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Current Advances in Solid Free-Form Techniques for Osteochondral Tissue Engineering

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Abstract:	Osteochondral (OC) lesions are characterized by defects in two different zones, the cartilage region and subchondral bone region. These lesions are frequently associated with mechanical instability, as well as osteoarthritic degenerative changes in the knee. The lack of spontaneous healing and the drawbacks of the current treatments has increased the attention from the scientific community to this issue. Different tissue engineering approaches have been attempted using different polymers and different scaffolds' processing. However, the current conventional techniques do not allow the full control over scaffold fabrication, and in this type of approaches, the tuning ability is the key to success in tissue regeneration. In this sense, the researchers have placed their efforts in the development of solid free-form (SFF) techniques. These techniques allow tuning different properties at the micro-macro scale, creating scaffolds with appropriate features for OC tissue engineering. In this review, it is discussed the current SFF techniques used in OC tissue engineering and presented their promising results and current challenges.
Corresponding Author:	Joaquim Miguel Oliveira, PhD "Universidade do Minho" Guimarães, PORTUGAL
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	"Universidade do Minho"
Corresponding Author's Secondary Institution:	
First Author:	João Bebiano Costa
First Author Secondary Information:	
Order of Authors:	João Bebiano Costa Joana Silva-Correia Rui Luís Reis Joaquim Miguel Oliveira, PhD
Order of Authors Secondary Information:	
Author Comments:	Dear Prof. Huayong Yang, Editor-in-Chief of Bio-Design and Manufacturing journal The 3B's Research Group - Biomaterials, Biodegradables and Biomimetics - develops its research activity in the interface between life sciences, materials science engineering, chemistry, and biotechnology. The multidisciplinary character of the work

	developed at the 3B's Research Group justifies the diverse range of scientific competences of its research team that includes researchers with engineering (materials, chemistry, polymer, textile, biological), as well biological (veterinary sciences, applied biology, biochemistry and chemistry) backgrounds. The advent of novel materials for successful tissue regeneration demands a hybrid research approach, combining high quality scientific activities in fields so diverse such as biotechnology, materials science and biology. In this regard, the 3B's Research Group aims to sustain its scientific growth by expanding and consolidating a multidisciplinary and highly competitive research team in the research domains of tissue engineering and regenerative medicine. The 3B's Research Group researchers were, for instance the pioneers on proposing starch based materials for applications related to bone orthopedics, such as bone replacement, bone cements, tissue engineering scaffolds, and carriers for control delivery of a range of bioactive agents. Moreover, the 3B's Research Group has a long experience in the development of scaffolds from natural origin biodegradable polymers using a wide range of non-conventional processing methodologies. The group is also involved in development of 3D in vitro models of disease for drug discovery and development, being well known internationally in the field of tissue engineering for its unique interdisciplinary research approach. As a result of our expertise, please find enclosed the review paper entitled "Current Advances in Solid Free-Form Techniques for Osteochondral Tissue Engineering" by authors J. B. Costa, J. Silva-Correia, R. L. Reis and J. M. Oliveira* to be considered for publication in Bio-Design and Manufacturing journal. Osteochondral (OC) lesions are characterized by defects in two different zones, the cartilage region and subchondral bone region. These lesions are frequently associated with mechanical instability, as well as osteoarthritic degenerative changes in the knee. The lack of spontaneous healing and the drawbacks of the current treatments has increased the attention from the scientific community to this issue. Different tissue engineering approaches have been attempted using different polymers and different scaffolds' processing. However, the current conventional techniques do not allow the full control over scaffold fabrication, and in this type of approaches, the tuning ability is the key to success in tissue regeneration. In this sense, the researchers have placed their efforts in the development of solid free-form (SFF) techniques. These techniques allow tuning different properties at the micro-macro scale, creating scaffolds with appropriate features for OC tissue engineering. In this review, it is discussed the current SFF techniques used in OC tissue engineering and presented their promising results and current challenges. The authors declare no competing financial interests in the publication of this work and that this is an original piece of writing, which has not been published or submitted for publication elsewhere. We therefore hope that this manuscript meets the quality to be published in the first issue of Bio-Design and Manufacturing journal. Yours sincerely, Joaquim Miguel Oliveira CORRESPONDING AUTHOR: Joaquim Miguel Oliveira Tel: +351 253 510931 Fax: +351 253 510909 Email: miguel.oliveira@i3bs.uminho.pt
Response to Reviewers:	Dear Prof. Huayong Yang Editor-in-Chief Journal of Bio-Design and Manufacturing The authors would like to inform you that the revised version of the manuscript “Current Advances in Solid Free-Form Techniques for Osteochondral Tissue Engineering” was submitted to the Journal of Journal of Bio-Design and Manufacturing. The changes made to the manuscript are highlighted by using red text color. The authors would also like to add that the manuscript has been completely gone over again to correct possible typographical, grammatical and bibliographical errors. Comments for BDMJ-D-18-00017

This review manuscript discussed the current SFF techniques used in OC tissue engineering and presented their promising results and current challenges. Three different SFF techniques, include stereolithography, fused deposition modeling and 3D Bioprinting, have been described and reviewed in the article. The article listed these technologies, and discussed their advantages and disadvantages. This article give an overview of the state of the art review for SFF techniques applications in OC tissue engineering. My recommendation is Minor revise. However, there are several issues that should be addressed before publication.
1.The title of the paper is too broad. Solid Free-Form techniques include much more techniques than the mentioned three techniques.

Answer: The authors understand the referee concern. However, the authors decided to address only these 3 techniques, considering that they are the most used in the OC field.

2.Generally speaking, Solid Free-Form techniques include other important techniques that are popularly used in bone tissue scaffold fabrication, e.g. Selective laser melting/sintering (SLM/SLS), electron beam melting (EBM), it is better to add this point in the manuscript.

- H Gong, K Rafi, H Gu, GDJ Ram, T Starr. et. al. Influence of defects on mechanical properties of Ti–6Al–4V components produced by selective laser melting and electron beam melting. Materials and Design, 2015, 86: 545-554.
- Boqing Zhang, Xuan Pei, Changchun Zhou. et.al. The biomimetic design and 3D printing of customized mechanical properties porous Ti6Al4V scaffold for load-bearing bone reconstruction, Materials and Design, 2018, 152, (15), 30-39.

