

Reviewing recently developed technologies to direct cell activity through the control of pore size: From the macro- to the nanoscale

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1        **Reviewing recently developed technologies to direct cell activity**  
2        **through the control of pore size: from the macro- to the nanoscale**

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13  
14        **Abstract**

15        Scaffold pore size plays a fundamental role in the regeneration of new tissue since it has been  
16        shown to direct cell activity *in situ*. It is well known that cellular response changes in relation with  
17        pores diameter. Consequently, researchers developed efficient approaches to realize scaffolds with  
18        controllable macro-, micro- and nanoporous architecture. In this context, new strategies aiming at  
19        the manufacturing of scaffolds with multiscale pore networks have emerged, in the attempt to  
20        mimic the complex hierarchical structures found in living systems. In this review we aim at  
21        providing an overview of the fabrication methods currently adopted to realize scaffolds with  
22        controlled, multisized pores highlighting their specific influence on cellular activity.

23  
24        **Keywords**

25        Scaffold pore size, cell activity, hierarchical structure, multiscale pore architecture, cellular  
26        response.

## 27 **1. Introduction**

28 In tissue engineering, cell fate can be modulated through several methods aiming at directing cell  
29 response to achieve the formation of a healthy new tissue. The composition, morphology and  
30 surface topography of scaffolds can furnish the right cues to guide cells in the generation of the  
31 newly developed tissue. Among others, pore size plays a fundamental role in defining topological  
32 features which contribute to obtain a functional interface between cells and material [1, 2]. Tuning  
33 scaffold pore size can serve to mediate cellular response *in situ* (similar to surface  
34 functionalization) through the tailoring of cell cytoskeleton arrangement. Cell membrane receptors  
35 interact with the multiscale topographical features of the scaffold inducing cytoskeleton  
36 deformation and assembly with a direct effect on cell functionalities (adhesion, proliferation, gene  
37 expression) and morphologies [3]. Hence, scaffold pores with controllable diameters over multiple  
38 length scales were developed to mimic complex living hierarchical structures. For instance, several  
39 studies investigated human bone topological features to resemble its unique hierarchical structure  
40 at different scales [4, 5]. Pore size affects the response of the hosting cells in a different way [1]:  
41 nanopores (< 300 nm in size) [6, 7] promotes cell adhesion increasing the surface area, micropores  
42 (0.3-100  $\mu\text{m}$  in size) [6, 8] enhance the permeability of the scaffold and facilitate cell migration  
43 while macropores (>100  $\mu\text{m}$  in size) [9, 10, 11] provide space for vascularization and tissue  
44 ingrowth, favor gas diffusion, nutrients supply and waste removal (Figure 1). The effect of pore  
45 size on cell activity has been extensively investigated (Table 1), as it represents an efficient mean  
46 to modify the tissue response *in vivo* by acting on geometrical features instead of compositional  
47 cues [12] [11]. However, gaining a full picture of how different cell types react to constructs with  
48 certain pore size is still a challenge for tissue engineering, as well as applying available  
49 technologies to produce scaffolds with hierarchical porous structure and high pore

50 interconnectivity [13]. Several techniques have been adopted to optimize the manufacturing and  
51 modelling of structures with controlled, engineered pore size across a variety of length scales.  
52 They include conventional fabrication techniques such as salt leaching, gas foaming, phase  
53 separation, freeze-drying, freeze-casting, solid-state porogen thermal decomposition, cell  
54 encapsulation, electrospinning [14, 15, 1]. Nonetheless, these traditional methods do not allow to  
55 obtain a precise control of scaffold architecture and to achieve reproducible size and shape of pores  
56 [16, 17]. Additive manufacturing (AM) techniques using computer-aided design (CAD) modeling  
57 introduced remarkable improvements in terms of repeatability and accuracy on scaffold micro-  
58 and macrotopography. Despite that, a strict control of nanopores is difficult to achieve with AM  
59 technologies. So far, stereolithography, selective laser sintering, selective laser melting, electron  
60 beam melting, 3D-bioprinting, direct laser writing, fused deposition modeling are the more  
61 common AM technologies applied in tissue engineering [18, 19]. Furthermore, the use of AM  
62 technologies allows to control not only the pore size but even the pore geometry which has been  
63 recently reported to be an effect on cell response [10, 20].

64 In this review we analyze recent papers published in the last five years, in which the specific effect  
65 of pore size on cell activity has been investigated. The influence of nano- micro- and macropores  
66 size on tissue response are illustrated in the following sections, together with the manufacturing  
67 techniques used (Table 1). Finally, we also report current examples of novel approaches applied  
68 to achieve hierarchical structures with multiscale pore architecture.

69

## 70 **2. Nanopores**

71 The scaffold nanofeatures are largely studied since cell-cell and cell-substrate interactions  
72 occurred at the nanoscale. Briefly, when cells interact with a substrate, they explore the

73 environment by expanding lamellipodia and filopodia [21]. When the substrate is suitable for the  
74 attachment, cells develop focal adhesions (FAs) that successively elongate and generate mature  
75 adhesions known as fibrillar adhesions (FBs) [22]. The evaluation and characterization of FAs and  
76 FBs allow analyzing the influence of nanotopography on cellular activity. Many approaches have  
77 been used to fabricate nanotextured surfaces from a large variety of materials with the aim to  
78 investigate the effects on cell behavior. Among others, the presence of nanopores can affect cell  
79 response and scaffold architecture can be optimized to achieve the wanted biological effect. For  
80 example, Zhang et al. [23] developed a porous hexagonal molybdenum sulfide ( $\text{MoS}_2$ )  
81 nanostructure, composed by many interconnected nanoflakes with a size of 5–8 nm. The authors  
82 fabricated the nanoporous architecture by a bottom-up hydrothermal method using the fluorine-  
83 doped tin oxide (FTO) coated glass as substrate [24]. This fabrication technique involves the use  
84 of high temperature (400 °C) and pressure for growing single crystals from an aqueous solution  
85 [25]. The high-resolution transmission electron microscopy (TEM) images showed the  $\text{MoS}_2$   
86 nanoflakes created a lattice spacing with a pore size of 0.63 nm. The effect of the nanostructured  
87  $\text{MoS}_2$  biointerface on mesenchymal stem cells (MSCs) attachment, spreading, and formation of  
88 focal adhesion was studied, by considering a flat substrate as control. Scanning electron  
89 microscopy (SEM) revealed the presence of more protrusions on cells grown on the nanoporous  
90  $\text{MoS}_2$  compared with that on control. Moreover, vinculin expression of cells on the flat substrate  
91 was low, whereas the vinculin intensity increased significantly for that on the nanostructured  
92  $\text{MoS}_2$ , demonstrating a higher concentration of FAs. Besides, the enhanced cell adhesion on the  
93 nanoporous surface, authors proved the nanotopography capability to induce the osteogenic  
94 differentiation of MSCs (Figure 2). Another recent study conducted by Greiner et al. [26]  
95 demonstrated for the first time that  $31.93 \pm 0.97$  nm pores present within endogenous collagen type

