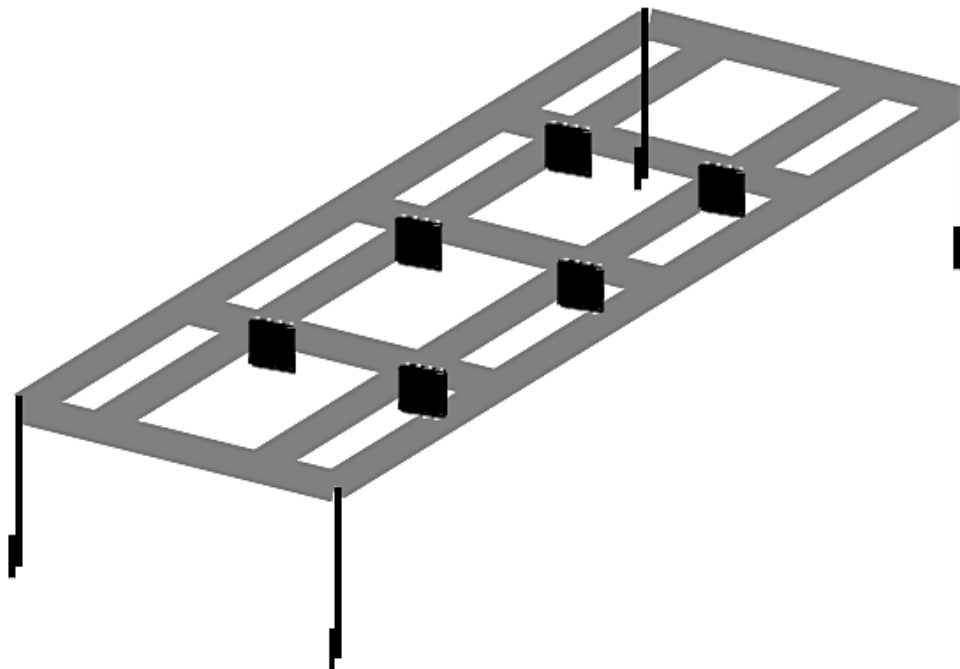


Figure 7.5 | Perspective view (from bottom) of the top plate to be used for allowing mechanical compression. The plate is composed by it's a) skeleton, b) the pieces adapted to entry in the caps of dual chambers and c) the legs that are attached by elastic to the dynamic plate.

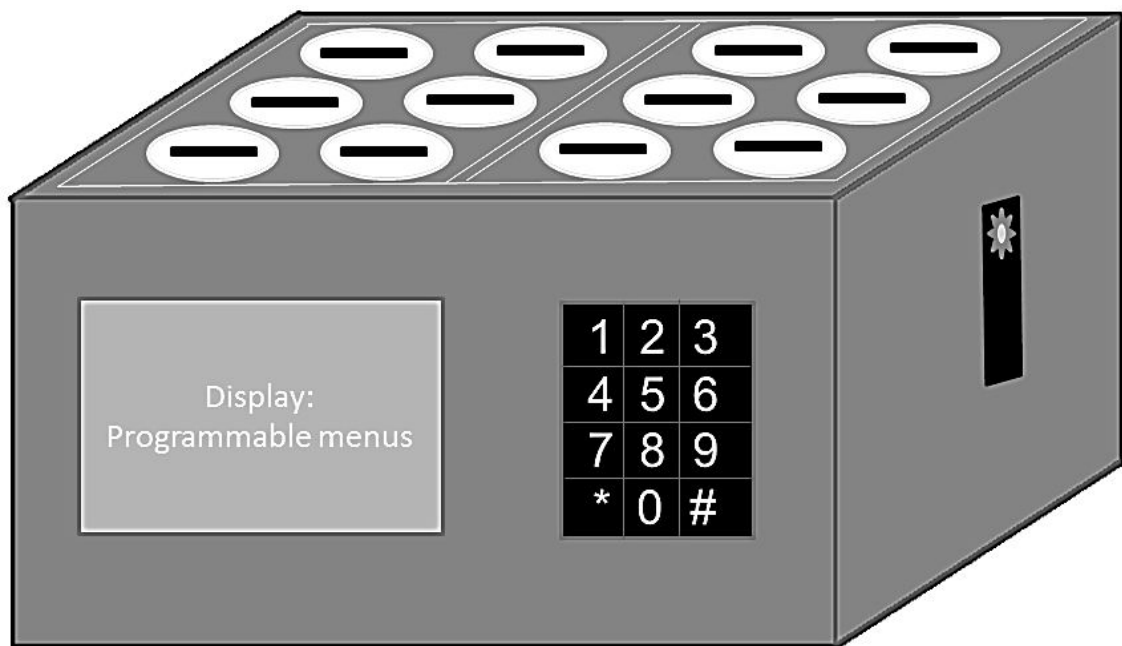


Figure 7.6 | Dynamic platform of the dynamic platform (2nd generation version 1) that performs the rotating movements (horizontal and vertical). This platform is attached to and suspended by the holder.

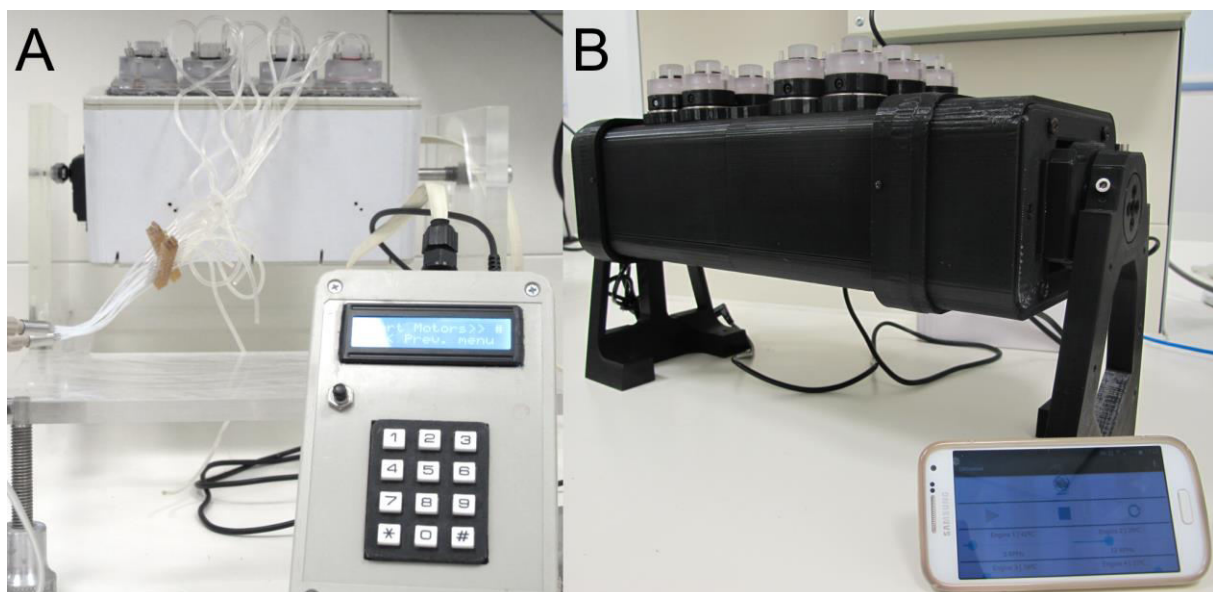


Figure 7.7 | 2nd and 3rd generation of the dynamic platform assembled with the dual-chambers on top (1st generation is not represented in this thesis). On the left: 2nd generation prototype, which was controlled with an external command. On the right: 3rd generation prototype controlled by mobile app through Bluetooth®.

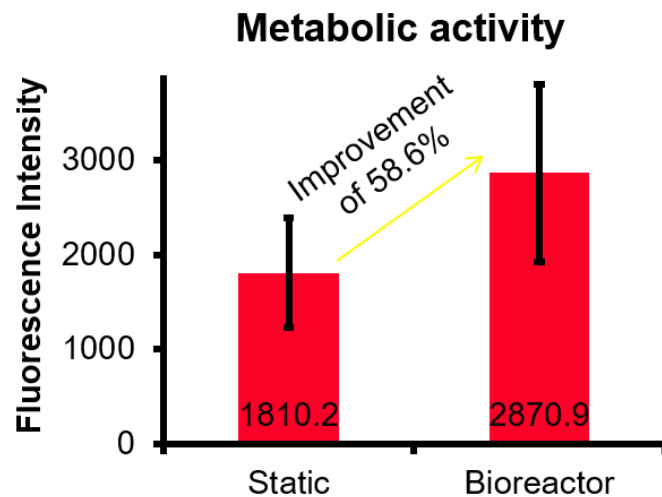


Figure 7.8 | Metabolic activity of cells cultured in a dual-chamber under horizontal rotation *vs* static. An increase of metabolic activity was observed over 3 days of cell culture, which was correlated with larger cell proliferation.

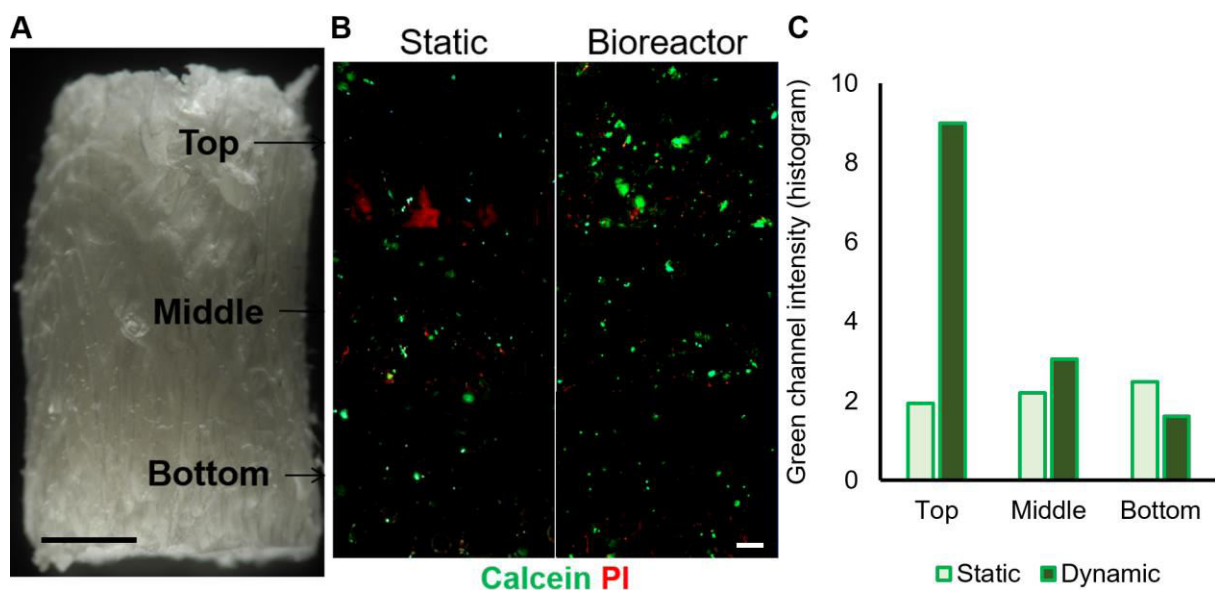


Figure 7.9 | Represents the metabolic activity obtained using the dual-chamber bioreactor of the present disclosure compared to a static culture condition (control). (A) A GG-gelatin 1:1 scaffold (monolayer) was used to assess the distribution/sedimentation of cells. A vertical rotation was performed every 30 min during the first 6 h, followed by a rotation every 2 h during 3 days of cell culture. The distribution of cells in the scaffold was reversed compared to the control (static chamber). (B) Live-dead assay (calcein-AM/PI) was performed to observe the distribution of cells in each condition (static *vs* vertical turning culture; scale bar = 200 μ m). (C) The green staining was quantified in a color histogram per condition and per region (top, middle and bottom).

7.2.6 Description of preferred embodiments

This invention refers to a rotational dual chamber bioreactor that is composed by a set of dual culture chambers, a multi-position magnetic stirrer plate, and flow pump(s).

The dual culture chambers present two independent culture medium entries and outputs, which permit induction of independent and different shear flows, and a central separator with a hole for insertion of the scaffold, whereby there is integration of the two chambers. The dual chambers have a magnetic bar attached to the bottom part.

The multiposition magnetic stirrer plate with 12 positions adapted for two 6 well tissue culture plates, which control independent horizontal movement for each position and vertical movement for all the plate.

7.2.6.1 Rotational dual chamber bioreactor

The dual chamber bioreactor is composed by a stirrer plate, which can be rotated vertically until 180°. In addition, the stirrer plate has 12 positions where can be placed, by magnetic attraction, 12 dual chambers. Each one of the 12 positions can be independently controlled to rotate until 180° with 10 different speeds ranging from zero to 0.12sec/degrees at no load.

7.2.6.2 Dual culture chambers:

The bioreactor can incorporate 12 dual culture chambers. The dual culture chambers have a central barrier with a hole to insert the bilayer scaffold. This kind of well serve to culture cells with different conditions in each chamber. The design of the dual culture chambers allows avoiding the mixture of the culture media. In the bottom part of the dual culture chamber is attached a magnetic bar to be attracted with the rotating position in the stirrer plate. Each chamber has detachable caps for the top and the bottom chambers. The top cap presents the possibly to compress the scaffold, allowing to test a compressive stimulus. The dual chambers have dimensions to adapt to commercial 6-well tissue culture plates (38.4 mm diameter, 17.5 mm height). This way, and being the top and bottom of the chambers detachable, the culture can be observed by microscopy. Microsensors can be added to the system to monitor several biochemical parameters, as oxygen tension, pH, temperature, or glucose and urea concentration and physical parameters like pressure. All of the pieces that compose the dual chambers are autoclavable.