Answer: The authors understand the referee observation and these two references were included in the manuscript. Also, the following text was included in the manuscript:

“Using the same principles, other laser-assisted techniques have been attempted in the OC field. Du et al. [21] have recently developed a new approach to produce bio-inspired multilayer osteochondral scaffold made of poly(ε-caprolactone) (PCL) and hydroxyapatite (HA)/PCL microspheres, through a selective laser sintering (SLS) technique. The results showed that the scaffolds revealed excellent in vitro biocompatibility, as well as great in vivo performance by inducing articular cartilage formation and subchondral bone regeneration in a rabbit model. In this sense, SLS can be a good alternative not only for OC tissue engineering, but also for the fabrication of bio-inspired multilayer scaffolds with well-designed architecture and gradient composition. Furthermore, a recent study performed by Fousová et al. [22] showed the comparison of other two laser-assisted techniques in the scaffold’s production. In this work, the authors compared the architecture and mechanical performance of solid free-form scaffolds composed by a Ti6Al4V alloy. The Ti6Al4V alloy is one of the most commonly used implant in orthopedic surgery and already showed promising results in terms of in vitro and in vivo performance [23]. The scaffolds were produced by selective laser melting (SLM) and electron beam melting (EBM). Interestingly, despite the results have revealed some similarities in terms of microstructure, due to differences in surface roughness and specific internal defects the fatigue strength of the EBM samples reached only half the value of the SLM samples. In short, this showed that the use of different solid free-form approaches could lead to different behaviors in terms of mechanical properties and architecture.”

[21] Y. Du, H. Liu, Q. Yang, S. Wang, J. Wang, J. Ma, I. Noh, A.G. Mikos, S. Zhang, Selective laser sintering scaffold with hierarchical architecture and gradient composition for osteochondral repair in rabbits, Biomaterials 137 (2017) 37-48.
[22] M. Fousova, D. Vojtech, K. Doubrava, M. Daniel, C.F. Lin, Influence of Inherent Surface and Internal Defects on Mechanical Properties of Additively Manufactured Ti6Al4V Alloy: Comparison between Selective Laser Melting and Electron Beam Melting, Materials (Basel, Switzerland) 11(4) (2018).
[23] B. Zhang, X. Pei, C. Zhou, Y. Fan, Q. Jiang, A. Ronca, U. D'Amora, Y. Chen, H. Li, Y. Sun, X. Zhang, The biomimetic design and 3D printing of customized mechanical properties porous Ti6Al4V scaffold for load-bearing bone reconstruction, Materials & Design 152 (2018) 30-39.

	3.In fact, the biomaterials for printing is extremely important, the material needs to be reviewed with the printing approaches. It is suggest to add a list of printed materials in Table 1. Answer: The authors understand the referee suggestion and a new column in Table 1 was included with some examples of the printed materials used in each solid free-from technique. 4.There is a lack of review in similar work conducted. How are the reviews from this study benchmark with the previous studies? Also, it is suggest to cite some new published article in Bio-design and manufacturing. - Xuan Pei, Liang Ma, Boqing Zhang. et.al. Creating hierarchical porosity hydroxyapatite scaffolds with osteoinduction by three-dimensional printing and microwave sintering, Biofabrication, 2017,9(4): 045008-045020 - Barba A, Diez-Escudero A, Maazouz Y, Rappe K, Espanol M, Montufar EB, et al. Osteoinduction by Foamed and 3D-Printed Calcium Phosphate Scaffolds: Effect of Nanostructure and Pore Architecture. Acs Appl Mater Inter. 2017;9(48):41722-36. Answer: The authors understand the referee observation. However, the suggested references are related only with bone regeneration. In this review, it is important to emphasize the works tackling osteochondral defects. In this sense, the authors believe that the references are not relevant for this review. 5.It is better to replace figure 4, the current figure did not provide any useful information. Answer: The authors understand the referee observation. However, the figure 4 shows a solid free-form approach with a printing step directly in the OC defect. In this sense, the authors believe that is important to show the possibility to create scaffolds by direct printing in the defect. In addition, this approach can be a very important step to tackle OC defects by using a single surgical procedure.

Current Advances in Solid Free-Form Techniques for Osteochondral Tissue Engineering

João Bebiano Costa[1,2,3], Joana Silva-Correia[1,2], Rui Luís Reis[1,2,3] and Joaquim Miguel Oliveira[1,2,3*]

[1]3B's Research Group, I3Bs – Research Institute on Biomaterials, Biodegradables and Biomimetics, University of Minho, Headquarters of the European Institute of Excellence on Tissue Engineering and Regenerative Medicine, AvePark, Parque de Ciência e Tecnologia, Zona Industrial da Gandra, 4805-017 Barco, Guimarães, Portugal;

[2]ICVS/3B's–PT Government Associate Laboratory, Braga/Guimarães, Portugal;

[3]The Discoveries Centre for Regenerative and Precision Medicine, Headquarters at University of Minho, Avepark, 4805-017 Barco, Guimarães, Portugal.

*e-mail: miguel.oliveira@i3bs.uminho.pt

Abstract

Osteochondral (OC) lesions are characterized by defects in two different zones, the cartilage region and subchondral bone region. These lesions are frequently associated with mechanical instability, as well as osteoarthritic degenerative changes in the knee. The lack of spontaneous healing and the drawbacks of the current treatments has increased the attention from the scientific community to this issue. Different tissue engineering approaches have been attempted using different polymers and different scaffolds' processing. However, the current conventional techniques do not allow the full control over scaffold fabrication, and in this type of approaches, the tuning ability is the key to success in tissue regeneration. In this sense, the researchers have placed their efforts in the development of solid free-form (SFF) techniques. These techniques allow tuning different properties at the micro-macro scale, creating scaffolds with appropriate features for OC

tissue engineering. In this review, it is discussed the current SFF techniques used in OC tissue engineering and presented their promising results and current challenges.

