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Original
Reviewing recently developed technologies to direct cell activity through the control of pore size: From the macro- to the nanoscale / Sgarminato, V., Tonda-Turo, C., Ciardelli, G.. - In: JOURNAL OF BIOMEDICAL MATERIALS RESEARCH. PART B, APPLIED BIOMATERIALS.. - ISSN 1552-4973. - ELETTRONICO. - (2020), pp. 1-10. [10.1002/jbm.b.34467]

Availability:
This version is available at: 11583/2781552 since: 2020-01-17T11:43:34Z

Publisher:
Wiley

Published
DOI:10.1002/jbm.b.34467

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10 October 2026

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

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Abstract

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

Keywords

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

1. Introduction

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

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

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

2. Nanopores

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

environment by expanding lamellipodia and filopodia [21]. When the substrate is suitable for the attachment, cells develop focal adhesions (FAs) that successively elongate and generate mature adhesions known as fibrillar adhesions (FBs) [22]. The evaluation and characterization of FAs and FBs allow analyzing the influence of nanotopography on cellular activity. Many approaches have been used to fabricate nanotextured surfaces from a large variety of materials with the aim to investigate the effects on cell behavior. Among others, the presence of nanopores can affect cell response and scaffold architecture can be optimized to achieve the wanted biological effect. For example, Zhang et al. [23] developed a porous hexagonal molybdenum sulfide (MoS_2) nanostructure, composed by many interconnected nanoflakes with a size of 5–8 nm. The authors fabricated the nanoporous architecture by a bottom-up hydrothermal method using the fluorine-doped tin oxide (FTO) coated glass as substrate [24]. This fabrication technique involves the use of high temperature (400 °C) and pressure for growing single crystals from an aqueous solution [25]. The high-resolution transmission electron microscopy (TEM) images showed the MoS2 nanoflakes created a lattice spacing with a pore size of 0.63 nm. The effect of the nanostructured MoS_2 biointerface on mesenchymal stem cells (MSCs) attachment, spreading, and formation of focal adhesion was studied, by considering a flat substrate as control. Scanning electron microscopy (SEM) revealed the presence of more protrusions on cells grown on the nanoporous MoS_2 compared with that on control. Moreover, vinculin expression of cells on the flat substrate was low, whereas the vinculin intensity increased significantly for that on the nanostructured MoS_2, demonstrating a higher concentration of FAs. Besides, the enhanced cell adhesion on the nanoporous surface, authors proved the nanotopography capability to induce the osteogenic differentiation of MSCs (Figure 2). Another recent study conducted by Greiner et al. [26] demonstrated for the first time that 31.93 ± 0.97 nm pores present within endogenous collagen type

I fibers are sufficient to induce the osteogenic differentiation of human stem cells. A collagen-like scaffold which mimics the collagen pore structures was developed by self-assembly of silicon dioxide (SiO_2) nanoparticles linked together by a thermally induced crosslinking reaction of oleic acid molecules [27]. The obtained substrates showed pore size of 34 ± 14 nm that directly lead to the successful osteogenic differentiation of adult neural crest-derived inferior turbinate stem cells (ITSCs). In contrast, nanocomposites with 18 ± 4 nm pores and flat glass substrates did not induce the differentiation of ITSCs. This SiO_2 porous nanocomposite could be employed as coating for micro- or macroporous scaffold to mimic the physiological bone architecture and guide the endogenous stem cells towards the osteogenic phenotype. The influence of a nanoporous structure on cell behavior was also analyzed by Merhie et al. [7] who used the electrochemical anodization process to produce the anodic porous alumina (APA) substrate. Anodizing is an electrolytic process which allows to obtain oxide coatings of 5 to 25 μm in thickness on a metallic component placed in acid solutions normally under DC voltages. Oxidation occurs at the surface, resulting in the formation of a porous oxide film that is adherent to the underlying metal substrate [28]. The porous architectures obtained presented various pore sizes (approximately 60, 80, 100 and 120 nm) depending on different exposition times of etching solution (0, 10, 20 and 30 min). The Neuro-2A (N2a) mouse neuroblastoma cell line seeded on each nanoporous substrate adhered and differentiated mainly on the substrate with small pores. Indeed, SEM and confocal fluorescence images showed neuron-like cell shape, with several neuritic extensions, whereas for substrates with larger pores, the cell cytosol appears with no preferred direction. Many studies reported the fabrication of nanopores on the titanium (Ti) surface applied in bone implants [29, 30, 31, 32, 33, 34, 35]. For instance, oxidative nanopatterning [36, 37] and electron-beam lithography (EBL) [3] can be applied to form nanopores on Ti alloys. The oxidative patterning/etching is a chemical

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

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

3. Micropores

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

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

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

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

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

4. Macropores

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

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

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

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

5. Multiscale Pore Architecture

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

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

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

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

6. Conclusions

Porous 3D scaffolds are typically used in tissue engineering applications since each pore size directly affects the cellular response in a different way. Depending on the pore diameters needed, 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

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

Figure 2 *– Scanning electron microscopy (SEM) image of MoS2 nanostructured surface (a) and immunofluorescent staining of the osteogenic markers osteocalcin (OPN) and osteopotin (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° (b) and 60° (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].*