7.2.6.3 Dynamic platform:

The 12 multiposition stirrer plate is adapted dimensionally to two standard 6 well tissue culture plates. In each of the 12 positions can be inserted one of the dual culture chambers by magnetic attraction. In each position can rotate until 180° (horizontal), being the rotations per minute controlled independently. The rotation is promoted by magnetic stirring, having magnets performing the attraction between the bottom part of the well and the stirring position in the plate. Vertical 180° movement can also be applied for all the chambers together.

The stirrer plate is controlled by a keyboard linked to a LCD display. The system is coordinated by an arduino (Atmel®) synchronized with a servo control module. The stirrer plate can also incorporate a wi-fi system to control the stirring at distance, using computer software.

All of the system can be placed inside an incubator.

7.2.6.4 Osteochondral tissue development using the dual chamber rotational bioreactor

To develop an OC analogue, which can be further use in as an *in vitro* 3-D tissue model, undifferentiated ASCs isolated from Fat Pad are cultured in a bilayered scaffold, aiming at *in situ* cell differentiation into chondrocyte and osteoblast-like cells. The bilayered scaffolds comprise the cartilage- and bone-like layers, which are composed of two layers of GG with a dispersed gradient of HAp.

In vitro mature and homogeneous OC tissue formation is achieved by culturing ASCs within the GG-HAp/GG bilayered scaffold by means of using the dual chamber rotational bioreactor. An optimized chemical mediation is provided at each compartment of the dual chamber, i.e. in one compartment is provided the osteogenic medium and in the second compartment is provided the chondrogenic medium. Chondrogenic differentiation culturing cocktail is performed based on well-established protocols. Although osteogenic differentiation of ASCs can require additional GFs, due to the presence of HA in the bone-like part of the scaffold, the conditions to maintain both cell types in co-culture needed to be optimized. The optimization, taking advantage of the dynamic culture system, accounts for the presence and the influence of osteogenic/chondrogenic mediators such as dexamethasone, L-ascorbic acid-2-phosphate, β -glycerophosphate, BMPs, FGF, platelet-derived GF and TGF- β .

The mixture of the different culture medium is prevented due to the independent flow through the two compartments of the chamber. This maximizes the differentiation potential of the ASCs towards each lineage in the respective scaffold layers. The system characterized by the rotational dual chamber bioreactor and the produced living tissue aims at being used as a 3-D *in vitro* OC model. These 3-D tissue

models make possible the continuous analysis of the GFs production and allow culture conditions optimization, thus holding a great promise for application in TE and RM, and screening of bioactive molecules or drugs (Figures 7, 8 e 9).

The present disclosure is of course not in any way restricted to the embodiment,s described and a person with ordinary skill in the art will foresee many possibilities to modifications thereof without departing from the basic idea of the invention as defined in the appended claims.

The following claims set out particular embodiment,s of the invention.

7.3 CLAIMS

- 1.** A bioreactor comprising a multiposition magnetic stirred plate on which are placed at least two dual chambers, a top plate fitting the top cap of each dual chamber and at least two independent flow pumps.
- 2.** The bioreactor according to claim 1, wherein the dual chambers are fixed to the stirred plate by magnetic attraction.
- 3.** The bioreactor according to claim 1, wherein the stirred plate has a rotational movement.
- 4.** The bioreactor according to the previous claim, wherein the rotational movement is horizontal and vertical.
- 5.** The bioreactor according to claim 4, wherein the horizontal rotational movement is applied independently for each dual chamber.
- 6.** The bioreactor according to the previous claim, wherein the horizontal rotational movement ranges from 0° to 180°.
- 7.** The bioreactor according to the previous claim, wherein the rotational movement comprises two or more programmable speed scales ranging from zero to 0.12sec/degrees.
- 8.** The bioreactor according to claim 4, wherein the vertical rotational movement is applied to all the chambers together.
- 9.** The bioreactor according to the previous claim, wherein the vertical rotational movement ranges from 0° to 180°.

- 10.** The bioreactor according to claims 1-2, wherein the dual chamber is a dual culture chamber.
- 11.** The bioreactor according to the previous claim, wherein the dual chamber comprises:
- a central barrier with a hole in which is inserted the multilayered scaffolds;
 - independent culture medium entries and outputs;
 - a magnet in the bottom of the chamber;
 - detachable caps
 - compressible caps; and
 - microsensors to register biochemical and physical parameters.
- 12.** The bioreactor according to any one of claims 9-10, wherein the dual chamber dimension adapts to the culture plate dimensions, preferably 38.4mm in diameter and 17.5mm in height.
- 13.** Use of the bioreactor described in claims 1-12 in medicine or RM, in particular in the biotechnology, pharmaceutical industry, biology or TE fields.
- 14.** Use of the bioreactor according to the previous claim, for the development of *in vitro* tissue models and TE of different organs.
- 15.** Use of the bioreactor according to the previous claim, for drug screening.
- 16.** Use of the bioreactor according to any one of the claims 13-14, to mimic the articular joint movement and other body mechanical stimuli.

CHAPTER 8

HIERARCHICAL CELL CULTURE UNDER CONTROLLED 3-D GRADIENTS AND POROSITY

Based on the following publication:

Raphaël F. Canadas, Alexandra P. Marques, Joaquim M. Oliveira, Rui L. Reis, Utkan Demirci.

Simultaneously co-differentiated stem cells applying 3-D structural and biochemical gradients matching interfaced tissues. (Submitted)

8. SIMULTANEOUSLY CO-DIFFERENTIATED STEM CELLS APPLYING 3-D STRUCTURAL AND BIOCHEMICAL GRADIENTS MATCHING INTERFACED TISSUES

8.1 Abstract

In nature, gradients of 3-D tissue interfaces organized in functional patterns are commonly found. However, mimicking these native features is still a challenge which directly affects numerous fields, such as drug development, micro-fabrication, and TE. Currently, material-based methods, micro-scale engineering, and microfluidic devices are being applied. But, there is still a need to have a robust control over the spatial composition and biochemical environment of these hierarchical structures, especially when heterogeneous cell differentiation has to occur simultaneously in a single construct. Here, we describe a customized technique to fabricate 3-D gradient constructs, with controllable composition and isotropic and anisotropic porosity. Hydrogel-like structures with random or vertically aligned pores and preserved micro-particles gradients were obtained through a strict control over crosslinking and temperature. Moreover, the patterned structure was biologically enhanced using an interconnected dual-chamber bioreactor that allowed a simultaneous perfusion of different cell culture media. As proof of concept, an *in vitro* 3-D OC tissue model was developed, being evaluated the co-differentiation of fat pad-ASCs onto the structures. Results clearly showed significant differences in the expression of cartilage and bone markers along the single construct. Additionally, it was also shown a control over angiogenesis in the 3-D space due to an improvement in pre-vasculature in half construct. So, the spatial-temporal control over the cellular environment achieved in this study, provides a new approach for interfaces and angiogenesis in TE with broad applications in drug testing and RM.

Keywords: Anisotropic; 3-D; neural model; tissue engineering; microfluidic

8.2 Introduction

Gradient structures present in living systems are a result of millions of years of evolution through natural selection which led to optimized heterotypic tissues and organs ¹. Interfaces are present in native tissues and different spatiotemporal gradient concentrations of bioactive signaling molecules are known to lead tissue formation and regeneration, as it is observed in embryonic development, aging, and even in degenerative diseases ²⁻⁴. Moreover, gradients in the cellular environment and extracellular matrix (ECM) accompany the biochemical gradients in native tissues ⁵. In particular, hierarchical tissues such as bone-tendon and OC interfaces, which are quite challenging to replicate due to the gradual calcification of their ECM ^{6,7}. For example, OC unit presents a gradient of calcified ECM from subchondral bone to cartilage, along with a decrease in the tissue's vascularity. Furthermore, OC tissue is also characterized by the specific anisotropic orientation of the subchondral bone and calcified cartilage region. Nevertheless, these spatial patterns are important regulators of cell adhesion, proliferation, migration, and differentiation ². Strikingly, none of these features has already been addressed together when trying to mimic tissue's interface *in vitro*.

Recently, two-dimensional (2-D) physicochemical gradient substrates ⁸ were enhanced through a transition to 3-D ⁹, which enabled the study of cell-protein-biomaterial interactions in a more native-like tissue environment ¹⁰. However, strategies related with this subfield usually face complex processes when applied in 3-D structures. So, the combination of specialized biomaterials along with spatially organized scaffolds composition, cell types and signaling molecules is highly required ¹¹. Current methods can create structural ¹², mechanical ¹³ or biochemical gradients ¹⁴, relying on material science ¹⁵, micro-scale engineering ^{16,17}, and microfluidics techniques ¹⁸. Although biomimetic and tailored microenvironments have been synthesized ^{19,20}, there is still a need for improved methods to control the gradient shape and stability ²¹, while coordinating the necessary biochemical and physical cues that will affect the fate of stem cells in a single gradient structure. Furthermore, guiding the porosity architecture, without affecting the structural gradient, is also important for 3-D cell distribution and, therefore, the improvement of the biological functionality of the construct. Likewise, user-friendly techniques must be developed and made accessible to a broad range of researchers, envisioning a scaling-up when translated to industry for clinical availability ^{21,22}.

In this study, we present a new strategy focused on three main aspects of the native tissue: a structural gradient, with a guided architecture, coordinated with a heterogeneous biochemical environment (figure 8.1). Through the combination of a material engineered strategy along with a bioreactor advanced design ^{23,24}, we obtained a construct with a continuous transition at both structural and cellular levels. Additionally,

a user-friendly and reproducible method to fabricate gradients with a guided porosity was developed. The method is based on a blending system of three phases: (1) GelMA plus (2) LAGG, as an ionically crosslinkable polymer, and (3) dispersed HAp. Focusing on concepts, as simple as, pre-polymeric solutions concentration adjustment, micro-particles distribution, crosslinking guidance, and ice crystals formation, we were able to control the gradient of bioactive signals, mechanical elasticity and porosity orientation in 3-D polymeric structures. Moreover, a micro-particle gradient formation with HAp was selected as a bioactive physicochemical stimulus. The quantitative correlations established between the structural orientations and mechanical properties were also investigated. Strikingly, by controlling the directionality of crosslinking and freezing temperature, we precisely controlled the porosity geometry without affecting the gradient of HAp. As a proof of concept, we designed an approach to create an *in vitro* 3-D OC tissue model, due to native tissue's complex architecture which, besides presenting an apatite gradient, is highly affected by aging or degenerative diseases such as OA²⁵. Overall, this study offers a feasible approach towards the generation of functional graded structures with possible applications in interfacial TE, with broad applications for tissue substitutes fabrication and drug screening in hierarchical 3-D tissue models.

surface. After crosslinking, the freezing step was also controlled to avoid losing the linearity obtained during the crosslinking step. Keeping the silicon mold and adding an external cover of styrofoam around the mold, a temperature gradient was created to force a linear ice crystals grow. (E) A bioreactor device was designed to culture cells in 3-D gradient constructs, combining the structural gradient with biochemical cues.

8.3 Results

To evaluate the 3-D architecture, composition and porosity profiles of the gradient structures, using micro-CT, a 3-D reconstruction of the architecture was performed. Cross-sections of the structures clearly showed the porosity orientation, either in isotropic or anisotropic arrangement (figure 8.2A-i and ii). Moreover, the ceramic phase was composed by particles conducted by the porosity orientation, demonstrating a specific distribution of bioactive patterns.

To characterize the fabricated 3-D gradients, quantitative data was graphically traced using micro-CT (figure 8.2B). A gradient of HAp was formed (figure 8.2B-i), all connected by pores (figure 8.2B-ii). The structure with randomly orientated pores, therefore isotropic structures, presented lower open porosity percentages when compared with the anisotropic structures (figure 8.2B-ii). Porosity diameter was controlled to obtain porous with sizes lower than 200 μm , adjusting the freezing step to $-196\text{ }^{\circ}\text{C}$, as it represented in the profile traced in figure 8.2B-iii. Additionally, while forming the 3-D structures, it was possible to control the pore orientation, as confirmed by the anisotropic degree quantification (figure 8.2B-iv). When closer to 1, a more anisotropic degree was obtained meaning that pores were more aligned and less randomly oriented, withstanding the fact that the structural gradient was not lost.