Keywords: Solid free-form, Osteochondral, Tissue Engineering, Scaffolds

1. Introduction

Osteochondral (OC) tissue engineering requires unique scaffolds with specific properties, which ideally promote individual growth of both cartilage and bone layers [1]. The OC defects are characterized by an injury in the cartilaginous region, as well as in the underlying subchondral bone, and are frequently related with mechanical instability of the joint. The lack of spontaneous healing and the associated osteoarthritic degenerative changes are leading to an increase awareness from the orthopedic field [2]. Therefore, in an ideal situation, OC repair strategies should: 1) comprise a substitute that is easy and quick to implant; 2) reduce surgical morbidity; 3) not require harvesting of other tissues (e.g. periosteum); and 4) allow an efficient and complete integration of the implant [2]. However, a complex structure that comprises a cartilage-bone interface requires a tissue engineering approach where implants are able to mimic the chondrogenic and osteogenic environment simultaneously. In other words, there must be a compromise between the temporary mechanical function provided and the architectural properties (i.e. pore shape, size, and interconnectivity) in order to pursue a better biological environment and tissue regeneration [3]. A paradigm shift is taking place in the field of orthopedic surgery, with the introduction of the use of synthetic or natural implants [4]. Despite being weaker and softer materials, natural polymers have the advantage of being flexible, thus presenting the capability to adapt their shape to the required forms. In addition, natural materials usually contain specific molecular domains that can support and guide cells, enhancing the biological interaction between the scaffold and the host tissue [1]. As example, Oliveira *et al.* [5] developed a hydroxyapatite/chitosan (HA/CS) bilayered scaffold by combining a sintering with a freeze-drying technique. Two distinct layers were obtained, a porous HA layer and a CS layer corresponding to bone and cartilage zones, respectively. The scaffolds were shown to present adequate porosities and mechanical properties. It was also shown that both layers provided support for cell attachment, proliferation and differentiation into osteoblasts and chondrocytes, respectively. Moreover, since collagen is the major component in the extra-cellular matrix, collagen-based scaffolds have been shown promising results in OC tissue engineering approaches. Levingstone *et al.* [6] fabricated a collagen layered structure using a novel “iterative layering” freeze-drying technique that allowed to control material composition, pore size and substrate

stiffness in each region of the construct. In the end, the authors obtained a gradient structure composed by a bone layer made of type I collagen and HA, an intermediate layer made of type I collagen, type II collagen and HA and a cartilaginous region made of type I collagen, type II collagen and hyaluronic acid. The scaffolds revealed an optimized environment for cell attachment and proliferation. In another study, Zhou *et al.* [7] developed also a collagen-based layered scaffold composed by a collagen and a collagen/HA part to mimic the cartilage and bone regions, respectively. Human mesenchymal stem cells were used to promote chondrogenic and osteogenic differentiation. The results showed that the collagen layer was more efficient at inducing chondrogenic differentiation, while the collagen/HA layer was superior in the promotion of osteogenic differentiation.

Unlike natural polymers, the synthetic polymers offer a wide range of chemistry and processing options and their production can be scaled up to industrial-scale manufacturing processing, which is a requirement for future clinical applications [1]. However, in general, they have limitations in terms of biocompatibility and bioactivity. As well as in natural polymers, synthetic polymers have been used in combination with HA and ceramics. Huang *et al*. [8] developed a novel amorphous calcium phosphate (ACP)/poly(L-lactic acid) (PLLA) material incorporating basic fibroblast growth factor (b-FGF) that showed good cartilage integration after 12 weeks implantation in a rabbit model. In another study, Jiang *et al.* [9] implanted a biphasic poly (DL-lactic-co-glycolide)/calcium phosphate construct into mini-pigs for 6 months. Despite the poor integration with the surrounding cartilage, histology revealed good bone integration and a tidemark was noted between cartilage and bone.

Nevertheless, despite conventional techniques (i.e. solvent casting, phase separation, electro-spinning, salt-leaching, freeze-drying) have some capacity to tune the scaffolds pore size and porosity, they will never be able to completely control the morphology and architecture of scaffolds in terms of pore size, geometry, interconnectivity and spatial distribution. As alternative, several researchers have recently changed their attention to solid free-form (SFF) technologies. Commonly known as SFF techniques, rapid prototyping (RP) or additive manufacturing (AM), rely on the use of computer-aided design (CAD) to build structures by selectively adding materials layer-by-layer [10]. Furthermore, medical scans such as,

Computerized Tomography (CT) or Magnetic Resonance Imaging (MRI,) can be used to create a personalized CAD model to produce patient-specific implants [11]. In other words, SFF techniques can be a huge help in OC tissue engineering, because allow the fine tuning of different materials' properties at the micro and macro levels, creating scaffolds with specific mechanical properties and with an appropriate biological environment for bone and chondral tissue differentiation [12]. For these reasons, and combining all these advantages with the high degree of reproducibility and homogeneity, SFF technique is considered the current "golden strategy" for the generation of scaffolds presenting significant benefits over conventional porous scaffold production technologies [13].

The schematics of solid free-form (SFF) techniques used in osteochondral (OC) tissue engineering is depicted in Figure 1.

The overview of the significant reports on SFF technologies in OC tissue engineering approaches using different methods for scaffolding fabrication is presented herein.

2. SFF techniques used in OC scaffold fabrication

2.1 Stereolithography

Considered a pioneer technique in SSF, stereolithography (SL) is a laser-based approach that follows basic principles. An ultraviolet (UV) laser irradiates the top of a bath composed by a photo-polymerizable liquid polymer material. As polymerization starts, the laser creates a solid layer by tracing the laser beam along the model boundaries and internal structure leading to the formation of a cross-sectional structure (layer). This polymerization process is repeated, creating overlapped layers that, after successive stacking, lead to the formation of the 3D construct. In the end, the platform is raised, and the excess of resin is drained. The resolution of this technique is not impressive (80-250 mm) and is dependent on the elevator layer resolution and laser spot size [14]. To overcome SL low-resolution values, micro-stereolithography (MSL) was developed to provide higher precision. This technique, based on the same principles of SL, has the capability to offer resolutions around 1-2 μm due to the presence of a focusing lens.