96 I fibers are sufficient to induce the osteogenic differentiation of human stem cells. A collagen-like  
97 scaffold which mimics the collagen pore structures was developed by self-assembly of silicon  
98 dioxide (SiO<sub>2</sub>) nanoparticles linked together by a thermally induced crosslinking reaction of oleic  
99 acid molecules [27]. The obtained substrates showed pore size of  $34 \pm 14$  nm that directly lead to  
100 the successful osteogenic differentiation of adult neural crest-derived inferior turbinate stem cells  
101 (ITSCs). In contrast, nanocomposites with  $18 \pm 4$  nm pores and flat glass substrates did not induce  
102 the differentiation of ITSCs. This SiO<sub>2</sub> porous nanocomposite could be employed as coating for  
103 micro- or macroporous scaffold to mimic the physiological bone architecture and guide the  
104 endogenous stem cells towards the osteogenic phenotype. The influence of a nanoporous structure  
105 on cell behavior was also analyzed by Merhie et al. [7] who used the electrochemical anodization  
106 process to produce the anodic porous alumina (APA) substrate. Anodizing is an electrolytic  
107 process which allows to obtain oxide coatings of 5 to 25  $\mu$ m in thickness on a metallic component  
108 placed in acid solutions normally under DC voltages. Oxidation occurs at the surface, resulting in  
109 the formation of a porous oxide film that is adherent to the underlying metal substrate [28]. The  
110 porous architectures obtained presented various pore sizes (approximately 60, 80, 100 and 120  
111 nm) depending on different exposition times of etching solution (0, 10, 20 and 30 min). The Neuro-  
112 2A (N2a) mouse neuroblastoma cell line seeded on each nanoporous substrate adhered and  
113 differentiated mainly on the substrate with small pores. Indeed, SEM and confocal fluorescence  
114 images showed neuron-like cell shape, with several neuritic extensions, whereas for substrates  
115 with larger pores, the cell cytosol appears with no preferred direction. Many studies reported the  
116 fabrication of nanopores on the titanium (Ti) surface applied in bone implants [29, 30, 31, 32, 33,  
117 34, 35]. For instance, oxidative nanopatterning [36, 37] and electron-beam lithography (EBL) [3]  
118 can be applied to form nanopores on Ti alloys. The oxidative patterning/etching is a chemical

119 surface treatment that produces nanoporous networks by exposing the substrate to oxide solutions  
120 [38]. In EBL, on the other hand, a resist layer is directly nanopatterned by directly writing on the  
121 surface with focused electron beams [39]. These nanofabrication techniques allow to enhance the  
122 anchorage of the implants by increasing adhesion, migration, proliferation and mineralization of  
123 osteo-like cells and MSCs.

124 Recent literature highlighted the role of nanopore size in mediating cell attachment, proliferation  
125 as well as differentiation and maturation. So far, nanopores are mainly applied for hard tissue  
126 devices [40] but the effect on other cell phenotypes has been recently investigated [7] thanks to  
127 novel nanofabrication technologies which offer the possibility to tailor nanometrical pore size on  
128 both metals and polymers in order to engineer the most promising conditions to each tissue  
129 engineering application.

130

### 131 **3. Micropores**

132 The microporous structure has a leading role in the interaction among small molecules and proteins  
133 as well as in the mechanical properties of the substrate at cellular level. Indeed, interconnected  
134 micropores directly influence the scaffold porosity, as porosity is related to the volume of empty  
135 pore space present in the construct. Therefore, micropores have a key role in scaffold permeability,  
136 protein adsorption and biodegradation rate. Furthermore, micropores induce a capillary force that  
137 anchors cells to the surface and drives them to migrate within the 3D structure [41]. Stachewicz et  
138 al., [18], compared the pore size produced by electrospinning polylactide-*co*-glycolide acid  
139 (PLGA) scaffolds in two configurations: aligned and randomly oriented nanofibers. Indeed, the  
140 electrospinning technique allows to fabricate nanofibrous mats by extruding a polymer solution  
141 contained into a syringe through a high potential difference between the metallic needle and a

142 collector [42]. The average pore sizes for the aligned and random fibers were  $0.92 \pm 0.57 \mu\text{m}$  and  
143  $2.30 \pm 1.33 \mu\text{m}$ , respectively. *In vitro* tests showed that the proliferation of MC3T3-E1 cells was  
144 much limited for aligned fibers as the size and circularity of pores were larger for the random  
145 fibers' construct. The electrospinning technique was also used by Abeyayehu's group [43] to  
146 fabricate fibrous scaffolds of various morphologies made from polydioxanone (PDO). Different  
147 pores and fibers diameters were obtained by varying the initial solution concentration: 60 mg/mL  
148 scaffolds featured fibers with a diameter of 400 nm and pores with an approximate diameter of 1.5  
149  $\mu\text{m}$ , while 140 mg/mL scaffolds contained fibers with a diameter of 2.4  $\mu\text{m}$  and pores with a  
150 diameter of 18  $\mu\text{m}$ . The authors examined how scaffold architecture affected both mast cell  
151 inflammatory response and angiogenesis. More specifically, they analyzed the only effect of pore  
152 size by altering pore diameters without changing fiber size. With this aim they used an air-flow  
153 mandrel approach, which increases the average pore size throughout the scaffold (from  
154 approximately 1.5  $\mu\text{m}$  to 4.5  $\mu\text{m}$ ). The bone marrow-derived mast cells (BMMC) were then seeded  
155 and the immune signals IL-33 and LPS were evaluated through the ELISA test. The results  
156 highlighted how the presence of micropores can modulate inflammatory cytokine secretion and  
157 the angiogenic response, thus demonstrating that large micropores reduce the inflammation and  
158 promote angiogenesis.