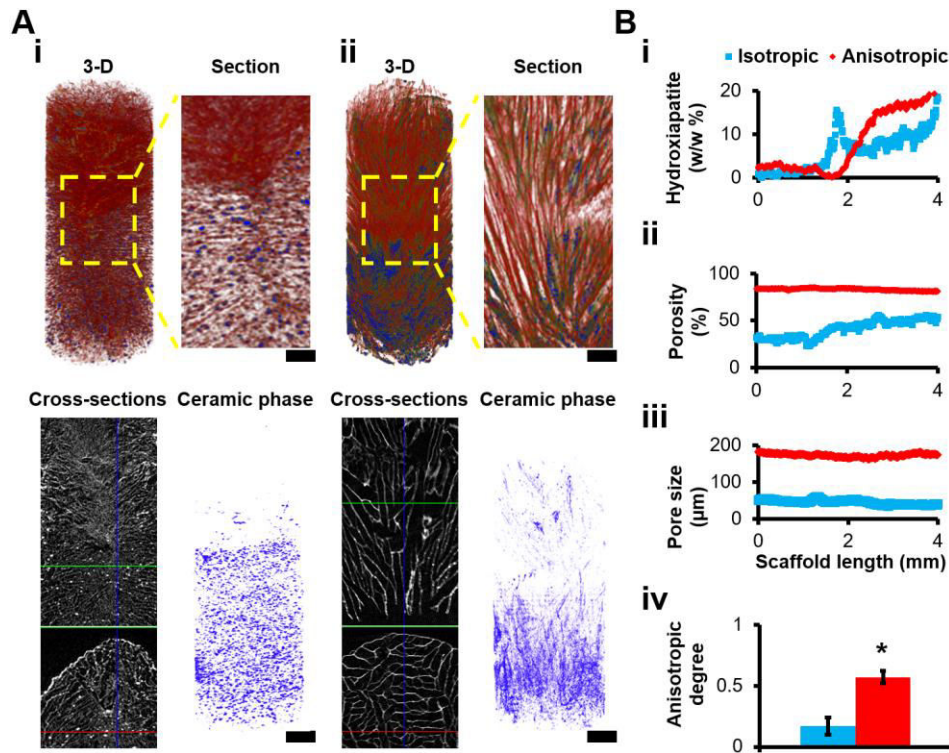


Figure 8.2 | Structural characterization of 3-D gradient structures. (A) X-ray micro-CT 3-D reconstruction of (i) random and (ii) linear porous gradient structures. Red color represents GelMA-MAGG blended polymers, blue color represents the HAp. Coronal and transaxial sections of the bilayered phased structures were represented, showing its continuous interface. The ceramic phase distribution is represented inside the structure's volume. Scale bar = 1 mm. (B) Profiles of: (i) HAp distribution, (ii) porosity percentage, (iii) mean pore size, were traced for each condition for linear and random pore orientation and (iv) the anisotropic degree was assessed for each porous architecture, showing the higher anisotropic shape of the linear porous structures. Values of anisotropic degree range from zero (isotropic) to 1 (anisotropic). Data expressed as (mean $\pm$ SEM). P value = 0.05, N=3.

To macroscopically observe the gradient of HAp, phosphate groups were stained by Alizarin red (figure 8.3A-i) showing the fabricated gradient in the structure. To confirm the correct formulation of HAp after processing the structures, the characteristic peaks of an amorphous HAp were revealed using X-ray diffraction (figure 8.3B). In addition, FTIR was performed showing a characteristic spectrum composed by peaks within wavelengths of the chemical groups PO_4^{3-} , OH , CO_3^{2-} , and HPO_4^{2-} , which are characteristic of non-stoichiometric HAp (figure 8.3C). PO_4^{3-} group forms intensive infrared absorption bands at 560 cm^{-1} and 600 cm^{-1} and from 1000 cm^{-1} to 1100 cm^{-1} . The CO_3^{2-} group intensive peaks between 1460 cm^{-1} and 1530 cm^{-1} were also present, showing that HAp kept incorporated after structure's processing, without losing its natural bioactive characteristics. Furthermore, the incorporation of the micro-particles

in the polymers walls was observed using SEM, which presented 2-4 μm diameter at the micro-scale (figure 8.3A-ii and 8.3A-iii).

Mechanical properties of isotropic and anisotropic porous structures (figure 8.3-D) showed a decrease in the G' of the anisotropic porous structure while it was kept a constant range of values for the isotropic porous structure (figure 8.3E-i), applying DMA. The absolute value of G' was lower for the isotropic porous structures meaning that these structures presented higher deformability. The decreasing slope of the anisotropic porous structures represented a lower elastic-like behavior in comparison with the isotropic ones. Assessing the $\tan \delta$ (figure 8.3E-ii), the isotropic porous structures presented higher absolute value, showing lower difference between the G' and the loss factor. This way, the isotropic porous structures presented higher capacity to dissipate energy than the structures with anisotropic porosity, being more viscous and less prone to mechanical deformation.

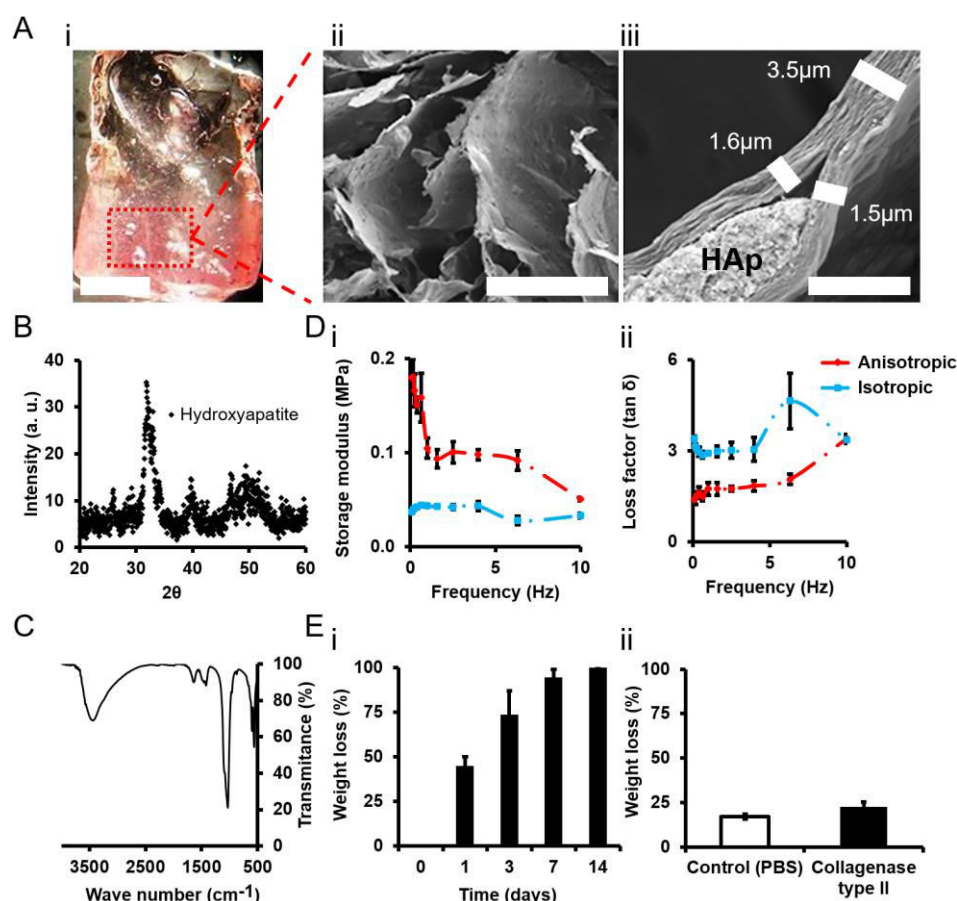


Figure 8.3 | Physicochemical characterization of gradient structures. (A) Image of gradient structure after freeze-drying process and (i) staining with alizarin red to show the HAp distribution profile (scale bar = 2 mm). SEM images at (ii) 300x (scale bar = 100 μm) and (iii) 5,000x magnification (scale bar = 5 μm) showed HAp incorporation in the polymeric structure after freeze-drying. (B) Typical

experimental HAp powder spectrum of X-ray diffraction and (C) FTIR. (D) DMA performed to the gradient structures. The stress was applied by forced set frequency to the samples to obtain deformation and a phase angle. These data allowed the calculation of: (i) G' and (ii) the $\tan \delta$ (δ), meaning damping, as well as complex modulus and viscosity properties. (E) Degradation tests were performed to GelMA and LAGG. (i) GelMA degraded over a period of 14 days in the presence of collagenase type I. (ii) LAGG had a 15% degradation by hydrolysis and reached 25% in presence of collagenase type I over a period of 30 days. Data expressed as (mean $\pm$ SEM). N=3.

Degradation rate of LAGG and GelMA using a solution of collagenase type I showed a complete degradation of GelMA (figure 8.3F-i). Contrarily, LAGG degraded about 15% in PBS by hydrolysis and reached about 25% of its weight loss when incubated with collagenase type I (figure 8.3F-ii).

The cell guidance pore orientation on was also assessed by culturing ASCs isolated from fat pad in the anisotropic and isotropic structures (figure 8.4A). At 21 days, osteogenic, chondrogenic and interfacial regions were quantified for cell orientation (figure 8.4B). Interestingly, a specific cell arrangement was observed in each condition. Cells cultured in anisotropic structure were organized in a specific orientation at higher frequency, as evidenced by the formed peak, which was absent in the isotropic condition.

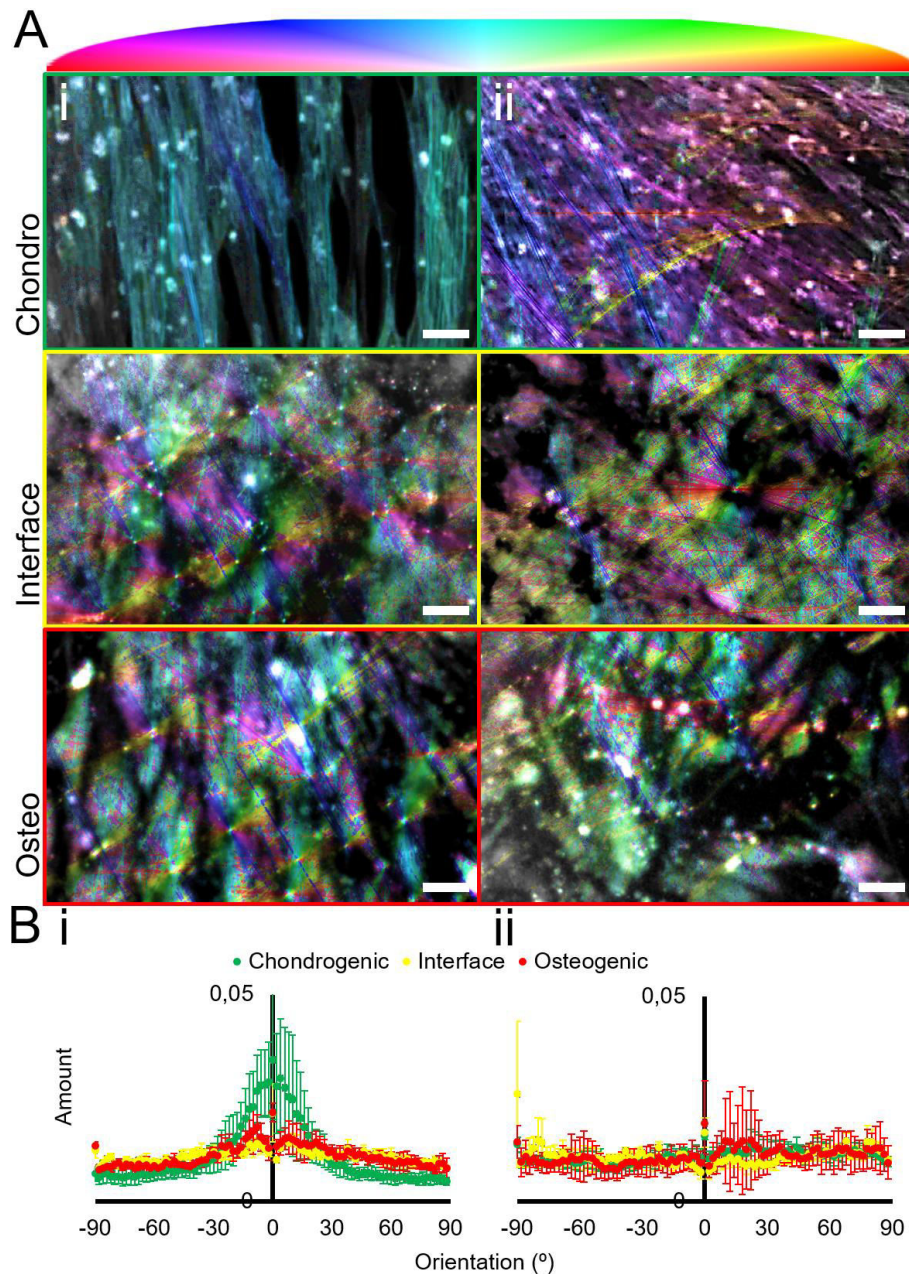


Figure 8.4 | Cell orientation assessment in 3-D gradient structures. (A) Cells were differentiated in a dual-chamber bioreactor device flowing osteogenic and chondrogenic media, which mix in the interface region creating a biochemical gradient. (i) Anisotropic and (ii) isotropic structures were used to seed the cells and the resulting constructs cultured for 21 days. Microscopic images were treated using Image J to assess cells orientation. A color map was designed according to cell orientation. (B) Cell orientation was quantified per condition in (i) anisotropic and (ii) isotropic constructs. The presence of a peak means that the frequency of alignment is superior for a specific angle. Data expressed as (mean $\pm$ SEM). N=5.