The performance of both techniques is highly dependent on the photo-polymerization reaction. In this sense, there is a limited choice in terms of biomaterials with good photo-polymerization capacity and with the adequate properties (e.g. biocompatibility, biodegradability, and mechanical properties) for tissue engineering applications. Many researchers opted to synthesize or modify existent polymers such as polypropylene fumarate, polyethylene glycol (PEG), polyvinyl alcohol (PVA) or polycaprolactone (PCL), in order to create biodegradable polymers [15]. Recently, Bian *et al.* [16] designed β-tricalcium phosphate (TCP)/collagen scaffolds for OC tissue engineering. In this work, the authors used SL to build a ceramic scaffold, where they subsequently added the cartilage zone (collagen) by gel casting. The final osteochondral scaffold presented fully interconnected pores (700–900 μm) and supported cell adhesion and proliferation up to 7 days of culturing. Later, Bian *et al.* [17] used histology, micro-computed tomography (micro-CT) and scanning electron microscopy (SEM) to investigate the microstructure of the cartilage-bone transitional structures in order to improve the biomimetic design of the OC scaffold. A new CAD model was developed, allow discovering that the subchondral bone plate is not an intact plate and the presence of some scattered defects allow the blood vessel invasion and nutritional supply.

In another study, Zhang *et al.* [18] fabricated PEG/β-TCP OC scaffolds using, as the in previous work, SL and gel casting. However, unlike the previous approach, the authors produced a β-TCP ceramic scaffold using the gel casting process, while for the chondral zone it was used SL. The PEG hydrogel was directly cured on the ceramic scaffolds giving origin to a bilayer OC scaffold. The scaffolds were implanted in rabbit trochlea model within a critical size defect. The animals were euthanized at 1, 2, 4, 8, 16, 24, and 52 weeks after implantation. This work revealed good outcomes and the authors concluded that subchondral bone migration is related with cartilage regeneration in critical size osteochondral defects. In a different and more advanced approach, Castro *et al.* [19] developed two biologically-inspired nanomaterials: (1) osteoconductive nanocrystalline hydroxyapatite (nHA) (primary inorganic component of bone) and (2) core-shell poly(lactic-co-glycolic) acid (PLGA) nanospheres encapsulated with the transforming growth-factor β1 (TGF-β1). The authors used a novel table-top SL 3D printer to fabricate a hierarchical scaffold with the aim to provide biological cues at nano- and micro-scales (Figure 2A). In the end, the

scaffolds were able to mimic the native tissue supporting cell adhesion, proliferation and osteochondral differentiation (Figure 2B).

However, the limited number of resins available for stereolithography applications, is one of the main drawbacks in this type of approach. Ronca *et al.* [20] developed an acrylic photocrosslinkable resin based on methyl methacrylate (MMA), butyl methacrylate (BMA) and poly(ethylene glycol) dimethacrylate (PEGDA) with different compositions. The resins were further characterized in terms of mechanical, thermal and biological behavior. The crosslinked materials revealed good mechanical properties and thermal stabilities; moreover, cytotoxicity tests confirmed their biocompatibility with no cytotoxic effect on cells metabolism. In addition, two different treatments have been proposed, using fetal bovine serum (FBS) and methanol (MeOH). The results showed that the samples threated with MeOH allowed cell adhesion and survival, promoting spreading, elongation and fusion of the cells.

Stereolithography, as a SFF technique, enables the production of personalized OC scaffolds controlling the morphology and architecture of the structures. Despite the promising outcomes obtained through *in vitro* analysis and *in vivo* animal studies, very few scaffolds fabricated by SL have been evaluated in clinical trials.

Using the same principles, other laser-assisted techniques have been attempted in the OC field. Du et al. [21] have recently developed a new approach to produce bio-inspired multilayer osteochondral scaffold made of poly(ε-caprolactone) (PCL) and hydroxyapatite (HA)/PCL microspheres, through a selective laser sintering (SLS) technique. The results showed that the scaffolds revealed excellent *in vitro* biocompatibility, as well as great *in vivo* performance by inducing articular cartilage formation and subchondral bone regeneration in a rabbit model. In this sense, SLS can be a good alternative not only for OC tissue engineering, but also for the fabrication of bio-inspired multilayer scaffolds with well-designed architecture and gradient composition. Furthermore, a recent study performed by Fousová et al. [22] showed the comparison of other two laser-assisted techniques in the scaffold's production. In this work, the authors compared the architecture and mechanical performance of solid free-form scaffolds composed by a Ti6Al4V alloy. The Ti6Al4V alloy is one of the most commonly used implant in orthopedic surgery and already showed promising results in terms

of *in vitro* and *in vivo* performance [23]. The scaffolds were produced by selective laser melting (SLM) and electron beam melting (EBM). Interestingly, despite the results have revealed some similarities in terms of microstructure, due to differences in surface roughness and specific internal defects the fatigue strength of the EBM samples reached only half the value of the SLM samples. In short, this showed that the use of different solid free-form approaches could lead to different behaviors in terms of mechanical properties and architecture.

2.2 Fused deposition modeling

The Fused Deposition Modeling (FDM) is one of the most famous and traditional SFF techniques. Briefly, a head-heated liquefier cartridge melts the filament and pushing it through a nozzle directly on the build platform. The melted thin filament is guided using a carriage that moves in the horizontal x,y plane and builds a layer-by-layer 3D construct. Once a layer is assembled, the build platform moves down in the z direction in a distance correspondent to the layer thickness and starts to deposit the next layer. This method does not require the use of harsh solvents; however, the polymers used in FDM techniques are restricted to thermoplastic materials, disabling cell encapsulation into the constructs during the fabrication process [10].