159 Finally, a scaffold with precise architecture and microporous structure was produced by using melt  
160 electrospinning technology [44] obtaining a 3D mesh with a pore size of 50  $\mu\text{m}$  from a top view  
161 perspective. Melt electrospinning technique forms well defined filaments with small diameters that  
162 can be deposited into 3D architectures using additive manufacturing principles (Figure 3) [45, 46].  
163 The effects of microfibrillar architectures on human skeletal stem cell (hSSC) behavior were  
164 investigated in terms of cell geometry and yes-associated protein (YAP) expression. An increase



165 in nuclear YAP expression, collagen formation and mineral deposition was observed at 24h post  
166 seeding. Moreover, cells appeared spread and elongated on the surface, demonstrating the  
167 influence of 3D fibrous extracellular matrix (ECM)-like architecture on hSSC behavior.

168 The possibility to modulate micropore size is a key strategy to improve the biological outcomes of  
169 tissue engineering device in terms of both cell regrowth and inflammatory process. To date, only  
170 few advanced processing technologies having a strict control of 3D pore size are available (e.g.  
171 electrospinning and melt-electrospinning) with a restricted number of processable materials.  
172 Therefore, the development of 3D architectures with optimized micropore size still remains a  
173 challenge for many applications and new technologies are required to process a wide variety of  
174 materials controlling their pore size at the microscale.

175

#### 176 **4. Macropores**

177 The diffusion of nutrients and oxygen is an important feature in the design of a bioengineered  
178 implant and can be modulated by tailoring macropores shape and size. Macroporous structures  
179 provide space for angiogenesis by allowing cellular infiltration and the development of vascular  
180 system within the scaffold. Indeed, a rapid vascular infiltration is needed to sustain tissue ingrowth  
181 *in vivo*, in addition to efficient gas diffusion and nutrients supply [47]. Recently, various studies  
182 were conducted to define the ideal macroporous patterns to direct cellular activity. For instance,  
183 Torstrick and coworkers [48] proposed the use of the salt leaching technique to realize a  
184 macroporous structure with pores size determined by salt particle diameters. In salt leaching  
185 method, salt crystals are blended with a polymer solution or placed into a mold and a polymer is  
186 then added to fill in the remaining spaces. The polymer is subsequently hardened, and the salt is  
187 removed via dissolution in a solvent such as water or alcohol [49]. This technique has been adopted

188 for many years as it allows to achieve a precise control of the pore size and pore morphology [50,  
189 51, 52]. In this study, the authors fabricated a porous polyetheretherketone scaffold (PEEK-SP)  
190 using sodium chloride with different sizes (200 to 312  $\mu\text{m}$ , 312 to 425  $\mu\text{m}$ , and 425 to 508  $\mu\text{m}$ ).  
191 The influence of pore size on cellular response was evaluated seeding human femoral osteoblasts  
192 and human MSCs (hMSCs) on PEEK-SP and comparing osteogenic differentiation of cells to  
193 smooth PEEK. The *in vitro* analysis proved the superior ability of PEEK-SP to induce bone cell  
194 proliferation and differentiation. The particulate leaching method was also adopted by Zhao et al.  
195 [53] who fabricated 3D porous PCL scaffolds with different macropore size to evaluate the hMSCs  
196 response to the macrotopography. Porous scaffolds were produced using paraffin microspheres  
197 (100–200  $\mu\text{m}$ , 200–300  $\mu\text{m}$  and 300–450  $\mu\text{m}$ ) as porogen. After porogen removal, the surface was  
198 functionalized through hydrolysis or aminolysis. The analysis indicated that the hydrolytically  
199 treated scaffolds, with a pore size of 200–300  $\mu\text{m}$ , better supported cell growth, while the  
200 aminolytic scaffolds performed best with a biggest pore size of 300–450  $\mu\text{m}$ . Regarding both the  
201 osteogenic and chondrogenic differentiation of hMSCs in these scaffolds, the deposition of  
202 minerals and glycosaminoglycans (GAG) suggested the successful differentiation mainly occurred  
203 in constructs with the largest pore size of 300–450  $\mu\text{m}$  despite the variation in surface chemistry.  
204 Walthers et al. [54] investigated the critical amount of angiogenesis necessary to sustain a  
205 population of implanted intestinal smooth muscle cells (SMCs) within multi-layered scaffolds.  
206 Macropores was fabricated by laser-cutting of PCL electrospun mats obtaining an interconnected  
207 network with 250  $\mu\text{m}$  pores. After 2 weeks of seeding, cell infiltration, vascular ingrowth, and  
208 survival of green fluorescent protein (GFP)-expressing SMCs were measured. The histologic  
209 sections of retrieved implants revealed a significant difference between porous and uncut scaffolds  
210 which showed little cellular penetration through the outermost layer, and the lack of nutrients

211 supply affected the vitality of the inner layer. In addition, blood vessels were more numerous into  
212 the porous rather than in smooth scaffolds.

213 In the last years, the rising of AM techniques has opened the ways to the development of macropore  
214 size-controlled scaffolds using a wide variety of materials and permitting a greater control of pore  
215 geometry [10, 55]. However, macropore can strongly affect the mechanical performance of the  
216 scaffold and the optimal pore size for cell response should be defined avoiding any structural  
217 damaging.