To be able to perform an *in vitro* cell culture having an environment identical to the structures gradient, a cell culture dual-chamber system was designed and fabricated (figure 8.5A). To monitor the mixing profile of two different solutions flowing through the system, pumping blue and red dyes resulted in a mix in the interface which created a yellow region (figure 8.5A-i). Gradient structures were this way successfully matured *in vitro* in a spatially controlled biochemical system (figure 8.5A-ii). To quantify the cell distribution in each half of the 3-D construct, Fat pad-ASCs were seeded and cultured for 3 days before starting differentiation. Metabolic activity studies, demonstrated a similar quantification in both halves (figure 5B-i), showing a homogeneous cell distribution in the gradient structure.

To mimic a native gradient tissue, OC phenotype was selected as a model. Chondrogenic and osteogenic differentiation were performed over a period of 21 days, flowing two different culture media over the 3-D construct. To evaluate the commitment of cells towards the osteogenic and chondrogenic lineages, the expression of osteogenic-and chondrogenic- related markers, e.g. Runt-related transcription factor 2 (RUNX2), osteocalcin (OCN) and osteopontin (OPN), and SOX-9, respectively, was studied. Three regions were created: top, interface and bottom parts of the construct (figure 8.5A-iii). Osteogenic markers were highly expressed in the HAp concentrated region, named osteogenic region (red), while the expression of SOX-9 chondrogenic marker, was significantly increased in the region with reduced or absent HAp concentration, named chondrogenic region (blue). Cells in the interface region presented significantly higher expression of SOX-9, but lower in comparison with the chondrogenic region. Furthermore, significantly lower expression of OCN, RUNX2 and OPN was observed in the interface when compared to the osteogenic region. Also, performing immunostaining and fluorescent intensity quantification to compare the effect of pore orientation on the expression of these proteins (figure 8.5C-I and 8.5C-iii), a significant increase was observed on the expression of SOX-9 and OCN on the chondrogenic region of the isotropic structures when compared to the anisotropic arrange (figure 8.5C-ii).

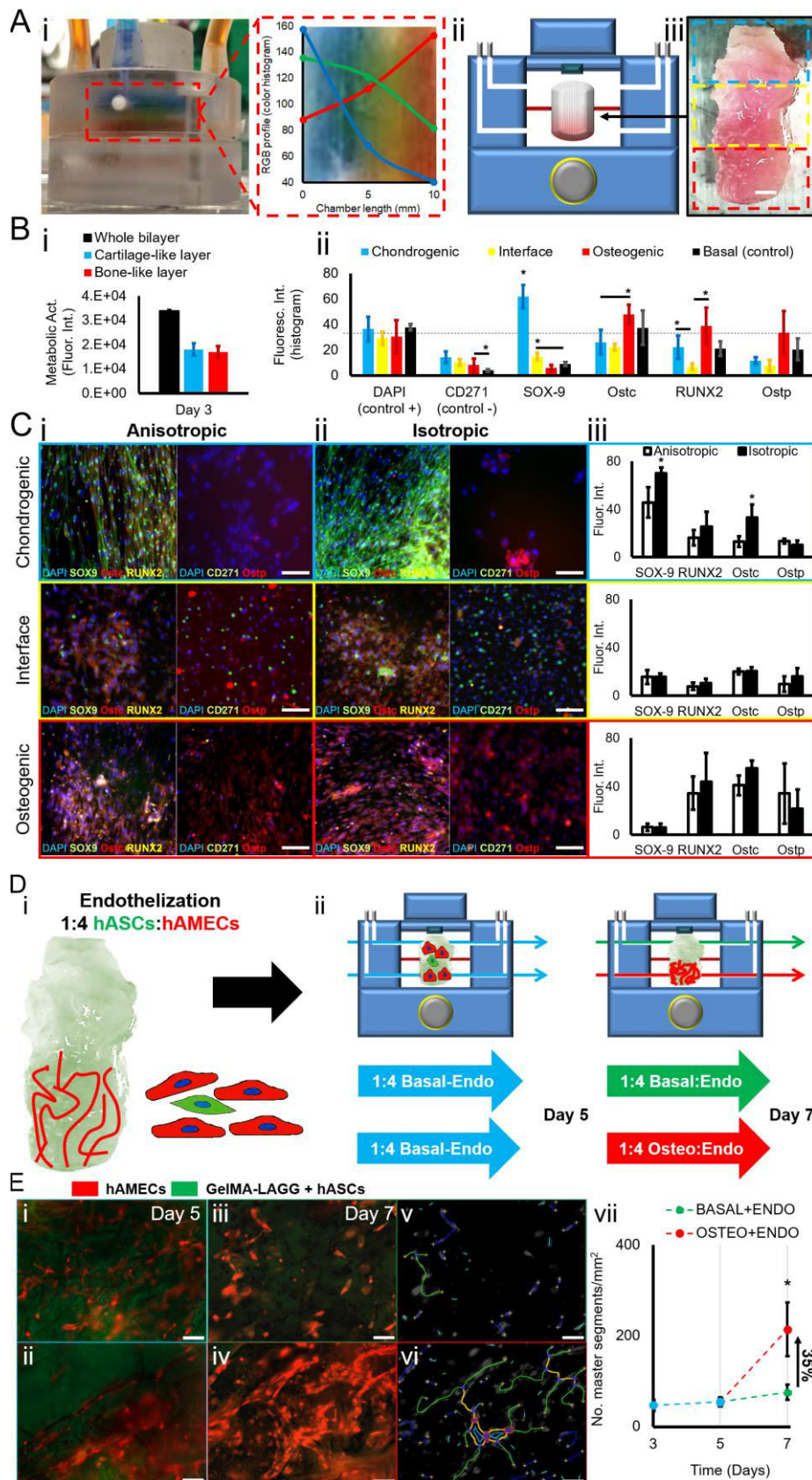


Figure 8.5 | Structural and biochemical gradients for OC differentiation in isotropic and anisotropic 3-D structures and endothelization. (A) The bioreactor device, designed for generating chemical gradients, perfused (i) two food dyes (red and blue) inside the dual-chamber, creating a yellow

interface. The color profile was traced showing the gradient formed in a RGB histogram. (ii) The bioreactor was designed to comprise two compartments in a dual-chamber, interfaced by a 3-D construct. (iii) The constructs were cultured for osteo- and chondrogenesis in the custom designed dual-chambers to coordinate the structure gradient along with the biochemical gradient (scale bar = 1mm). (B) Fat pad-ASCs were cultured in the gradient structures. (i) Each layer was quantified for metabolic activity three days after seeding in order to assess the cell distribution through the whole scaffold. (ii) Immunostaining was performed and quantified by fluorescent intensity to assess the expression of osteogenic and chondrogenic markers. RUNX2, osteocalcin and osteopontin were selected as osteogenic-related markers and SOX-9 as a chondrogenic-related marker. The quantification of these markers was evaluated against a non-differentiated control of hASCs cultured under basal medium. (C) hASCs were seeded onto (i) anisotropic and (ii) isotropic structures and cultured under OC conditions over 21 days (scale bar = 100 μ m). Immunostaining with the previous markers was performed to assess cell organization and markers expression. (iii) Quantification was performed to each marker for each 3-D condition. (D) hASCs isolated from fat pad were co-cultured with hAMECs over 7 days. (i) The seeding was performed onto the structures in a ratio of 1:4, respectively (scale bar = 1mm). (ii) Two interfaced culture media perfused the dual-chamber. (i) The culture media mixture was prepared as 4:1 endo-basal or endo-osteogenic, accordingly to the maintenance or differentiation conditions and perfused after 5 days in basal culture medium. (E) hAMECs were stained red with a live staining allowing a continuous tracking. Scaffold and hASCs are represented as green (bright field) in the microscopic images (scale bar = 100 μ m). The whole scaffold was cultured over 5 days in basal culture conditions, and the representative images for: (i) top and (ii) bottom regions represented. At day 5, basal condition kept being perfused on the top region (iii) and the osteogenic mix perfused on the bottom region (iv). The top (v) and bottom (vi) regions of the scaffolds were analyzed to identify the tubular segments created per region. (vii) The tubular segments identified in the microscopic images were quantified per area and the formation profile was traced using three time-points, 3, 5 and 7 days using Angiogenesis Analyzer tool for Image J. Data expressed as (mean $\pm$ SEM). N = 5, P value = 0.05.

Finally, to assess the endothelization in the gradient structures, hAMECs with Fat pad-ASCs (figure 8.5D-i) were co-cultured with two different media cocktails, being shown a spatial-temporal control over pre-vasculature formation (figure 8.5D-iii). Moreover, cell adhesion, shape and distribution of hAMECs stained before seeding (figure 8.5E), demonstrated a cell morphology change from day 5 to 7 (figure 8.5E-i to iv). Furthermore, the endothelial cells dispersed at single cell in the endo-basal condition (figure 8.5E-iii), and

were arranged in an elongated shape creating networks in the endo-osteogenic medium (figure 8.5E-iv). The tubular-like segments were identified in both conditions (figure 8.5E-v and 8.5E-vi) and quantified, showing a significant increase of about 35% in the region under endo-osteogenic cocktail condition (figure 8.5E-vii).

8.4 Discussion

Human tissue interfaces are known by their complex hierarchical structure, complex composition, heterotypic cellular environment, and, importantly, gradient of topographical, mechanical and biochemical cues. The structures developed in this study comprised both micro- and macro-pores and microcrystals (figures 8.2A, 8.3A and 8.3-D). An example of such structures in physiological settings are nanocrystals in the form of HA in the bone microstructure part. Additionally, aligned cells are also prevalent in the transition to the hyaline cartilage until it reaches the top surface which is free of HA²⁶. To address this challenging architecture and physical gradient, 3-D oriented porous structures were fabricated using convection as driving force to create a gradient of HAp.

A simple, versatile, and controllable methodology was developed to produce 3-D gradients with guided porosity. The use of two different polymers with physicochemical antagonist characteristics blended in two solutions, at controlled temperatures, allowed the use of convective forces to disperse micro-particles in the 3-D space, creating a gradient. Strikingly, controlling the polymeric solution 10 °C above its gelation temperature and using the second polymeric solution 5 °C lower than the first one, generated a continuous interface tuned by both convection and gelation. The physicochemical model behind thermal gradients in fluidic systems was recently demonstrated in the case of lattes formed by pouring espresso into a glass of warm milk²⁷.

Porosity guidance was obtained through crosslinking and freezing strategies. Thus, iso- and anisotropic structures were fabricated without losing the HAp gradient (figure 8.2). Then, a UV-crosslinking was performed which created strong covalent bonds holding the oriented polymeric chains. Before lyophilization, freezing cycle was applied to direct ice crystal formation. A range of temperatures from -80 °C to RT promoted an aligned ice crystal growth in the direction of this gradient. Interestingly, at the same concentration, these polymers showed, not only differences on elastic and degradation profiles (figure 8.3E), but also in cell adhesion and proliferation (supplementary information (SI), figure S8.9 and S8.10). Using this polymeric blend, gradients of several features can potentially be created allowing a simultaneous control over degradation rate and elasticity.

In addition to the achievement of isotropic and anisotropic porous structures, pore diameter and wall thickness were in the range of the native porosity and trabeculae size in subchondral bone²⁶, which is fundamental while controlling stem cell fate in a 3-D environment, as it was recently demonstrated²⁸. Nevertheless, biological tissues are hierarchically organized from nanometer-to-centimeter scale. The produced 3-D structures showed a micro- and macro-porosity in this range (figure 8.2 and 8.3). During bone mineralization, the HA crystals form micro- and nanocomposites with collagen fibers. Thus, preparing biomimetic surfaces which present synergistic effects of micro- and nanostructures is expected to provide additional advantages. Moreover, the structures produced in the present study showed a bioactive phase promoted by the HAp and a non-bioactive phase where HAp particles were absent (SI, figure S8.5). This behavior was controlled by the fabricated gradient.

The development of physiologically relevant models requires an understanding of the tissue architecture, physiology, and pathophysiological responses to biochemical or biophysical insults. This phenomenon is observed in the OC interface, where the two main tissues, cartilage and bone, differ substantially. Although bone and cartilage are heterogeneous tissues as many other interfaces in nature, they were proven to be generated from one continuous developmental lineage²⁹. So, using a single stem cell type required a spatial control of the 3-D environment, which was achieved by the custom-made system designed for biochemical gradients. After induction in the three-layered environment made of osteogenic, chondrogenic media, and their mixed interface, phenotypic changes were observed in the ASCs cultured in the different regions of the construct for 21 days of culture. Chondrogenic- and osteogenic-related markers were differently expressed by the cells in the osteogenic, chondrogenic and interface regions. For instance, cells in the interface evidenced a decrease in SOX9 immunolocalization as compared to cells in the chondrogenic zone but still significantly higher than the osteogenic region. Moreover, here we observed that cells cultured in isotropic porous structures significantly increased SOX9 immunolocalization when compared to those cultured in anisotropic porous structures.