PCL is a thermoplastic that has been constantly used in OC tissue engineering. However, although PCL can generate mechanically stable constructs, the lack of osteoconductive factors such as TCP or HA, has led to its combination with ceramics. In 2003, Endres *et al.* [24] assessed the osteogenic potential of human adipose stem cells in PCL-HA constructs. In that work, the authors reported encouraging results showing cell proliferation toward and onto the PCL-HA scaffolds surfaces. Heo *et al.* [25] developed nano- and micro-sized HA-PCL composite three-dimensional scaffolds with potential for bone tissue engineering applications. These potential was proved later in an *in vivo* study, since 8 weeks after implantation in a rabbit tibial segmental defect model, dense bone formation was observed throughout the constructs [26]. Swieszkowski *et al.* [27] developed a biphasic OC scaffold by FDM. The scaffolds were composed of a PCL-TCP phase for the bone region, and a PCL-Fibrin phase for the cartilage region. Bone marrow-derived mesenchymal cells were isolated and seeded into the scaffolds that were subsequently implanted in medial condyle critical size defects of the rabbit model. Micro-CT analysis

revealed significant regeneration in the bone phase. Moreover, the fast degradability of the fibrin restrained the cartilage healing in the PCL-Fibrin region. More recently, Ding *et al.* [28] developed also a biphasic scaffold, which comprised a cartilage region made of polylactic acid-coated polyglycolic acid (PGA/PLA) and a bone region made of PCL/HA. As usual in SFF approaches, CAD technology helped the authors to produce a scaffold suitable for the regeneration of goat femoral head. Chondrocytes and bone marrow stromal cells (BMSCs) were seeded into the scaffolds for cartilage and bone regeneration, respectively, and subsequently implanted subcutaneously in nude mice. After 10 weeks, the regenerated femoral heads presented smooth, continuous and homogeneous articular cartilage layer and a good subchondral bone integration. Recently, Holmes *et al.* [29] created novel osteochondral scaffolds with both excellent interfacial mechanical properties and biocompatibility for facilitating human bone marrow mesenchymal stem cell (MSC) growth and chondrogenic differentiation. In this sense, the authors designed and printed a series of innovative bi-phasic 3D models that mimic the osteochondral region of articulate joints. The mechanical testing results showed suitable mechanical properties under compression (a maximum Young's modulus of 31 MPa) and shear (a maximum fracture strength of 5768 N/mm^2) when compared with homogenous designs. In addition, in order to improve their biocompatibility, the authors modified the surface of the scaffolds with acetylated collagen. The biological assays revealed that the surface modification enhanced MSC proliferation up to 5 days of *in vitro* culturing. A 2-weeks' chondrogenic differentiation was also performed with the cells presenting good indication of chrondrogenic differentiation. Santis *et al.* [30] used two solid free-form technologies to fabricate PCL- and PEG-based magnetic nanocomposite scaffolds. These scaffolds were fabricated using fused deposition modeling and stereolithography approaches in order to produce a hybrid scaffold. The viscoelastic properties under compression were investigated at 37°C, spanning a range frequency of four decades. The results suggested that hybrid scaffolds adequately reproduce viscoelastic properties of subchondral bone and articular cartilage tissues, respectively. By means of combining FDM and SL it was possible to produce a hybrid scaffold suitable for osteochondral tissue regeneration.

Using different polymers, Woodfield *et al.* [31] fabricated porous scaffolds from a poly(ethylene glycol)-terephthalate poly(butylene terephthalate)

(PEGT/PBT) block co-polymer. The influence of different PEGT/PBT compositions and pore geometries in the scaffolds' mechanical behavior was tested, as well as the scaffolds capability to support cell adhesion and proliferation. The scaffolds revealed good biological performance showing its ability to support cell proliferation and matrix synthesis. Later, the same group also revealed better biological results when compared FDM produced scaffolds with more traditional particulate leached scaffolds [32]. The author's hypothesized that the superior nutrient and oxygen diffusion caused by the orientated pores of the FDM scaffold improved cell viability in the central region of the scaffolds. The effectiveness of porous polyethylene-oxide-terephthalate/polybutylene-terephthalate (PEOT/PBT) scaffolds (Figure 3A) seeded with MSC was also evaluated in an osteochondral defect using a rabbit model [33]. Regarding chondrogenesis, the results showed evidence of GAG accumulation in the empty defect and around the scaffolds struts of the cell-free scaffold (Fig. 3B-D). Chondrocyte cells were observed in their lacunae above the tidemark in the cell-seeded scaffolds (Fig. 3Dii–iii). There was evidence of hypocellularity in the cell-free scaffolds (Fig. 3Ciii–iv). In addition, chondrocyte clusters were observed in the cell-seeded constructs (Fig. 3Div) and the adjacent cartilage at the margin of the defect in the empty defects (Fig. 3Biii–iv). Succinctly, the FDM scaffolds provided both biological cues and mechanical support and enabled to obtain enhanced hyaline-like tissue repair.

Recently, the same group developed advanced strategies to better mimic the native tissue. Different pore size gradients revealed stimulation of different cell behaviors. In this sense, the authors showed the capability to tune the scaffolds' architecture using FDM to achieve an improved induction of mesenchymal stem cells chondrogenesis and osteogenesis [34,35].

Although FDM technique already showed promising results, there is still the need to see if the *in vitro* improvements will be translated into enhanced OC regeneration *in vivo*.

2.3 3D Bioprinting

In the previous two SFF techniques, the control of cell and growth factors distribution inside the scaffold was not possible, which limits the provision

of an ideal microenvironment for cell migration and differentiation. In this sense, new strategies have been developed, namely approaches based on the 3D Bioprinting. This SFF technique differs from the others due to the capability to produce cell-leaden scaffolds with specific cell types, cell densities or with a specific growth factor. In other words, besides the possibility to tune the architecture and morphology of the scaffolds, it is possible to mimic as much as possible the anatomical cell arrangement of the native tissues enabling optimal conditions for the regeneration of specific tissues.

Fedorovich *et al.* [36] developed a 3D fiber deposition (3DF) technique for the fabrication of heterogeneous hydrogel constructs. This novel technique allowed the control of fiber spacing and deposition angle, as well as the capability to dispense cells. The cell-leaden scaffolds were composed by two sections: 1) alginate with encapsulated chondrocytes and 2) alginate supplemented with biphasic calcium phosphate and HA with encapsulated bone marrow stem cells. The authors confirmed heterogeneous tissue formation, but, as expected, the use of alginate will not confer sufficient mechanical strength in future OC applications. Shim *et al.* [37] developed a multi-head tissue/organ building system (MtoBS) capable of dispensing a wide range of relevant biomaterials to produce 3D tissues or organs. That complex system was composed by six nozzles: two nozzles were used to dispense molten PCL, while four nozzles dispensed a liquid alginate hydrogel encapsulated with human osteoblast-derived cells or chondrocytes derived from human nasal septum. Therefore, PCL was used to enhance the mechanical properties of the constructs and the cell-leaden hydrogel was to confer biological cues to induce the regeneration process. The *in vitro* biological assays revealed cell viability maintenance of printed cells up to 7 days.