218

## 219 **5. Multiscale Pore Architecture**

220 In order to develop biomimetic scaffolds that resemble the complex living hierarchical structures,  
221 constructs having pore size on multiple length scales can be obtained. To achieve this ambitious  
222 goal Chen et al. [56], prepared porous gelatin scaffolds using the freeze-drying technique, which  
223 involves the sublimation of frozen water directly into the gas phase, resulting in pore formation.  
224 The pore sizes of the scaffolds fabricated are largely dependent on the ratio of water to polymer  
225 solution and on the emulsion viscosity [57, 58]. To analyze cellular contraction, proliferation and  
226 synthesis of ECM, bovine articular chondrocytes were seeded on gelatin substrates with round  
227 macropores and interconnected micropores on their walls. Chondrocytes resulted more infiltrated  
228 into scaffolds prepared using high concentrations of ice particulates while they deposited on the  
229 surface of the control with less interconnected pores. Hierarchical structures for tissue engineering  
230 applications were also recently realized by several groups [15, 59, 60, 61, 62, 8, 63, 64]. Jakus and  
231 coworkers [15], for example, used the 3D-painting process, a new form of 3D-printing combined  
232 with salt leaching. Like fused deposition modeling, this new method extrudes fused thermoplastic  
233 polymers through a nozzle to build complex structures through a layer by layer approach directly

234 from a computer aided design (CAD) model [65]. The difference with 3D-printing technique is  
235 the material processed, made almost entirely out of water-soluble salt. The resulting polymeric  
236 structures are highly porous and contain a low percentage of solid material [15]. More specifically,  
237 authors synthesized a 3D-printable ink using PLGA and a water-soluble salt as porogen; then the  
238 salt was dissolved and removed from the printed structure, obtaining a multiscale pore architecture  
239 formed by controlled macropores and interconnected micropores on the filaments surface (F-  
240 PLGA). F-PLGA scaffold increased hMSCs attachment, viability, proliferation and matrix  
241 synthesis capabilities when compared to 3D printed PLGA construct. Kim et al. applied a novel  
242 modified electrohydrodynamic direct-jet printing (EHDP) to fabricate a hierarchical 3D structure  
243 composed by collagen nanofibers assembled into 3D macroporous structures [59]. In this  
244 processing technique designed by authors, the machine moved automatically according to the path  
245 designed by a CAD model. As a target, EtOH was used as media with a grounded copper plate  
246 immersed in the bath. After dispensing the 3D fibrous structures, the EtOH was removed with  
247 water [66]. The *in vitro* analysis, performed by culturing MSCs, proved that this hierarchical  
248 collagen structure provided a suitable biomimetic environment to efficiently induce the cell-cell  
249 and cell-substrate interactions. The group of Novotna and coworkers, on the other hand, developed  
250 hierarchical 3D porous calcium phosphate scaffolds with high pore interconnectivity by using *in*  
251 *situ* polyurethane foaming technique [60]. With this method the foam is formed by carbon dioxide  
252 bubbles generated *in situ* via reaction of water with isocyanate groups [67, 68]. The pore size can  
253 be controlled by optimizing the reactant composition, namely by modifying the water,  
254 diisocyanate, polyol, and hydroxyapatite ratio. The study showed these *in situ* foamed scaffolds  
255 were well supportive to proper attachment and viability of normal human cells and can potentially  
256 be used in bone tissue engineering applications. Furthermore, Hu et al. designed bioactive

257 nanoparticle/PCL (BNPCL) hierarchical porous scaffolds with tunable performance and well-  
258 defined pore size [61]. With this aim, authors employed the solvent evaporation of 3D printed  
259 water-in-oil high internal phase emulsion (HIPE) templates, containing hydrophobically modified  
260 hydroxyapatite and silica nanoparticles in the oil phase. This innovative approach allowed to  
261 achieve a multiscale pore architecture with macropores formed by 3D-printing and micropores  
262 from HIPE templates (Figure 4). The in vitro biomineralization study suggested that the BNPCL  
263 scaffolds possessed excellent apatite formation ability (bioactivity). Another additive  
264 manufacturing technique, stereolithography, was employed by Sherborne et al. [8]. This method  
265 is based on the spatially controlled solidification of a liquid resin by photo-polymerization, in a  
266 layer-by-layer manner. A pre-defined pattern is illuminated on the surface of a resin using a  
267 computer-controlled laser beam or a digital light projector with a computer-driven building stage.  
268 As a result, the illuminated resin solidified to a defined depth, causing it to adhere to a support  
269 platform [69]. Authors photo-polymerized a 3D scaffold from a polymeric emulsion known as  
270 High Internal Phase Emulsions (PolyHIPEs) and produced hierarchical and repeatable pore  
271 structures [8]. Indeed, micropores with diameters of 1-50  $\mu\text{m}$  governed by emulsion templating  
272 and macropores (100  $\mu\text{m}$  size) dictated by additive manufacturing, were obtained. PolyHIPE  
273 scaffolds were compared, in terms of cell viability, to a commercial product that had a similar  
274 macroscopic architecture but lacked the internal micropores of the PolyHIPE construct. MLO-A5  
275 cells, a murine osteoblast cell line, improved their proliferation capability and deposited  
276 significantly greater amounts of mineralized ECM when seeded on PolyHIPE, demonstrating the  
277 beneficial effects of the hierarchical structure onto cell activity. Hierarchical architectures of  
278 primary (macroscale) and secondary (microscale) pores was also developed [63, 64]. For instance,  
279 Morgan et al. [63] designed a multicompartamental scaffold with a precise 3D microporous