Currently, there is increasing evidence from *in vivo* and clinical studies that subchondral bone lesions may precede cartilage degeneration. So, OA is considered an OC disease and possibly bone dependent because of the inflammatory factors delivered by the vasculature present in the subchondral bone³⁰. Blood vessels from the subchondral bone invade the articular cartilage promoting the progression of OA and forming osteophytes. To target this feature, ASCs from fat pad were co-cultured with hAMECs. This approach aimed at providing solutions not only to mimic the lesion condition *in vitro*, but also to improve osteogenesis. ECs secrete factors such as FGF, IL-1, and IL-6, and nitric oxide that influence osteoclast behavior, thereby regulating bone formation and resorption as previously shown^{31,32}. In turn, synergistically

ECs are the target of many bone-derived signals, such as parathyroid hormone, insulin-like GFs types 1 and 2, basic FGF, platelet-derived growth factor, and vascular endothelial growth factor. Although these events are well studied and reported for 2-D *in vitro* conditions, 3-D reality still requires a better understanding and control. Using a gradient of culture media from osteogenic to basal conditions, the co-culture promoted endothelization in the region perfused by the osteogenic-endothelial media cocktail. This can potentially be explained by the differentiation of ASCs towards osteogenesis, which possibly induced the secretion of VEGF, stimulating the formation of endothelial vascular networks.

Overall, the presented fabrication technique and biological results have a direct impact on the TE field for the fabrication of hierarchically organized tissues, such as the OC unit, with broad applications in RM and *in vitro* tissue modelling for drugs screening and discovery.

8.5 Materials and methods

Materials. LAGG (Gelzan™ CM, F=1,000,000), gelatin (from porcine skin, gel strength 300, Type A), and methacrylic anhydride were obtained from Sigma-Aldrich (USA). Unless otherwise stated all the reagents were purchased from Sigma-Aldrich.

Hydroxyapatite microparticles synthesis (HAp). HAp were prepared by precipitation in an aqueous system at RT. In the precipitation reaction, solutions of calcium hydroxide (Riedel-de Haën, Germany) and ortho-phosphoric acid 85% (Panreac, Spain) were gently mixed and brought to pH 11 with concentrated ammonium hydroxide (Riedel-de Haën, Germany). The formed ceramic was pounded and sieved at different sizes ranges. Particles with less than 263 µm were collected for this study.

Methacrylation of gelatin (GelMA). Gelatin was methacrylated by reaction with methacrylic anhydride. A solution of 2 g of gelatin in 20ml of PBS (1X, Invitrogen) was prepared by dissolution at 50°C. The reagent methacrylic anhydride was added (1.6 mL) at a flow rate of 0.1 mL/min during 16 min. After complete addition of methacrylic anhydride, the solution kept reacting for 2 h at 45°C. Then, the reacted solution was dialyzed against distilled water at 45°C to avoid gelation, for 5 days, changing water twice a day. The dialyzed solution was transferred to falcon tubes and lyophilized to obtain dried GelMA. GelMA foam was stored at -80°C until further use. The reagent TNBSA was used to quantify the free amino groups in GelMA which correlate to the polymeric modification yield. Absorbance and concentration present a linear relationship up to 1 mg/mL. The modification of gelatin by methacrylation

is inversely proportionally to the absorbance of the solutions at 335 nm and was quantified using a standard curve of concentrations for calibration.

Preparation of GelMA and LAGG 3-D gradient structures. LAGG and GelMA were dissolved at a total polymer concentration of 2% (w/v) in two separated beakers. First, LAGG was dissolved at 80 °C for 1 h in distilled water. Then, the solution was cooled down to 50 °C and 0.5% (w/v) 2-hydroxy-1-[4-(2-hydroxyethoxy)phenyl]-2-methyl-1-propanone (Irgacure 2959, Ciba Specialty Chemicals) was added to the polymeric solution and dissolved for 1 h. GelMA was then added for dissolution for 1 h at 45 °C. HAp were added at 30% (w/w) and dispersed under stirring in one of the two beakers with the blend solution. After complete HAp dispersion, 800 μ L of this solution was added into a mold of 10x7 mm at 55 °C. A second injection of 800 μ L was added on top of the first half at 50 °C within 30 s after pipetting the first gel. Afterwards, hydrogels were obtained by immersing the gradients in α -MEM medium without FBS for 24h for total ionic-crosslinking of LAGG molecular chains. For the isotropic structures fabrication, the silicon molds were removed after 1h in medium solution. For the fabrication of anisotropic structures, a guided ionic crosslinking was performed. To guide the ionic crosslinking, cylindrical molds were covered on the bottom part with Parafilm™ and immersed in culture medium solution. After 24 h at 37 °C, the ionic-crosslinked hydrogels were exposed to UV light (320-500 nm, 7.14 mW/cm², EXFO OmniCure S2000) for 60s each side of the mold. After photo-crosslinking, the anisotropic structures were fabricated using the formed hydrogels and applying freezing gradient temperature, reaching the -80 °C. To create the temperature gradient, a custom made styrofoam mold involving the silicone mold was used covering the polymeric solution all around except the top, where a frozen plate at -80 °C was directly placed for 2 h. After lyophilization, the fabricated structures were punched and sliced in cylinders of 4x3 mm. The structures were submitted to 3 cycles of 5 min of oxygen plasma treatment for sterilization and surface hydrophilicity cure before cell seeding.

Dynamic mechanical analysis (DMA). The viscoelastic measurements were performed using a TRITEC8000B dynamic mechanical analyzer (Triton Technology, UK) in the compressive mode. The measurements were carried out at 37 °C. Samples were cut into cylindrical shapes of approximate 8 mm diameter and 6 mm thickness (measured each sample accurately with a micrometer). The scaffolds were always analyzed whilst immersed in a liquid bath placed in a Teflon reservoir. The scaffolds had previously been immersed in PBS until equilibrium was reached (37 °C overnight). The geometry of the samples was then measured and the samples were clamped in the DMA apparatus. 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 a 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.

Scanning electron microscopy analysis. The 3-D gradient structures were analyzed to assess wall surface morphology and HAp incorporation by SEM/EDS (NanoSEM-FEI Nova 200, USA). Prior to the microstructure analysis, specimens were coated with gold using a Fisons Instruments Coater (Quorum/Polaron E6700, UK) with a current set at 18 mA, for a coating time of 120 s.

FTIR analysis. Infrared spectra of the HAp were obtained by FTIR (IRPrestige 21, Shimadzu, USA). For this purpose, HAp were mixed with KBr in the proportion of 1/150 (by weight) and pressed into a pellet. To assess the amorphous state of HAp in the structures, FTIR-ATR was performed instead. Each infrared spectrum was on the average between 32 to 40 scans collected at 2 cm^{-1} resolution at RT.

X-ray diffraction analysis. The qualitative and quantitative analyses of crystalline phases presented on the powders and scaffolds were obtained by X-ray diffraction (XRD) using a conventional Bragg–Brentano diffractometer (Bruker D8 Advance DaVinci, Germany) equipped with $\text{CuK}\alpha$ radiation, produced at 40 kV and 40 mA. Data sets were collected in the 2θ range of $10\text{--}70^\circ$ with a step size of 0.04° and 2s for each step.

The phase composition of the powders was calculated on the basis of XRD patterns by means of Rietveld analysis with TOPAS 5.0 software (Bruker, Germany). Refined parameters were scale factor, sample displacement, background as Chebyshev polynomial of fifth order, crystallite size, and lattice parameters.

Assessment of degradation rate. GelMA and LAGG polymers were assessed for degradation over 14 and 30 days, respectively. Weighed hydrogels ($n=6$) were incubated in a 24 ng/mL collagenase type I solution at 37°C . The samples were removed, the excess of water adsorbed, and then freeze-dried and weighed again to determine weight loss.

$$\text{equation 7.1. weight loss (\%)} = (m_0 - m_f) / m_0 \times 100\%$$

where m is mass, 0 is initial, and f is final.

3-D architecture characterization of 3-D porous structures and HAp profile. Freeze-dried structures were analyzed by micro-CT. The gradient structures were acquired by X-ray diffraction, and then reconstructed for qualitative and quantitative analyses. HAp profile, porosity, mean pores size, and anisotropic degree were quantified. 3-D cross-sections of isotropic and anisotropic structures were designed. Samples were acquired with a SkyScan 1272 scanner (v1.1.3, Bruker, Boston, USA), with a pixel size of 10 μm . Approximately 300 projections were acquired over a rotation range of 180°, with a rotation step of 0.6°. Data sets were reconstructed using standardized cone-beam reconstruction software (NRecon 1.6.10.2, Bruker). The output format for each sample was bitmap images. Representative data set of the slices was segmented into binary images with a dynamic threshold of 30–255 for soft polymer analysis and 120–2255 for ceramic phase analysis (grey scale values). Then, the binary images were used for morphometric analysis (CT Analyser, v1.13, Bruker) and to build the 3-D models.

Live/dead cell viability assay. The effect of each formulation on cell viability was assessed after 7 days of culture of NIH-3T3 fibroblasts (ATCC). Cells were seeded within the 30 min after sterilization in a 24-well suspension cell culture plate (VWR) and kept in the CO₂ incubator. 1 M cells were suspended in 50 μL culture medium and seeded in both ends of each structure. After 1h, 500 μL of culture medium was added. NIH-3T3 constructs were cultured in α -MEM (Life Technologies) supplemented with 10% of FBS (Thermo-Fisher Scientific) and 1% penicillin-streptomycin (A/A, Life Technologies) at 37 °C, in a humidified environment (37°C, 5% CO₂). Cell viability in the 3-D structures was characterized after 7 days of culture, staining cells with Live/Dead calcein AM and ethidium homodimer-1 in PBS (Life Technologies) for 20 min. All formulations were performed in triplicate. Cell culture on tissue culture polystyrene well plates were used as a positive control.

Alamar blue assay. NIH-3T3 fibroblasts were seeded and cultured as previously described. After 3h, the constructs were moved to new 24-well suspension culture plate and incubated with 900 μL of basal medium (α -MEM supplemented with 10% FBS, 1% A/A. The previously defined medium was used and changed at least twice weekly. Metabolic activity was evaluated after 1 and 7 days of culture with AlamarBlue® CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI, USA) (n = 9), according to the manufacturer's instructions. Briefly, the medium was removed, samples washed and incubated in 10% (v/v) AlamarBlue solution in basal medium, at 37 °C. The obtained supernatant was transferred to a 96-well plate and the fluorescence measured in a microplate reader. Then, the medium was removed by aspiration and the constructs were washed with fresh medium three times and kept in

culture until the end of the assay. Scaffolds without cells were used as a negative control for fluorescent intensity correction.

Isolation and culture of Fat Pad-ASCs. ASCs were isolated from human tissues obtained from Hoffa's body removal during arthroscopic surgeries on male and female donors with ages between 19 and 32 years, after informed consent, under cooperation protocols established with Centro Hospitalar Póvoa do Varzim and Clínica Saúde-Atlântica. The study has been approved by the Ethical Committee of Centro Hospitalar Póvoa do Varzim, Grupo Saúde-Atlântica and University of Minho. All experiments were carried out according to animal care standards set forth by the National Institute of Health and were approved by the Institutional Animal Care and Use Committee (IACUC) at Stanford University.

All the samples were processed within 24 h after the arthroscopic procedure to the knee and hASCs were enzymatically isolated from Hoffa's body. hASCs were isolated following an enzymatic digestion-based method with type II collagenase (Sigma) from Hoffa's body human tissue. The extracted tissue was placed in PBS solution and washed several times with 1% PBS/AA (v/v) solution, until blood or other bodily contaminants were totally removed, and cut into small pieces. Tissue digestion was performed by incubating, at 37°C in a humidified atmosphere of 5% CO₂ for 1-2h, the small pieces with 10-20 mL of a mixture composed by cell medium MEM alpha medium (Gibco), supplemented with 10% FBS and 1% A/A mixture, and type II collagenase (1:1). The digested tissue was filtered and cell suspension centrifuged at 1220 rpm for 5 min. The isolated cell populations were expanded in MEM alpha medium supplemented with 10% FBS and 1% of A/A mixture until reaching confluence.

Fat pad ASCs were cultured in bilayered structures for 3 days, before starting cell differentiation. The cells were seeded in a concentration of 1x10⁶ cells per scaffold, having these scaffolds 6x4 mm high and diameter, respectively. At day 3, the two layers of each structure were divided and measured the metabolic activity of each layer versus the whole scaffold, in order to assess cell distribution in each layer.

Co-culture of fat pad ASCs with Human Adipose Microvascular Endothelial Cells (hAMECs) using a dual-chamber bioreactor. hAMECs were stained with Dil live staining (Thermo Fischer Scientific) before mixed with ASCs for seeding. A mixed population of ASCs and hAMECs were seeded in a ratio of 1:4, respectively, with infiltration on top and bottom parts of the isotropic porous 3-D structures inside the dual-chamber bioreactor. Basal MEM alpha supplemented with 10% FBS and 1% A/A was mixed with endothelium growth medium (Lonza) containing 2% FBS and VEGF in a ratio of 1:4, respectively, and equally perfused in both compartments of the dual-chamber. The bioreactor chamber

was perfused with 5 $\mu\text{L}/\text{min}$ at 37 °C in a humidified 5% CO_2 atmosphere. After 5 days of perfusion, the cell culture medium of the bottom chamber was changed by the mix of osteogenic differentiation medium (Lonza) with endothelial growth medium (1:4) and perfused for 2 days more. Construct were imaged on top and bottom regions at day 5 and 7 by fluorescent microscopy. FIJI plugin Angiogenesis Analyzer was applied to evaluate the major segments formation. Bright field and fluorescent images were individually analyzed and their outcomes were combined in average to reduce potential artefacts occurring in each image type (n=5).