In another study, Cui *et al.* [38] combined a solution of poly(ethylene glycol) dimethacrylate (PEGDMA) with human chondrocytes to print scaffolds for osteochondral defects. This technique is based on a photo-polymerization reaction, where a photolytic cross-linker was used to form a hybrid cell-containing hydrogel. The cell-leaden structure revealed good integration with the surrounding tissue and the capability to maintain cell phenotype. Recently, cell-leaden osteochondral constructs composed of gelatin methacrylated hydrogel were fabricated. In this approach, scaffolds with high cell density and viability were achieved by the addition

of mesenchymal stromal cells encapsulated in polylactic acid microcarriers. Additionally, the microcarrier encapsulation increased the stiffness of the printed constructs, as well as the cell adhesion and osteogenic differentiation. In a different approach, Cohen *et al.* [39] have developed an approach for *in situ* fabrication of an alginate scaffold. This novel strategy requires an adaptive system capable of performing real-time imaging, registration and path planning in order to directly print the material in the defect. In this study, the alginate cross-linking with calcium sulfate was initiated inside the printing cartridge and subsequently printed with the specific size and shape of defects formed in an *ex vivo* bovine femoral condyle. In a similar approach, Li *et al.* [40] applied 3D scanning and bioprinting for repairing osteochondral defects (Figure 4). In that work, two different photopolymerized hydrogels were used as bioinks to fully restore the osteochondral defects. As well as in the previous study, the results suggested that 3D scanning and 3D bioprinting could provide a useful strategy for osteochondral regeneration. *In situ* SFF techniques have great potential for clinical applications. However, there are considerable challenges that need to be addressed regarding the material processing, printing resolution and printing conditions.

Despite the challenges that still exist in SFF technique, 3D bioprinting can be considered a powerful tool for the development of cell-laden tissue constructs with suitable characteristics for OC tissue engineering.

In this review, three different SFF techniques (i.e. stereolithography, fused deposition modeling and 3D Bioprinting) that have been widely used in OC tissue engineering applications are discussed. In our opinion, it cannot be said that there is a better or a worse technique, but we can mention that each one of the three SFF techniques present some advantages and limitations (Table 1), and it is thus important to choose the most adequate technique for the envisioned application, which will also depend on the type of processing and material that will be used.

3. Conclusions

The SFF approaches have been revolutionizing scaffolds fabrication techniques in the OC tissue engineering field. In this review, three different SFF techniques have been described showing the most promising features for future applications, being one of these features the capability offered in

terms of design control. Pore architecture, pore geometry, total porosity and cell density are some of the aspects that can be tuned using these SFF approaches. Probably, the 3D Bioprinting is the most promising technique among the three, due to the possibility to control the cell spatial distribution in order to produce more complex constructs. Concerning the polymers used, over the last few years, a wide group of materials has been investigated, with the natural materials taking advantage over the synthetic materials due to their biocompatibility. Despite the important advances in this field, further investigation concerning material processing, printing resolution and polymers biocompatibility is necessary to translate the in vitro validated results to the clinics.

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Conflict of Interest

The authors declare no conflict of interest.

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Figure Legends

Figure 1 - Schematic diagram of solid free-form (SFF) techniques used in osteochondral (OC) tissue engineering.

Figure 2 – A) (i–iii) 3D CAD model (bottom, top and side view) of the three-layer osteochondral scaffold design with 60% in-fill density. SEM images of (iv–vi) control scaffolds without nHA (bottom and top images); and (vii–ix) osteochondral scaffolds with graded nHA (vi is the bottom, vii is the top; viii is 10% nHA layer and ix is 20% nHA layer). B) Three- (i,ii,iii,iv) and five-day (v,vi) hMSC spreading morphology on 3D printed scaffolds containing spatially distributed nHA (graded) when compared to controls. After three days of culture, hMSCs display excellent spreading when compared to the spherical morphology of hMSCs seeded upon control scaffolds. In addition, increased cell growth density is observed through DAPI staining of cell nuclei. Scale bars: A) 100 µm (iv,v,vii,viii), 2 µm (vi and ix). B) 2 mm (i,ii), 100 µm (iii-vi). Reprinted with permission from reference [19].

Figure 3 - A) SEM images showing the 3D scaffold architecture from (i) top view and (ii) cross-section. Representative images showing toluidine blue staining for chondrogenesis and GAG accumulation in (B) an empty defect; (C) a cell-free PEOT/PBT scaffold, and (D) a rabbit MSC-seeded scaffold, with insets taken at higher magnifications of ×4 and 10× to show tissue repair at the edge and the center of the defects as highlighted by dotted black boxes. Dotted red box shows original defect site areas. Reprinted with permission from reference [33].

*Figure 4 - Process of 3D bioprinting and photopolymerization on osteochondral defect. Photopolymerization was taken at the end of printing. (**A**) Repair of osteochondral defect through in situ 3D bioprinting with alginate hydrogel. (**B**) Exposure to UV light. (**C**) Alginate hydrogel that was printed to repair the osteochondral defect was transparent before photopolymerization. (**D**–**F**) The color of alginate hydrogel turned milky white after being exposing to UV light in few seconds. Reprinted with permission from reference [40].*

Tables

Table 1 – Advantages and limitations of SFF thechniques and examples of materials used in these thechniques.