280 framework. In particular, muscle and vascular templates were constructed from a novel slowly  
281 degrading elastomer, poly(limonene thioether) (PLT32i), and were connected via an oxygen  
282 permeable vascular-parenchymal interface constructed from rapidly biodegrading poly(glycerol  
283 sebacate) (PGS). The macroporous structure constituted by microchannels and grids was  
284 fabricated by casting the PLT32i prepolymer onto sintered spheres of poly(methyl methacrylate)  
285 (PMMA) within precisely patterned molds followed by photocuring, de-molding, and leaching out  
286 the PMMA. The behavior of human umbilical vein endothelial cell (HUVEC) and heart cell seeded  
287 on this scaffold was evaluated, by demonstrating the improvements in perfusion and heart cell  
288 alignment given by the grids and the enhanced heart cell retention conferred by microscale pores.  
289 Finally, we report the study of Chen et al. where controllable and reproducible extrusion deposition  
290 and porogen foaming processes were applied to generate highly porous hierarchical scaffolds [62].  
291 Particularly, the authors produced three kinds of hydroxyapatite scaffolds varying the particles size  
292 of graphite used as porogen (HA-G, HA-nG, HA- $\mu$ G). The hierarchical structures were  
293 advantageous in terms of biological performance, including biodegradation, proliferation,  
294 adhesion, and differentiation. Indeed, SEM analysis indicated that myoblasts adhered much more  
295 freely on HA-G, in contrast to the restricted adhesion on normal scaffolds. Moreover, cell  
296 interactions and cellular functionalities were further improved with the HA-nG and HA- $\mu$ G  
297 constructs.

298

## 299 **6. Conclusions**

300 Porous 3D scaffolds are typically used in tissue engineering applications since each pore size  
301 directly affects the cellular response in a different way. Depending on the pore diameters needed,  
302 and the type of material used, several conventional and AM techniques can be employed. However,

303 the current main goal among research groups worldwide is to develop a hierarchical scaffold with  
304 pore size over multiple length scales, which can introduce significant improvements in terms of  
305 biomimetic structure, interconnectivity of pores and final mechanical properties of the scaffold.  
306 The overview described in this review clearly indicates that the most performing techniques to  
307 obtain a controlled and hierarchical pore architecture are additive manufacturing methods in  
308 combination with traditional technologies. Indeed, AM techniques allow to achieve highly  
309 interconnected and controlled macro- and micropores, while the conventional methods provide  
310 pore size at the nanoscale. In this scenario, the use of melt electrospinning technology is very  
311 promising technique as it combines conventional (electrospinning) and AM techniques in one  
312 single system providing a nanofibrous matrix with a complex geometry and controlled micro- or  
313 macroporous architecture. Furthermore, recent findings highlighted as not only the pore size is  
314 pivotal to modulate cell fate but even the pore geometry have a role in controlling cell/structure  
315 interactions. So far, few recent studies [70, 71] reported the effect of pore geometry on cell  
316 behavior and the implementation of melt-electrospinning devices having a strict control on both  
317 pore size and geometry could be a promising strategy to combine pore size and geometry to gain  
318 further insights in the knowledge of cell response on different architectures to improve the design  
319 of bioinductive scaffolds.

320

## References

- [1] Q. L. Loh and C. Choong, "Three-Dimensional Scaffolds for Tissue Engineering Applications: Role of Porosity and Pore Size," *TISSUE ENGINEERING: Part B*, vol. 19, pp. 485-502, 2013.
- [2] G. Bellod., A. Fouillen, A. Badia and A. Nanci, "A nanoporous titanium surface promotes the maturation of focal adhesions and formation of filopodia with distinctive nanoscale protrusions by osteogenic cells," *Acta Biomaterialia*, vol. 60, pp. 339-349, 2017.
- [3] D. Garoli, L. Lovato, G. Della Giustina, M. Oliverio, M. Francardi, E. Zanchetta, G. Brusatin and F. De Angelis, "Directly nanopatternable nanoporous titania – Application to cell growth engineering," *Microelectronic Engineering*, vol. 155, pp. 102-106, 2016.

- [4] X. Y. Zhang, G. Fang, L. L. Xing, W. Liu and J. Zhou, "Effect of porosity variation strategy on the performance of functionally graded Ti-6Al-4V scaffolds for bone tissue engineering," *Materials and Design*, vol. 157, pp. 523-538, 2018.
- [5] H. Yuan, Z. Yang, Y. Li, X. Zhang, J. De Bruijn and K. De Groot, "Osteoinduction calcium phosphate biomaterials," *J. Mater. Sci.: Mater. Med.*, vol. 9, pp. 723-726, 1998.
- [6] M. E. Cox and D. C. Dunand, "Bulk gold with hierarchical macro-, micro- and nano-porosity," *Materials Science and Engineering A*, vol. 528, pp. 2401-2406, 2011.
- [7] A. E. Merhie, M. Salerno, C. Toccafondi and S. Dante, "Neuronal-like response of N2a living cells to nanoporous patterns of thin supported anodic alumina," *Colloids and Surfaces B: Biointerfaces*, vol. 178, pp. 32-37, 2019.
- [8] C. Sherborne, R. Owen, G. C. Reilly and F. Claeysens, "Light-based additive manufacturing of PolyHIPeS: Controlling the surface porosity for 3D cell culture applications," *Materials and Design*, vol. 156, pp. 494-503, 2018.
- [9] A. Salerno, D. Guarnieri, M. Iannone, S. Zepetelli and P. A. Netti, "Effect of Micro- and Macroporosity of Bone Tissue Three-Dimensional-Poly(e-Caprolactone) Scaffold on Human Mesenchymal Stem Cells Invasion, Proliferation, and Differentiation In Vitro," *TISSUE ENGINEERING: Part A*, vol. 16, pp. 2661-2673, 2010.
- [10] D. A. Zopf, C. I. Flanagan, A. G. Mitsak, J. R. Brennan and S. J. Hollister, "Pore architecture effects on chondrogenic potential of patient-specific 3-dimensionally printed porous tissue bioscaffolds for auricular tissue engineering," *International Journal of Pediatric Otorhinolaryngology*, vol. 114, pp. 170-174, 2018.
- [11] I. Bruzauskait, Bironaitė D., E. Bagdonas and E. Bernotiene, "Scaffolds and cells for tissue regeneration: different scaffold pore sizes—different cell effects," *Cytotechnology*, vol. 68, pp. 335-369, 2016.
- [12] H. Jeon, C. Simon and G. Kim, "A mini-review: Cell response to microscale, nanoscale, and hierarchical patterning of surface structure," *Biomed Mater Res Part B*, vol. 102B, pp. 1580-1594, 2014.
- [13] L. Du, W. Li, Z. Jiang, L. Wang, D. Kong and B. Xu, "Hierarchical macro/micro-porous silk fibroin scaffolds for tissue engineering," *Materials Letters*, vol. 236, pp. 1-4, 2019.
- [14] B. Kundu, R. Rajkhowa, S. C. Kundu and X. Wang, "Silk fibroin biomaterials for tissue regenerations," *Advanced Drug Delivery Reviews*, vol. 65, pp. 457-470, 2013.
- [15] A. E. Jakus, N. R. Geisendorfer, P. L. Lewis and R. N. Shah, "3D-printing porosity: A new approach to creating elevated porosity materials and structures," *Acta Biomaterialia*, vol. 72, pp. 94-109, 2018.
- [16] P. Danilevicius, L. Georgiadi, C. J. Pateman, F. Claeysens, M. Chatzinikolaidou and M. Farsari, "The effect of porosity on cell ingrowth into accurately defined, laser-made, polylactide-based 3D scaffolds," *Applied Surface Science*, vol. 336, pp. 2-10, 2015.
- [17] A. Salerno, E. Di Maio, S. Iannace and P. A. Netti, "Tailoring the pore structure of PCL scaffolds for tissue engineering prepared via gas foaming of multi-phase blends," *J Porous Mater*, vol. 19, pp. 181-188, 2012.
- [18] U. Stachewicz, P. Szewczyk, K., A. Kruk, A. H. Barber and A. Czyrska-Filemonowicz, "Pore shape and size dependence on cell growth into electrospun fiber scaffolds for tissue engineering: 2D and 3D analyses using SEM and FIB-SEM tomography," *Materials Science & Engineering C*, vol. 95, pp. 397-408, 2019.
- [19] H. A. Zaharin, A. M. Rani, F. I. Azam, T. L. Ginta, N. Sallih and A. Ahmad, "Effect of Unit Cell Type and Pore Size on Porosity and Mechanical Behavior of Additively Manufactured Ti6Al4V Scaffolds," *Materials*, vol. 11, p. 2402, 2018.