Immunofluorescence staining. Cells in constructs were fixed with 4% paraformaldehyde for 20 min at RT. Fixed cells were washed with PBS, non-specific binding was blocked with 1% BSA (Sigma) and permeabilized with 0.3% Triton-X 100 (Sigma) for 2h at RT. Cells were incubated with rabbit anti-osteocalcin (Millipore), mouse anti-SOX9 (Santa Cruz Biotechnology), and goat anti-RUNX2 (Novus Biologicals) overnight at 4 °C. Afterwards, samples were washed and incubated with secondary goat anti-mouse Alexa Fluor 568, Alexa Fluor 647 and goat anti-mouse Alexa Fluor 488 antibodies (Life Technologies) for 2h at RT. Mouse anti-Osteopontin (Abcam) was visualized with using the secondary antibody Alexa Fluor 568 (Life Technologies). CD271-APC (BioLegend) was used as a negative control staining. Finally, cell nuclei were counterstained with 4,6-diamidino-2-phenylindole dilactate (DAPI, Invitrogen). All samples were visualized by fluorescence microscopy (Zeiss LSM710 inverted confocal microscope).

Statistical analysis. All results expressed as mean $\pm$ SD or confidence interval. Student's t test was used to compare group means, and a p -level < 0.05 was considered statistically significant. Statistical analyses were performed with Microsoft Excel 2010 and GraphPad Prism 5 software for Windows. Not-paired two-tailed Student's t-test and nonparametric Mann-Whitney test were used.

8.6 References

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8.7 Acknowledgments

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8.8 Supplementary information

8.8.1 Supplementary methods

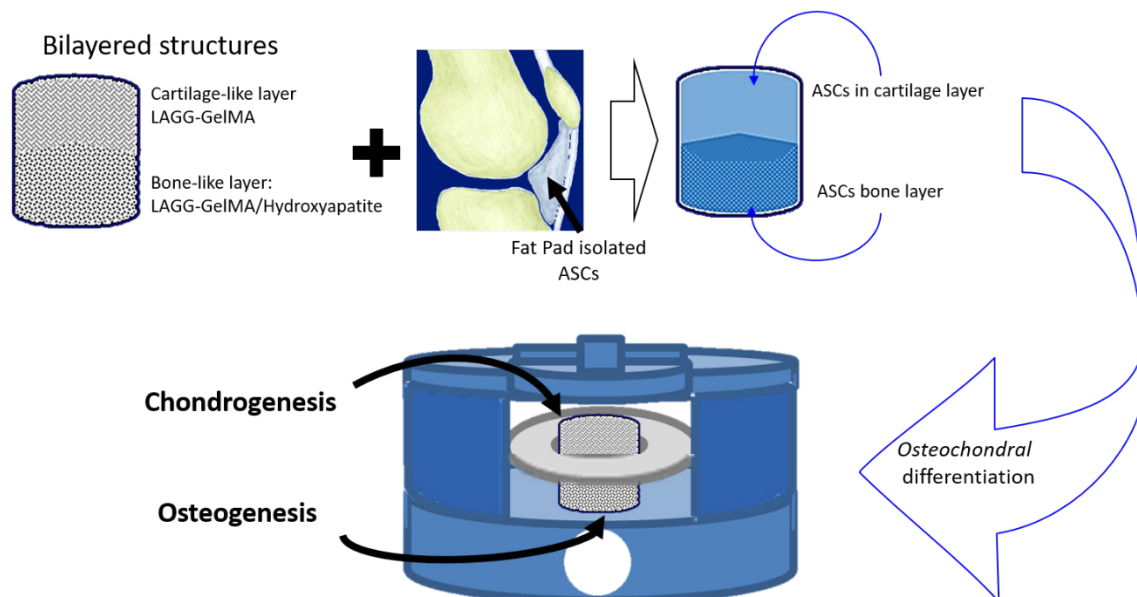


Figure S8.1 | Scheme of the strategy for 3-D OC *in vitro* tissue development. Gradient scaffolds seeded with fat pad-derived ASCs for OC differentiation in a dual-chamber bioreactor.

8.8.2 Supplementary results

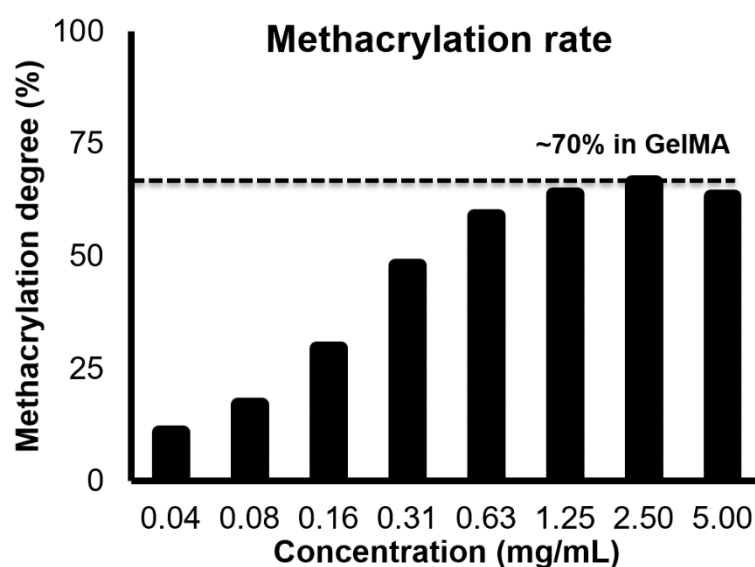


Figure S8.3 | Gelatin modification by methacrylation. Characterization by TNBSA quantified ~70% methacrylation of gelatin.

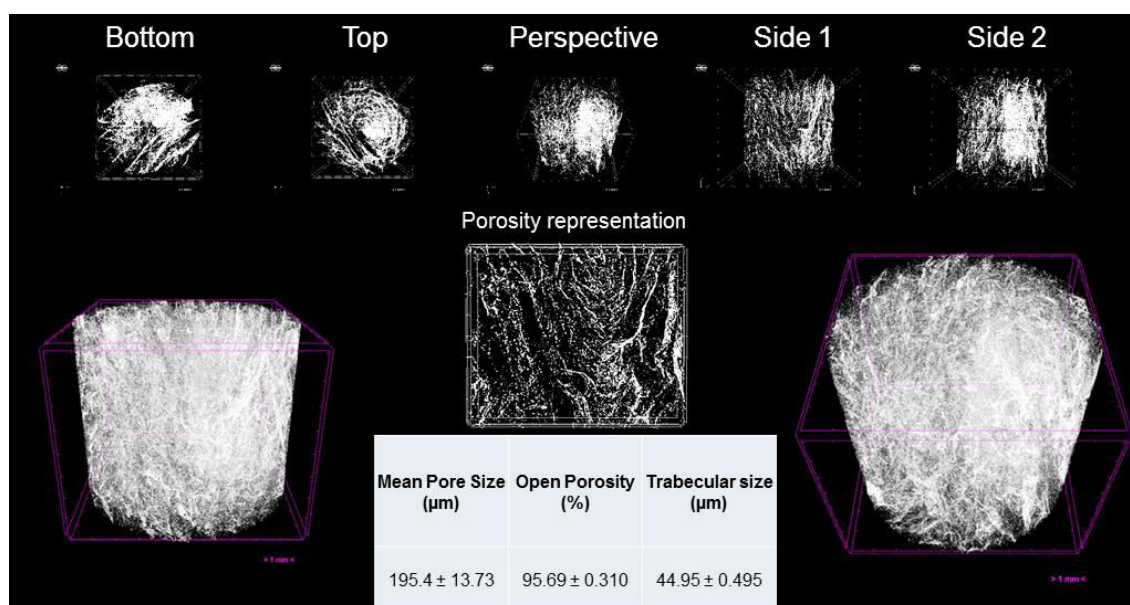


Figure S8.4 | X-ray microtomographic analysis of lyophilized GelMA-GG structures. The formulation of 3:1 GelMA:MAGG, 5 mm x 5 mm, was characterized addressing porosity and trabecular size. The measurement by x-ray microtomography showed a mean pore size of 195.4μm. Open porosity was quantified in 95.69%. The mean trabecular size was evaluated in 44.95 μm.

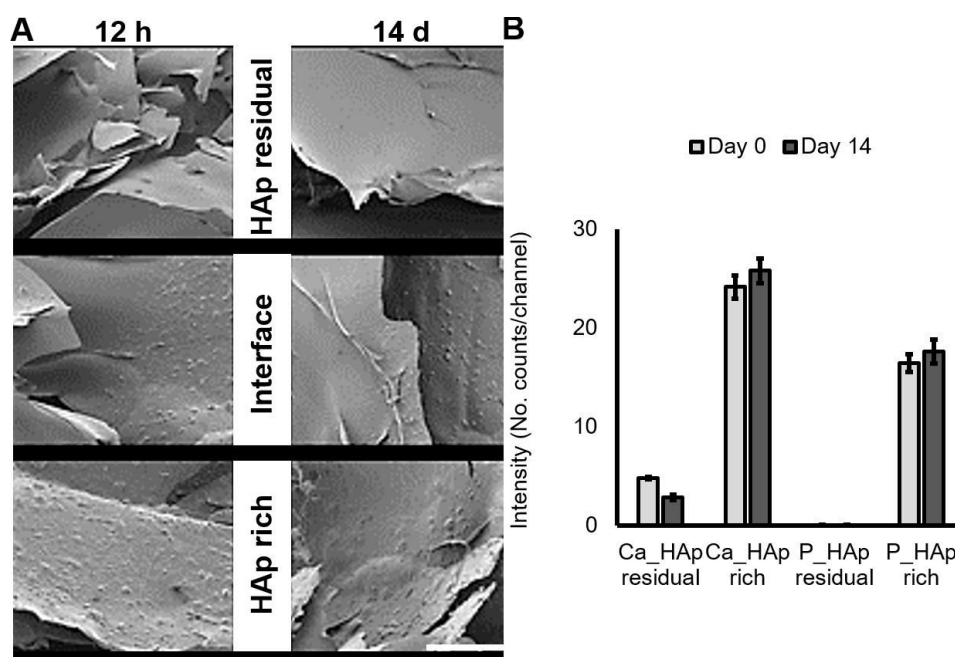


Figure S8.5 | Bioactivity assay on GelMA-GG 3:1 scaffolds. (A) The transition from rich to residual HAp regions is represented by SEM images (scale bar = 400 μ m). (B) Calcium (Ca) and phosphorus (P) were quantified per region at day 0 and 14 by EDS. The apatite layer disappears from the HAp rich to the HAp residual region, as required for OC differentiation (osteogenesis and chondrogenesis, respectively).

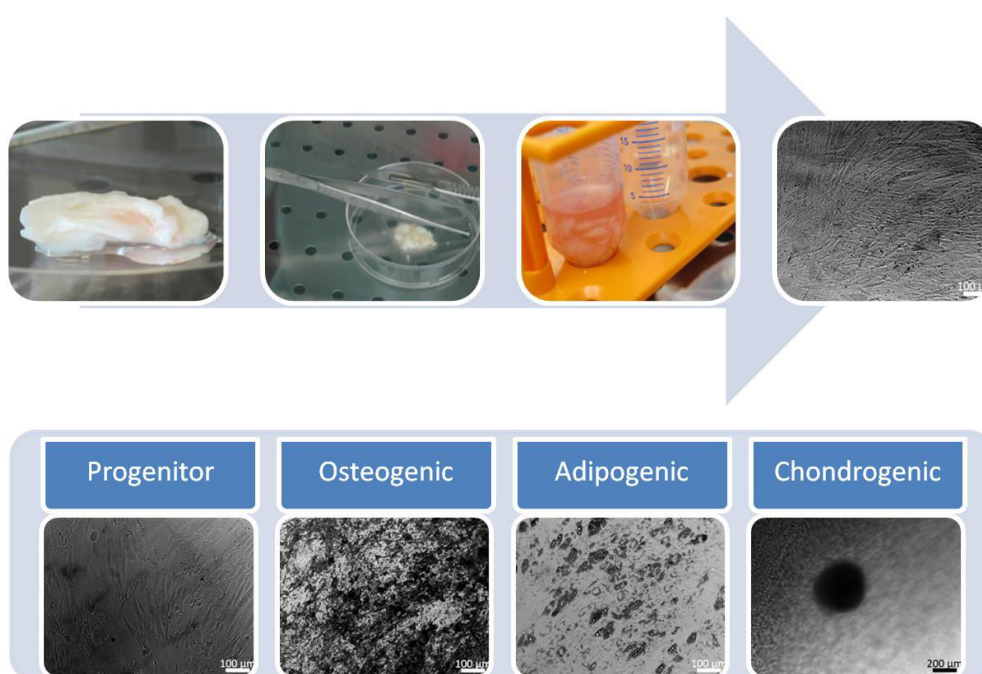


Figure S8.6 | Multipotency of ASCs isolated from Hoffa's body. Tissue was sliced, then digested with collagenase type II and finally adherent cells were selected. Progenitor ASCs have multi-lineage potential, with strong osteogenic and chondrogenic differentiation.

If we consider that the surgeries that would be performed for future introduction of implantable constructs, Hoffa's body can be used for ASCs isolation, thereby recycling the tissue removed during arthroscopy to produce a patient customized OC engineered tissue.