	Advantages	**Limitations**	**Materials**
Stereolithography	• High detailed constructs • Good surface finish	• Requires post-curing • Possibility of shrinkage and curl • Limited biomaterials (Photo polymers) • The need of support • In some cases, the difficulty of removing the support structures	• Modified polypropylene fumarate • Modified polyethylene glycol (PEG) • Modified polyvinyl alcohol (PVA) • Modified polycaprolactone (PCL) • Methyl methacrylate (MMA) • Butyl methacrylate (BMA)
Fused deposition modeling	• No post curing • Variety of biomaterials • Easy to change the biomaterial • Economic	• Low detailed constructs • Surface finish • Support structures are needed depending on the model design • In some cases, the difficulty of removing the support structures	• Polycaprolactone (PCL) • Polyethylene glycol (PEG) • Polylactic acid (PLA) • Polyglycolic acid (PGA) • Poly(ethylene glycol)-terephthalate Poly(butylene terephthalate) (PEGT/PBT) • Polyethylene-oxide-terephthalate/Polybutylene-terephthalate (PEOT/PBT)
3D Bioprinting	• Controllable cell spatial distribution	• Low detailed constructs • Expensive	• Polycaprolactone (PCL) • Polyethylene glycol (PEG) • Gelatin

	• Better biological properties • Variety of biomaterials	• Sterilization of the printing environment • Ethical issues	• Alginate

Figure 1

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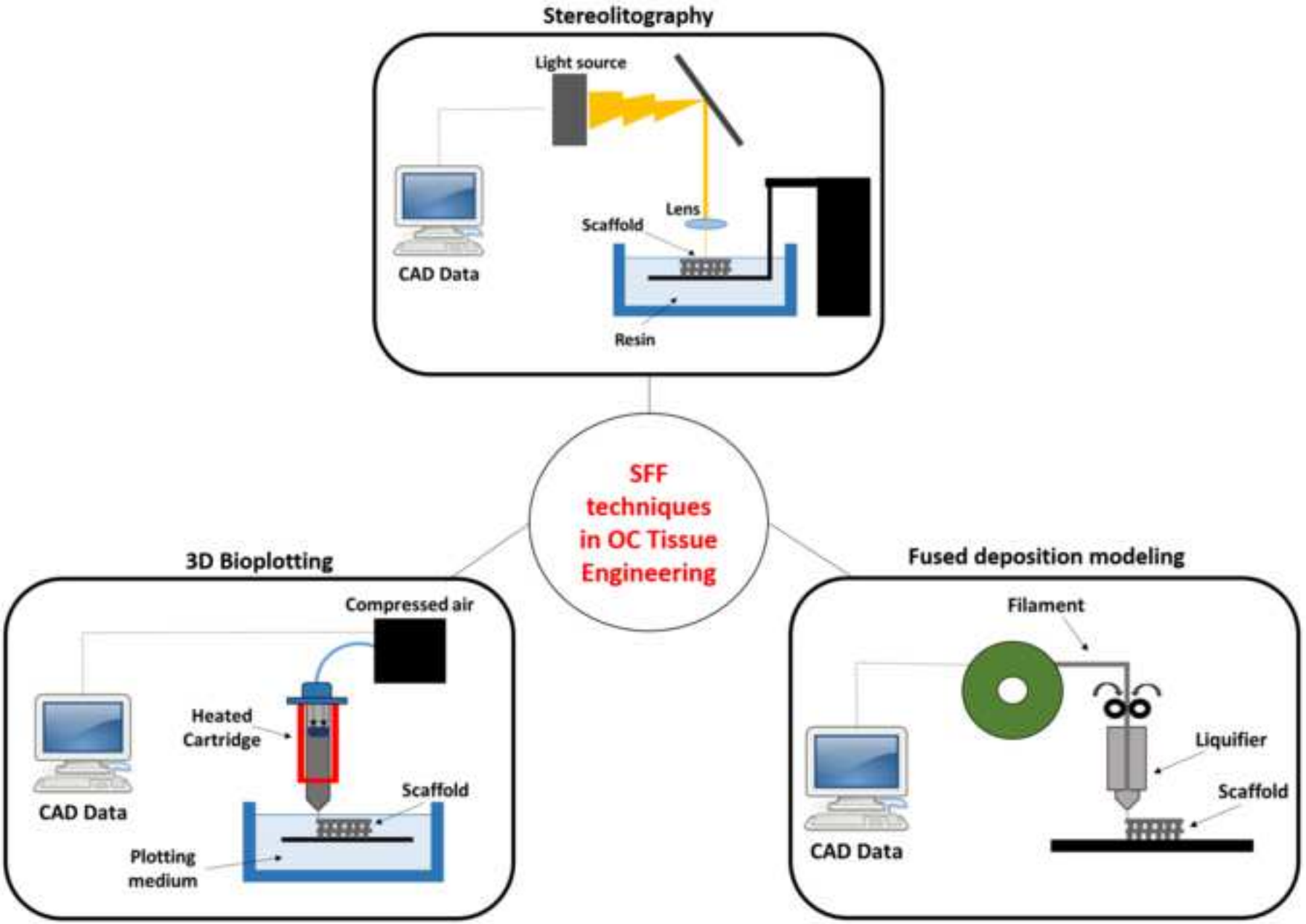