- [20] X. Wang, S. Xu, S. Zhou, W. Xu, M. Leary, P. Choong, M. Qian, M. Brandt and Y. Xie, "Topological design and additive manufacturing of porous metals for bone scaffolds and orthopaedic implants: A review," *Biomaterials*, vol. 83, pp. 127-141, 2016.
- [21] J. O. Gallagher, K. F. McGhee, C. D. Wilkinson and M. O. Riehle, "Interaction of animal cells with ordered nanotopography," *IEEE Transactions on NanoBioscience*, pp. 24-28, 2001.
- [22] R. Zaidel-Bar, C. Ballestrem, Z. Kam and B. Geiger, "Early molecular events in the assembly of matrix adhesions at the leading edge of migrating cells," *Journal of Cell Science*, vol. 116, pp. 4605-4613, 2003.
- [23] X. Zhang, J. Nie, X. Yang, Z. Liu, W. Guo, J. Qiu, S. Wang, X. Yu, Y. Guan, H. Liu and L. Li, "Nanostructured molybdenum disulfide biointerface for adhesion and osteogenic differentiation of mesenchymal stem cells," *Applied Materials Today*, vol. 10, pp. 164-172, 2018.
- [24] W. Zhou, Z. Yin, Y. Du, X. Huang, Z. Zeng, Z. Fan, H. Liu, J. Wang and H. Zhang, "Synthesis of Few-Layer MoS<sub>2</sub> Nanosheet-Coated TiO<sub>2</sub> Nanobelt Heterostructures for Enhanced Photocatalytic Activities," *Small*, vol. 9, pp. 140-147, 2013.
- [25] G. Yang and S. Park, "Conventional and Microwave Hydrothermal Synthesis and Application of Functional Materials: A Review," *materials*, vol. 12, p. 1177, 2019.
- [26] J. Greiner, M. Gottschalk, N. Fokin, B. Büker, B. P. Kaltschmidt, A. Dreyer, T. Vordemvenne, C. Kaltschmidt, A. Hütten and B. Kaltschmidt, "Natural and synthetic nanopores directing osteogenic differentiation of human stem cells," *Nanomedicine: Nanotechnology, Biology and Medicine*, vol. 17, pp. 319-328, 2019.
- [27] A. Dreyer, A. Feld, A. Kornowski, E. Yilmaz, H. Noei, A. Meyer, T. Krekeler, C. Jiao, A. Stierle, V. Abets, H. Weller and G. A. Schneider, "Organically linked iron oxide nanoparticle supercrystals with exceptional isotropic mechanical properties," *Nature Materials*, vol. 15, pp. 522-528, 2016.
- [28] S. Shrestha and B. Dunn, "Plasma electrolytic oxidation and anodising of aluminium alloys for spacecraft applications," *Surface Engineering of Light Alloys*, pp. 603-641, 2010.
- [29] G. Louarn, L. Salou, A. Hoornaert and P. Layrolle, "Nanostructured surface coatings for titanium alloy implants," *Journal of Materials Research*, pp. 1892-1899, 2019.
- [30] A. Ketabchi, K. Komm, M. Miles-Rossouw, D. A. Cassani and F. Variola, "Nanoporous Titanium Surfaces for Sustained Elution of Proteins and Antibiotics," *PLoS One*, vol. 9, pp. 1-9, 2014.
- [31] X. Zhuang, B. Zhou and K. Yuan, "Role of p53 mediated miR-23a/CXCL12 pathway in osteogenic differentiation of bone mesenchymal stem cells on nanostructured titanium surfaces," *Biomedicine & Pharmacotherapy*, vol. 112, p. 108649, 2019.
- [32] X. Shen, M. Al-Baadani, H. He, L. Cai, Z. Wu, L. Yao, X. Wu, S. Wu, M. Chen, H. Zhang and J. Liu, "Antibacterial and osteogenesis performances of LL37-loaded titania nanopores in vitro and in vivo," *Int J Nanomedicine*, vol. 14, pp. 3043-3054, 2019.
- [33] X. Pan, Y. Li, A. O. Abdullah, W. Wang, M. Qi and Y. Liu, "Micro/nano-hierarchical structured TiO<sub>2</sub> coating on titanium by micro-arc oxidation enhances osteoblast adhesion and differentiation," *R. Soc. open sci.*, vol. 6, p. 182031, 2019.
- [34] Y. Sun, J. Liu, C. Wu and H. Huang, "Nanoporous surface topography enhances bone cell differentiation on Ti-6Al-7Nb alloy in bone implant applications," *Journal of Alloys and Compounds*, vol. 643, pp. S124-S132, 2015.
- [35] M. Schürmann, A. Wolff, D. Widera, S. Hauser, P. Heimann, A. Hütten, C. Kaltschmidt and B. Kaltschmidt, "Interaction of adult human neural crest-derived stem cells with a nanoporous titanium surface is sufficient to induce their osteogenic differentiation," *Stem Cell Res*, vol. 13, pp. 98-110, 2014.