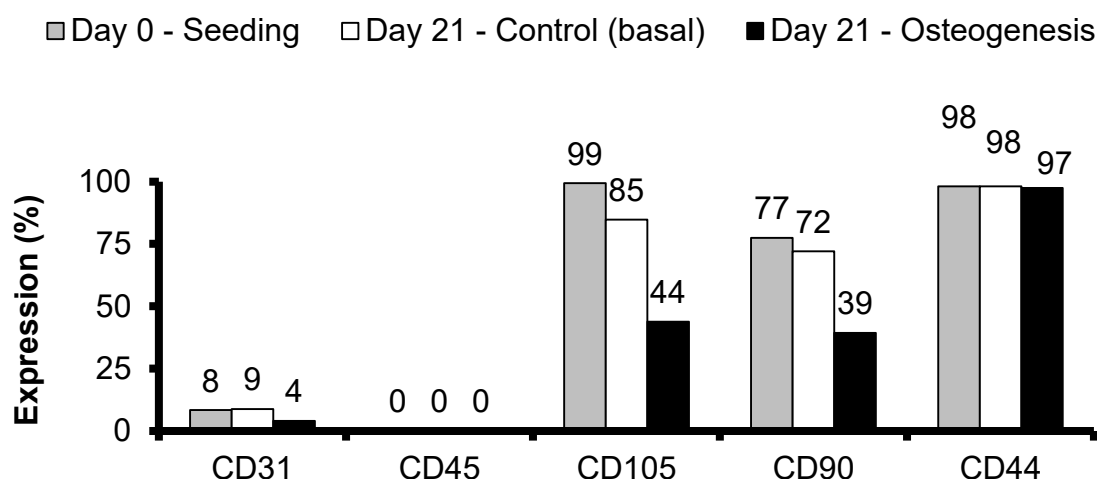


Figure S8.7 | Flow cytometry analysis of fat pad-ASCs over 21 days. Mesenchymal markers were analysed at day zero (seeding) and after 21 days of osteogenic differentiation. Basal medium was used as a control over time.

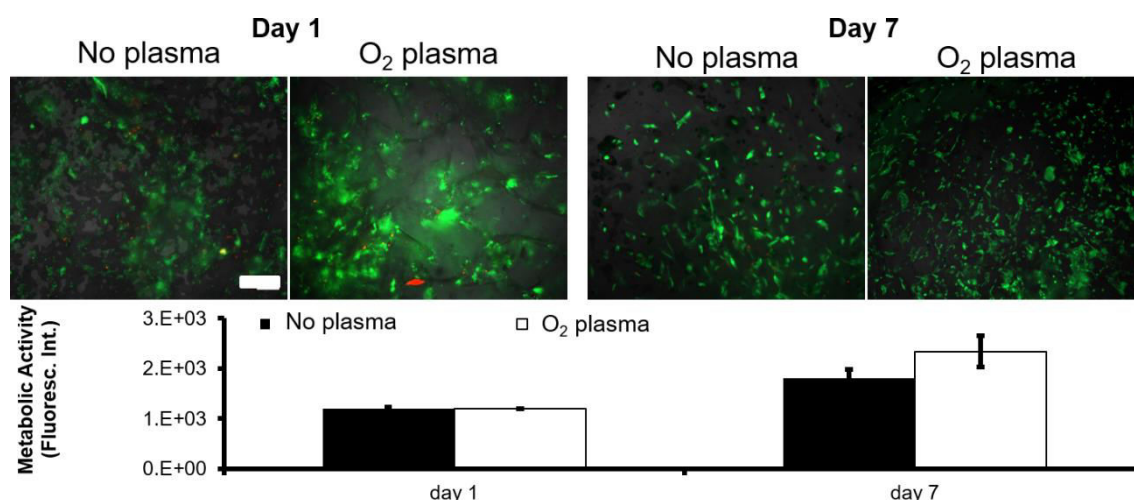


Figure S8.8 | Scaffolds sterilized by O₂ plasma. 3 cycles of 5min exposure were performed. Metabolic activity was assessed to confirm the cell viability cultured on scaffold sterilized by plasma treatment.

The sterilization of 3-D structures is an important concern. The use of polymeric medical devices has stimulated the development of new sterilization methods. The traditional techniques rely on ethylene oxide, but there are many questions concerning the carcinogenic properties of the ethylene oxide residues adsorbed on the materials after processing. Another common technique is the gamma irradiation process, but it is costly, its safe operation requires an isolated site and it also affects the bulk properties of the polymers. The plasma promotes an efficient inactivation of the micro-organisms, minimizes the damage to the materials and presents very little danger for personnel and the environment. Pure oxygen reactive plasma was applied, showing efficient structures sterilization. In this assessment, some the samples not submitted to oxygen plasma showed contamination (data not shown), while all the samples that passed by 3 cycles of 5 min under oxygen plasma were free of contamination. Furthermore, knowing that this plasma treatment can change the surface of the produced structures, we tested the cell viability, adhesion and metabolic activity over 7 days. The scaffolds submitted to oxygen plasma showed improved cell spreading through the structures and higher metabolic activity at 7 days.

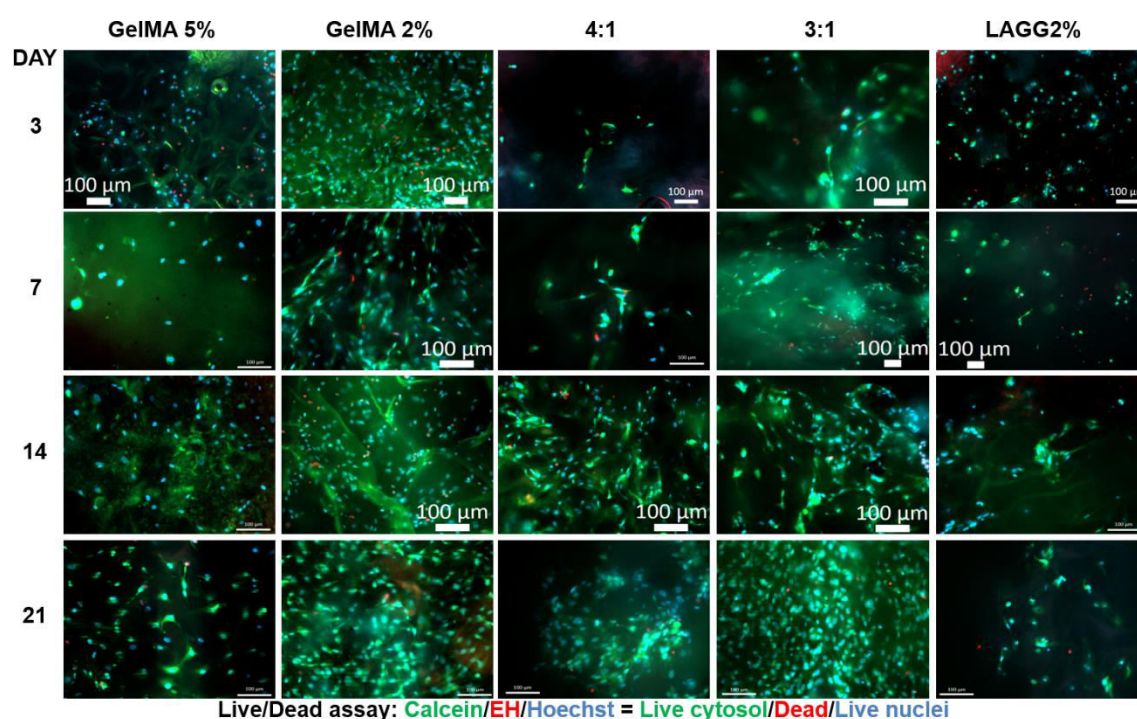


Figure S8.9 | Adhesion, viability and proliferation of fat pad-ASCs in GelMA-GG formulations. Samples were assessed over 21 days of culture in formulations of GelMA and GG. Live/Dead assay was performed with Calcein/EA/Hoechst (Live cytosol/Dead/Live nuclei). Fat pad-ASCs were seeded in a density of 1M per scaffold.

Different polymeric formulations were produced and cultured with human fat pad-ASCs. Two different concentrations of GelMA were tested (5 and 2%) as well as GG at 2%. Keeping the 2% of GelMA, the produced blends of 4:1 and 3:1 GelMA:MAGG were also assessed.

GelMA 2% presented the best cell adhesion, being confluent at day 3 and keeping the cell confluence over 21 days. However, these structures became loose and degraded over time, losing their integrity. GelMA 5% and GG2% showed poor cell adhesion and almost no cell proliferation. The cells cultured in GG2% scaffolds were roundish. Cell death was also observed over the first 3 days. As the days passed, the cells in GelMA5% started spreading in the structure, fact that should be correlated with the change of mechanical properties of these structures as their degradation occurs overtime in culture medium, becoming more soft. The blends of 4:1 and 3:1 showed about 50% cell adhesion reaching the confluence in the second week during the second week of culture. Taking into account the improved mechanical characteristics provided by GG, the formulation of 3:1 GelMA:MAGG was selected to proceed for OC differentiation in bilayered structures.

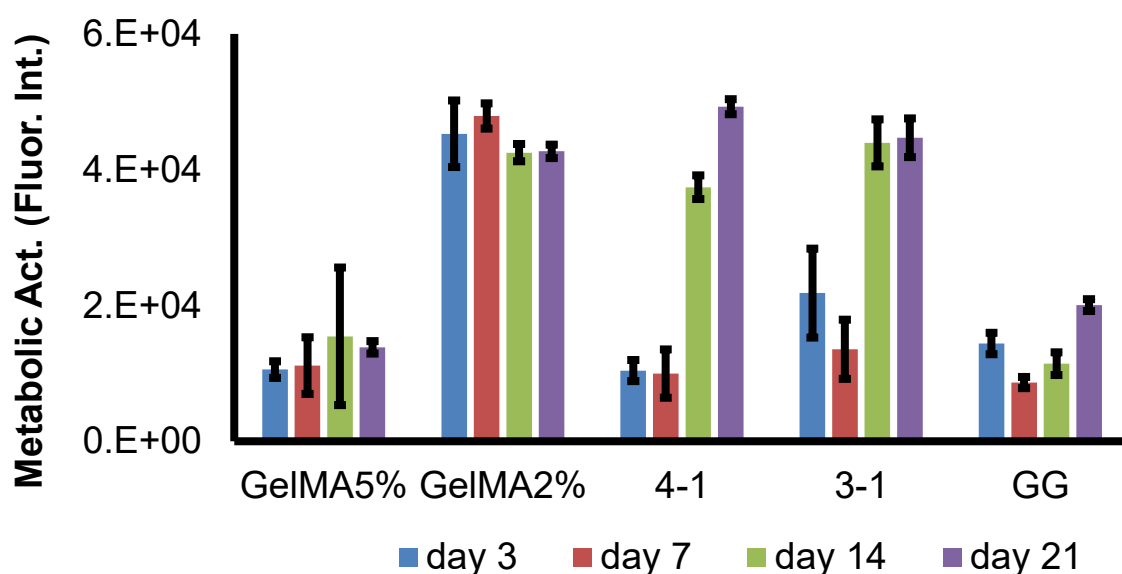


Figure S8.10 | Metabolic activity of fat pad-ASCs cultured in GelMA-GG formulations. The metabolic activity was assessed over 21 days of culture in GelMA and GG scaffolds formulations.

SECTION III

FINAL REMARKS

CHAPTER 9

GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

9. GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

9.1 Conclusions

Three decades ago, in 1987, the term “Tissue Engineering” was introduced in medicine. This concept boosted the evolution of medical technologies based on engineering concepts. Despite the evident potential demonstrated by TE approaches to be applied in the replacement and regeneration of damaged tissues, their translation from the bench lab to the clinic has faced several obstacles. Firstly, the complexity of the majority of the human tissues, which are mainly arranged in a 3-D configuration thus require advanced strategies to control diverse hierarchical environments in a single structure. Secondly, the coordinated control of the vascularization, essential for tissue’s viability and phenotype, and of the fabrication of the hierarchical architecture. Thirdly, and probably the most important, the up-scaling of these methodologies due to lack of simplified strategies for *in vitro* tissue fabrication/maturation.

Therefore, the work described in this thesis was planned focusing on the development of simple strategies for advanced needs, such as the control over gradients and porosity for hierarchical tissues. The works were projected considering an easy industrial translation, such as the use of FDA-approved natural origin materials (GG as a food additive), and simple manufacturing techniques, based on the control of temperature and crosslinking methodologies to generate structures with gradients and guided porosity. Moreover, a bioreactor and a microfluidic system were designed to achieve spatial control over different culture media simultaneously perfused, which could facilitate the maturation/differentiation of hierarchical tissues such as OC unit and a technological solution for the upscale production of engineered tissues. Such upscale is dependent on the standardization of production methods, as the market analysis described in chapter 2 revealed. The performed survey showed that, simple but efficient methods, mainly adaptable to the current cell culture protocols, could accelerate this transition. In any case, this is an urgent need belonging to a growing market. A paradigm transition is ongoing, which is an opportunity for new technologies and related-startups to appear. The outcomes from 3-D cell culture have clearly shown an enhancing relevance of outcomes regarding biological studies. Taking these indicators into consideration, simple but advanced strategies were the focus of the PhD work plan here described for 3-D TE and cell culture systems development.