Figure 2

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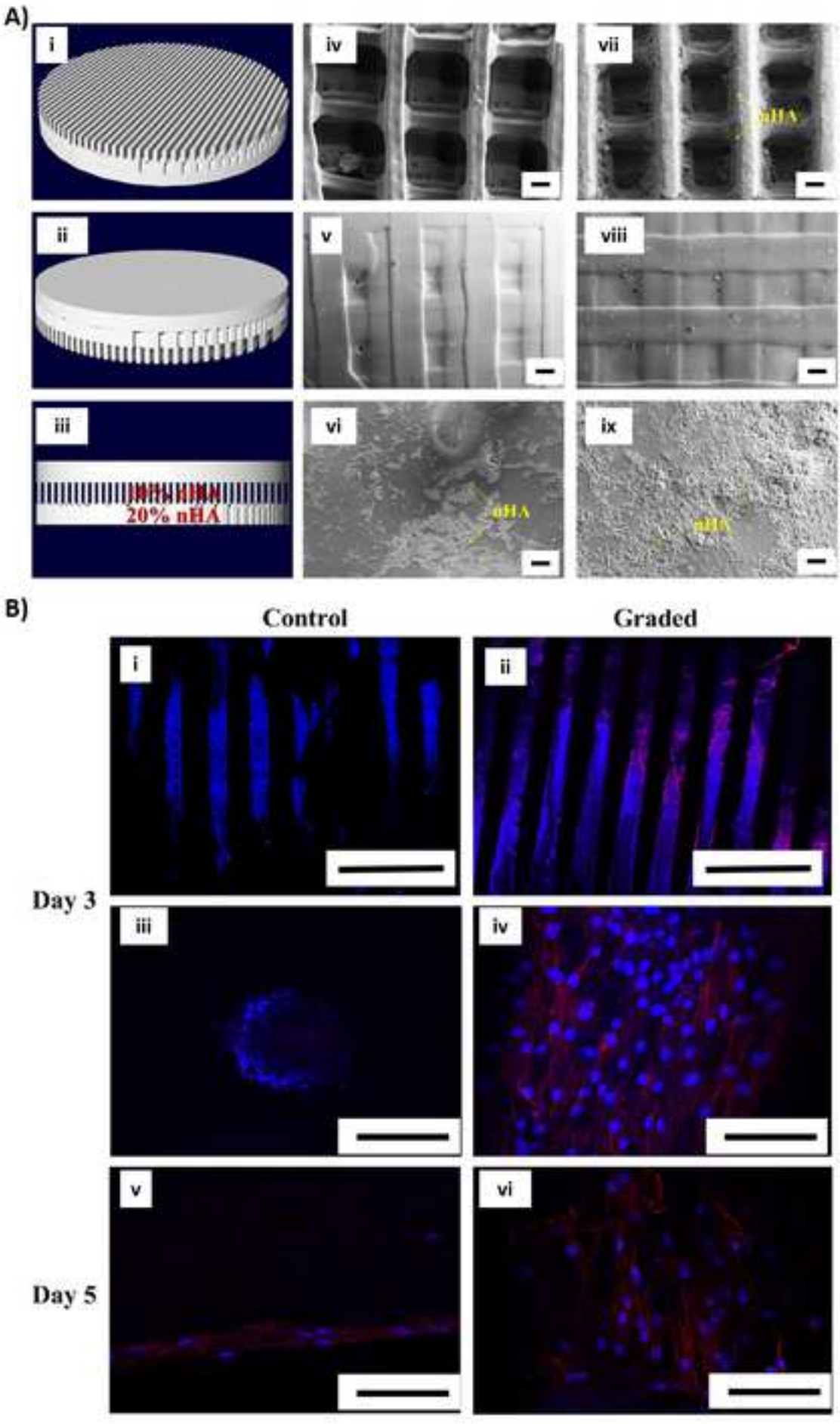

Figure 3

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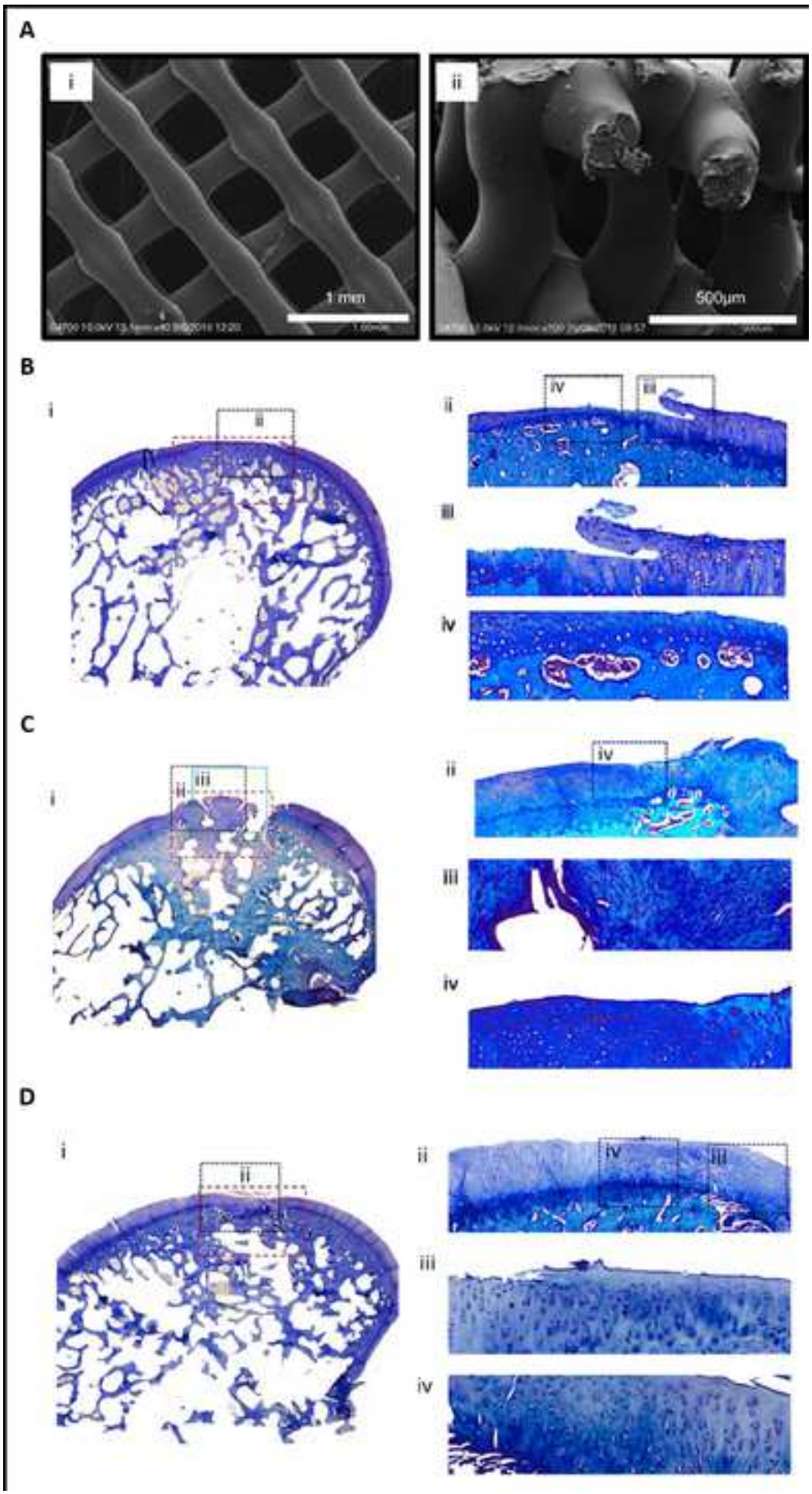

Figure 4

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