- [36] D. Guadarrama Bello, A. Fouillen, A. Badia and A. Nanci, "A nanoporous titanium surface promotes the maturation of focal adhesions and formation of filopodia with distinctive nanoscale protrusions by osteogenic cells," *Acta Biomaterialia*, vol. 60, pp. 339-349, 2017.
- [37] I. Lauria, T. N. Kutz, F. Boke, S. Rutten, D. Zander and H. Fisher, "Influence of nanoporous titanium niobium alloy surfaces produced via hydrogen peroxide oxidative etching on the osteogenic differentiation of human mesenchymal stromal cells," *Materials Science and Engineering: C*, vol. 98, pp. 635-648, 2019.
- [38] J. Yi, C. Bernard, F. Variola, S. Zalzal, J. Wuest, F. Rosei and A. Nanci, "Characterization of a bioactive nanotextured surface created by controlled chemical oxidation of titanium," vol. 600, pp. 4613-4621, 2006.
- [39] S. Prakash and J. Yeom, "Advanced Fabrication Methods and Techniques," *Nanofluidics and Microfluidics*, pp. 87-170, 2014.
- [40] T. Gong, J. Xie, J. Liao, T. Zhang, S. Lin and Y. Lin, "Nanomaterials and bone regeneration," *Bone Research*, p. 15029, 2015.
- [41] K. Zhang, Y. Fan, N. Dunne and X. Li, "Effect of microporosity on scaffolds for bone tissue engineering," *Regen Biomater*, vol. 5, pp. 115-124, 2018.
- [42] A. Senthamizhan, B. Balusamy and T. Uyar, "Electrospinning: A versatile processing technology for producing nanofibrous materials for biomedical and tissue-engineering applications," *Electrospun Materials for Tissue Engineering and Biomedical Applications*, pp. 3-41, 2017.
- [43] D. Abebayehu, A. J. Spence, M. J. McClure, T. T. Haque, K. O. Rivera and J. J. Ryan, "Polymer scaffold architecture is a key determinant in mast cell inflammatory and angiogenic responses," *Journal of Biomedical Materials Research Part A*, vol. 107, pp. 884-892, 2019.
- [44] K. F. Eichholz and D. A. Hoey, "Mediating human stem cell behaviour via defined fibrous architectures by melt electrospinning writing," *Acta Biomaterialia*, pp. 140-151, 2018.
- [45] L. M. Muerza-Cascante, D. Haylock, D. W. Hutmacher and P. D. Dalton, "Melt Electrospinning and Its Technologization in Tissue Engineering," *TISSUE ENGINEERING: Part B*, vol. 21, pp. 187-202, 2015.
- [46] T. Brown, P. Dalton and D. Hutmacher, "Direct Writing By Way of Melt Electrospinning," *Advanced Materials*, vol. 23, pp. 5651-5657, 2011.
- [47] T. S. Karande, J. L. Ong and C. M. Agrawal, "Diffusion in Musculoskeletal Tissue Engineering Scaffolds: Design Issues Related to Porosity, Permeability, Architecture, and Nutrient Mixing," *Annals of Biomedical Engineering*, vol. 32, pp. 1728-1743, 2004.
- [48] F. B. Torstrick, N. T. Evans, H. Y. Stevens, K. Gall and R. E. Guldberg, "Do Surface Porosity and Pore Size Influence Mechanical Properties and Cellular Response to PEEK?," *Clin Orthop Relat Res*, vol. 474, p. 2373-2383, 2016.
- [49] S. Lee, Y. Kim, M. Chong, S. Hong and Y. Lee, "Study of gelatin-containing artificial skin V: fabrication of gelatin scaffolds using a salt-leaching method," *Biomaterials*, vol. 26, pp. 1961-1968, 2005.
- [50] R. Goodall, J. F. Despois and A. Mortensen, "The plasticity size effect in replicated microcellular aluminium," *Scripta Mater.*, vol. 69, pp. 469-472, 2013.
- [51] J. Osorio-Hernández, M. Suarez, R. Goodall, G. Lara-Rodriguez, I. A. Lopez and I. A. Figueroa, "Manufacturing of open-cell Mg foams by replication process and mechanical properties," *Materials & Design*, vol. 64, pp. 136-141, 2014.