In chapter 4, a gradient structure was fabricated by controlling the distribution patterns of HAp in interfaced gels. The mix was experimentally modulated through the combination of different temperatures applied in two solutions of GG. When different temperatures were applied the corresponding convective

lines guided the HAp through the GG phase, while each GG solution showed minimal mixing at the interface. When the difference of temperatures between solutions increases, the Brownian motion is superseded by convection and the transport of heat matched the transport of HAp. We found that this phenomenon was dependent on the temperature of each GG solution, which relates to its viscosity. Additionally, two different dyes were incorporated to understand the mixing of the two gel solutions at different temperatures. Methylene blue was selected as a model drug, allowing to modulate drug incorporation and investigate its spatial diffusion along the gradient structure. The dye was spatially confined to half hydrogel, meaning that a spatial control is achievable when small molecules are incorporated. This was confirmed when, GG modified with FITC was used to compare the location of the dyes with the mixing of the polymer itself, and similar profiles were observed even under the combination of solutions at different temperatures. This way, the distribution of different small molecules can be spatially controlled in each solution of GG while a distribution pattern of HAp can be obtained by programming the gradient of temperatures. This phenomenon can have important applications in drug delivery, TE, tissue modelling, or even in broader areas of materials engineering. The ideal conditions, temperature, concentration of microparticles, and volumes, for OC regeneration were selected based on the representability of the tissue by the obtained properties of the structures. The gradient of HAp resulted in structures with bioactive-to-non-bioactive properties as supported with hASCs cultures and the differences in ALP activity. Moreover, this feature was further confirmed in an OC regeneration model with full closure of the critical size defect. The structures were crucial to keep distinguishable ECM deposition and tissue organization in the bone-to-cartilage transition. The supported formation of hyaline-like neo-cartilage occurred at the same time as the penetration of fibrous tissue into the subchondral bone region was avoided. The spatial control over microparticles or bioactive factors in general has been a challenging need not only in the TE field, but also in most of materials engineering areas. The work developed in chapter 4 provide interesting solutions for the control over microparticles spatial distribution in hydrogels with application in OC TE, as well as other interfaced tissues, and in broader areas of materials engineering.

In chapter 5, 3-D structures with controlled porosity were attained by the creation of temperature gradients during the freezing process, before freeze-drying. This freezing resulted in the formation of linear ice crystals nucleation and growth that correspond to the linear pores observed after the ice removal by freeze-drying. A control over porosity is of major importance in TE applications since the pore geometry can determine both cell adhesion and orientation/organization which directly impacts on tissue formation and function. As proof-of-concept, the fabricated anisotropic structures were applied to control neurites

outgrowth of primary neurons since it is known that in neocortex neurons alignment is directly linked to anisotropic synaptic events organized in meaningful functional patterns. The generated porosity enabled not only the guidance of neurites, but also the synaptic junctions throughout the 3-D environment. The pore size of the anisotropic structures permitted to control the width of the neural columns, being within the range observed in the native dimensions found in the human cerebral cortex - approximately 2 mm tall and diameter of 0.5 mm. In order to add a complementary functionality to the developed 3-D structures, a microfluidic device was designed to flow two cell culture media creating gradients of bioactive molecules in the interface region of the 3-D structures. Endothelial and neural culture media were flowed through the anisotropic structures creating a perfused endo-neural interface. Another challenging feature is the control over porosity formation in a 3-D structure.

Chapters 6 and 7 describe two patents related with a continuous development of two technological components that are combined in a bioreactor system. In chapter 6, a dual-chamber bioreactor was designed and fabricated, which is attachable to the dynamic platform developed in chapter 7. The dual-chamber was conceived to stimulate stem cell co-differentiation in 3-D constructs, considering the specific need of generating interfaced tissues. Therefore, the chambers of the bioreactor were designed to hold 3-D porous structures where two different cell culture media could be simultaneously perfused. The dual-chamber was designed to match the sizes of commercially available 6-well culture plates, allowing an easy adaptation of the device to standard laboratory cell culture disposables. This permits its use under static in addition to dynamic conditions. Additionally, the bioreactor can support two-dimensional (2-D) and three-dimensional (3-D) approaches, as membranes interfacing two environments or 3-D tissues and interfaces as OC unit. As proof of concept, the developed dual-chamber bioreactor was used to recreate what happens during embryonic development of the brain. By embedding NSCs in Matrigel® hydrogels and flowing two molecular signals, Reelin for NSCs differentiation and fibroblast GF 2 (FGF2) for NSCs proliferation, a proliferative region adjacent to a differentiated neural region was promoted. This observation proved the relevance of the dual-chamber bioreactor as a system to create interfaced regions of the same tissue, which can have a relevant impact on tissue development studies, and broader areas of TE and drug testing.

The dynamic platform was projected to solve issues related with 3-D cell culture such as sedimentation which impaired a uniform cell distribution in 3-D, and nutrients diffusion. So, having this in mind, the dynamic platform was designed to attach the bioreactor dual-chamber performing rotational movements. In this platform, all the dynamic rotations were controlled with a wires-free interface, and a feedback on the temperature of each motor was collected by sensors, enabling an easy industrial translation.

Nevertheless, the platform was projected to have a user-friendly interface. The metabolic activity of fat pad-ASCs cultured in a GG-based 3-D scaffold was assessed under horizontal rotation of the dual-chambers attached to the dynamic platform by magnetic attraction, resulting in an increase of metabolic activity when compared to the static condition. This metabolic activity was correlated with the rotational stimulus which directly affected the diffusion of culture medium inside the chamber. Moreover, a vertical rotation was performed, which was strategically planned to control cell sedimentation in the 3-D structures. A spongy-like hydrogel made of GG was used as scaffold, which was seeded with a low number of fat pad-ASCs (500k cells/scaffold). The vertical turn of the cell culture in 180°C resulted in an inverted cell distribution when compared to the static cell culture. Strikingly, this results clearly demonstrated the potential use of the vertical turn to control the cell distribution in 3-D scaffolds, which can also be homogenized through the optimization of the time delay of the vertical turning movement. Moreover, cell adhesion time is affected by the variety of materials that may compose a scaffold in TE. These features provided by the developed dynamic platform, can contribute to optimize either laboratory or industrial processes using *in vitro* tissue maturation.

An overall strategy described in Chapter 8 combined the diverse features addressed in the different works showing that using the dual-chamber bioreactor a coordination between a gradient of GFs and HAp in the constructs lead to simultaneous but spatially controlled osteo- and chondrogenic differentiations. Moreover, the control of structures porosity was achieved keeping the microparticles gradient, which also influenced markers expression. An OC-like construct in which the expression of bone markers was significantly increased in the bone-like region, and an inverted gradient expression of osteogenic and chondrogenic markers was detected through the subsequent layers (interface and cartilage-like layer). Moreover, isotropic and anisotropic architectures were compared regarding bone and cartilage markers expression. A significantly increased expression of SOX-9, a chondrogenic marker, was observed in the isotropic structures when compared to the anisotropic one. Finally, pre-vasculature formation was controlled by flowing a pro-angiogenic medium in one region of the construct when co-culturing fat pad-ASCs with endothelial cells. The formation of major pre-vascular segments was significantly increased in the pro-angiogenic region of the construct in the dual-chamber. The developed study clearly demonstrated the potential use of the spatial control of biochemical gradients to simultaneously induce the co-differentiation of stem cells in different regions of a single 3-D structure. Moreover, the creation of a gradient with control over porosity is of major importance with possible application in materials engineering and RM, such as tissue *in vitro* modelling and drug discovery.

As final remark, the work developed in the scope of this PhD thesis has contributed to further unveil the great potential of materials engineering and cell culture technological solutions to control gradients and 3-D oriented porosity for the development of living tissues, such as the OC interface and anisotropic neural tissue. The developed work has also contributed with the design of advanced approaches for TE particularly because it combines different areas such as: the use of microfluidic and bioreactor devices, biomimetic structures made of natural origin biomaterials, and stem cells. Altogether, in the near future, these technologies will enable the application of substantial degrees of programmable relevant features in automated systems. Moreover, the close control over hierarchical tissues formation by TE approaches will in turn become much more reproducible and scalable. Additionally, the use of 3-D approaches will enable relevant solutions for *in vitro* models of human mimetic tissues, which are urgent in pharmaceutical industry for drug discovery. The materials engineered approaches together with the automated cell devices can potentiate the acceptance and integration of such strategies in the pharmaceutical industry as a solution to fill the existing the gap between the current cell culture paradigm and the clinical trials. Regarding clinical application, new solutions for integrated interfaced tissues and complex tissue patterns were provided, which can result in tissue grafts with improved functionality and regenerative potential.

9.2 Future perspectives

The work developed in the scope of this PhD thesis provides simple solutions to control complex patterns existing in nature, namely gradients, intricate architectures and arrangements of cells, which result in specified functions. First, the control over colloidal systems, as described in Chapter 4, provides a set of features to control the distribution patterns of particles in hydrogels, which can be programmed to target different tissues such as hard-to-soft interfaces. An interesting approach, with potential application for *in situ* drug delivery, could focus on the integration of GFs in one of the layers of the structure. So, as a future approach, a chondrogenic GF, such as TGF- β 3, could be integrated in the top layer of the construct for the enhancement of stem cell differentiation towards cartilage, while a gradient of HAp would be distributed throughout the layers for subchondral bone and calcified cartilage development. The efficacy of such strategy, *versus* a cell-based approach or even *versus* structures with distribution patterns of GFs or cytokines for OC regeneration would potentially allow to speed-up the regeneration of bone and cartilage interface, increasing the integration with host tissue and stratification of the bone-to-cartilage interface. Moreover, endothelial cells and pro-angiogenic GFs integrated in the bone-like layer could improve bone

angiogenesis, while anti-angiogenic GFs spatially distributed in the cartilage-like layer could keep the avascular characteristic of the hyaline cartilage.

Using fluid convection as a mechanism to pattern hydrogels can also be explored for the development of other *in vitro* models. Cells adhered to microparticles, or even cell spheroids, can be patterned in hydrogels applying these principles. To achieve such goal, lower concentrations polymeric concentrations have to be used to keep the solution in a liquid state at physiologic temperature, decreasing its sol-gel transition point. Based on this concept to control cell distribution, a combination of horizontal layers of neurons to mimic the neocortex architecture vertically crossed by linear porosity could be developed to mimic neocortex, which could potentially be used to study a neurodegenerative condition, such as Alzheimer's disease. So, a better understanding of the effect of the tissue architecture on the synaptic performance would be of great interest. When clarified, this knowledge can be further applied to create specific disease models of the neocortex.

Importantly, engineering vasculature could also profit from the tubular geometry of the pores of anisotropic structures. Endothelial capillaries can create a perfusable pre-vascular structure for implantation or to host surrounding *in vitro* tissues. If combined with the previously described approach, a vascularized neocortex mold could be simulated for drug testing.

Furthermore, several different approaches, which were not attempted in this PhD thesis, either for tissue graft development or *in vitro* tissue modelling, can be explored using the dual-chamber bioreactor. As an example, the establishment of an air-liquid interface for the maturation of skin epidermis, while the dermis part of substitute is perfused and nourished underneath certainly contribute to a faster and better maturation of the whole construct. Additionally, many other interfaced tissues can be matured by flowing two different media on top and bottom chambers, to mimic as close as possible the environment found in: tendon-to-bone interface, dermis-epidermis, blood-brain-barrier, lung or intestinal epitheliums, among others. Nevertheless, the same concept of chamber can be used to design a triple-chamber bioreactor for the creation of more complex tissues, such as bone-tendon-bone, hypodermis-dermis-epidermis, cartilage-calcified cartilage-subchondral bone.

Ultimately, two dual-chambers can also be connected, meaning that, the outlet of a first chamber can be linked to the inlet of a second chamber. The efficacy of a drug candidate in a disease model could be tested in the first chamber, while cytotoxicity assessment could precede in the second chamber, using for example a hepatic 3-D model. The same design could also be used to assess the ability of a drug candidate or any bioactive molecule to cross a tissue barrier. For example, the blood brain barrier could be tested in a first chamber, followed by its assessment on a tissue model cultured in a second chamber,

such as a brain-like model. Similarly, a cancer model could also be designed using interconnected chambers to test the formation of metastasis in a second tissue. Similar approaches can be developed in the microfluidic platform fabricated in Chapter 5, which allows reducing in size the scaffolds, and cells in number when compared to the bioreactor.

The combination of the dynamic platform with the dual-chambers, was useful to proof the concept of cell homogenization in 3-D scaffolds. However, this system could also be potentially useful to solve other problems such as the heterogeneous formation of organoids. Using the rotational movements, which can reduce the effect of gravity over cell culture, would potentially lead to a controlled formation of organoids and spheroid tissues. Moreover, varied horizontal rotation speeds and vertical turning times can be independently achieved which increase the versatility of the system and its utility to diversified application, such as constructs maturation, spheroids increased in size and homogenization, living explants extended viability, and stem cell differentiation potentiated by physical stimuli.

Generally, the strategies were proved, but their potential utility in a broader range of applications, as for example for *in vitro* disease and cosmetic models and graft development for *in vivo* implantation, still need more exploration. The applicability of this future work could proof the efficiency of the technological platform as a system for drug discovery and screening, as well as a potential industrial system for hierarchical tissue grafts development, which would enable the implementation of these technologies in the clinic and pharmaceutical industry.