- [52] G. Jia, Y. Hou, C. Chen, J. Niu, H. Zhang, H. Huang, M. Xiong and G. Yuan, "Precise fabrication of open porous Mg scaffolds using NaCl templates: Relationship between space holder particles, pore characteristics and mechanical behavior," *Materials & Design*, vol. 140, pp. 106-113, 2018.
- [53] Y. Zhao, K. Tan, Y. Zhou, Z. Ye and W. Tan, "A combinatorial variation in surface chemistry and pore size of three-dimensional porous poly( $\epsilon$ -caprolactone) scaffolds modulates the behaviors of mesenchymal stem cells," *Materials Science and Engineering: C*, vol. 59, pp. 193-202, 2016.
- [54] C. Walthers, A. K. Nazemi, A. K. Patel, B. M. Wu and J. C. Dunn, "The effect of scaffold macroporosity on angiogenesis and cell survival in tissue-engineered smooth muscle," *Biomaterials*, vol. 35, pp. 5129-5137, 2014.
- [55] V. Tran and X. Wen, "Rapid prototyping technologies for tissue regeneration," *Rapid Prototyping of Biomaterials*, pp. 97-155, 2014.
- [56] S. Chen, Q. Zhang, T. Nakamoto, N. Kawazoe and G. Chen, "Gelatin Scaffolds with Controlled Pore Structure and Mechanical Property for Cartilage Tissue Engineering," *Tissue Engineering Part C Methods*, pp. 189-198, 2015.
- [57] A. Mikos and J. Temenoff, "Formation of highly porous biodegradable scaffolds for tissue engineering," *Electronic Journal of Biotechnology*, vol. 3, pp. 114-119, 2000.
- [58] E. Sachlos and J. Czernuszka, "Making tissue engineering scaffolds work. Review: the application of solid freeform fabrication technology to the production of tissue engineering scaffolds.," *European cells & materials*, vol. 5, pp. 29-39, 2003.
- [59] M. Kim, Y. Choe and G. Kim, "Injectable hierarchical micro/nanofibrous collagen-based scaffolds," *Chemical Engineering Journal*, vol. 365, pp. 220-230, 2019.
- [60] L. Novotna, L. Kucera, A. Hampl, D. Drdlik, J. Cihlar and J. Cihlar, "Biphasic calcium phosphate scaffolds with controlled pore size distribution prepared by in-situ foaming," *Materials Science and Engineering: C*, vol. 95, pp. 365-370, 2019.
- [61] Y. Hu, J. Wang, X. Li, X. Hu, W. Zhou, X. Dong, C. Wang, Z. Yang and B. Binks, "Facile preparation of bioactive nanoparticle/poly( $\epsilon$ -caprolactone) hierarchical porous scaffolds via 3D printing of high internal phase Pickering emulsions," *Journal of Colloid and Interface Science*, vol. 545, pp. 104-115, 2019.
- [62] Z. Chen, X. Zhang, Y. Yang, K. Zhou, N. Wragg, Y. Liu, M. Lewis and C. Liu, "Fabrication and characterization of 3D complex hydroxyapatite scaffolds with hierarchical porosity of different features for optimal bioactive performance," *Ceramics International*, vol. 43, pp. 336-344, 2017.
- [63] K. Y. Morgan, D. Sklaviadis, Z. L. Tochka, K. M. Fischer, K. Hearon, T. D. Morgan, R. Langer and L. E. Freed, "Multi-Material Tissue Engineering Scaffold with Hierarchical Pore Architecture," *Advanced functional materials*, vol. 26, pp. 5973-5883, 2016.
- [64] M. Prakasam, A. Chirazi, G. Pyka, A. Prokhardtseva, D. Lichau and A. Largeteau, "Fabrication and Multiscale Structural Properties of Interconnected Porous Biomaterial for Tissue Engineering by Freeze Isostatic Pressure (FIP)," *Journal of Functional Biomaterials*, vol. 9, p. 51, 2018.
- [65] S. Masood, "Advances in Fused Deposition Modeling," *Comprehensive Materials Processing*, pp. 69-91, 2014.
- [66] M. Kim and G. Kim, "Electrohydrodynamic Jet Process for Pore-Structure-Controlled 3D Fibrous Architecture as a Tissue Regenerative Material: Fabrication and Cellular Activities," *Langmuir*, vol. 30, pp. 8551-8557, 2014.
- [67] E. Sharmin and F. Zafar, "Polyurethane: An Introduction," *Polyurethane*, 2012.

- [68] S. Sartori, V. Chiono, C. Tonda-Turo, C. Mattu and G. Ciardelli, "Biomimetic polyurethanes in nano and regenerative medicine," *Journal of Materials Chemistry B*, vol. 2, pp. 5128-5144, 2014.
- [69] F. Melchels, J. Feijen and D. Grijpma, "A review on stereolithography and its applications in biomedical engineering," *Biomaterials*, vol. 31, pp. 6121-6130, 2010.
- [70] B. Green, M. Panagiotakopoulou, F. Pramotton, G. Stefopoulos, S. Kelley, D. Poulikakos and A. Ferrari, "Pore Shape Defines Paths of Metastatic Cell Migration," *Nano Letters*, vol. 18, pp. 2140-2147, 2018.
- [71] K. P. Fuller, D. Gaspar, L. Delgado, A. Pandit and D. Zeugolis, "Influence of porosity and pore shape on structural, mechanical and biological properties of poly  $\epsilon$ -caprolactone electrospun fibrous scaffolds," *Nanomedicine*, vol. 11, pp. 1031-1040, 2016.

**Figure 1** – Schematic representation of different pore size surfaces and their influence on physical properties and cell behavior. Nanopores ( $< 0.3 \text{ nm}$ ) promotes cellular attachment by inducing cells to develop FAs; micropores ( $0.3 - 100 \text{ }\mu\text{m}$ ) improves the permeability of the scaffold and facilitate cell migration; macropores ( $> 100 \text{ }\mu\text{m}$ ) provide space for vascularization, nutrients supply, waste removal and gas diffusion.

**Figure 2** – Scanning electron microscopy (SEM) image of MoS<sub>2</sub> nanostructured surface (a) and immunofluorescent staining of the osteogenic markers osteocalcin (OPN) and osteopontin (OCN) expressed by rat bone marrow mesenchymal stem cells (MSCs) after 14 days in vitro. Reprinted with permission from [23].

**Figure 3** – Melt electrospinning setup (a) and scanning electron microscopy (SEM) images of PCL scaffolds obtained by melt electrospinning with fibrous layers oriented at  $90^\circ$  (b) and  $60^\circ$  (c). Reprinted with permission from [46].

**Figure 4** – Example of hierarchical porous structures basing on 3D-printing of Pickering HIPE templates. Reprinted with permission from [61].

