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Breath figures in tissue engineering and drug delivery: state-of-the-art and future perspectives

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Abstract

The breath figure (BF) method is an easy, low-cost method to prepare films with a highly organized honeycomb-like porous surface. The particular surface topography and porous nature of these materials makes them valuable substrates for studying the complex effects of topography on cell fate, and to produce biomimetic materials with high performance in tissue engineering. Numerous researchers over the last two decades have studied the effects of the honeycomb topography on a variety of primary and immortalized cell lines, and drew important conclusions that can be translated to the construction of optimal biomaterials for cell culture. The literature also encouragingly shows the potential of honeycomb films to induce differentiation of stem cells down a specific lineage without the need for biochemical stimuli.

Here, we review the main studies where BF honeycomb films are used as substrates for tissue engineering applications. Furthermore, we highlight the numerous advantages of the porous nature of the films, such as the enhanced, spatially controlled adsorption of proteins, the topographical cues influencing cellular behavior, and the enhanced permeability which is essential both *in vitro* and *in vivo*. Finally, this review highlights the elegant use of honeycomb films as drug-eluting biomaterials or as reservoirs for distinct drug delivery systems.

Keywords

Breath figure method; honeycomb films; surface topography; contact guidance; tissue engineering; drug delivery

1. Introduction

1.1. Cell adhesion to biomaterials

In their natural tissue environment, cells are surrounded by the extracellular matrix (ECM), a dynamic and complex network of secreted soluble and insoluble molecules, which provides cells with structural support and signaling cues affecting cell morphology, movement, function, and fate [1]. In tissue engineering, an appropriate cellular response to the biomaterial is critical for successful tissue regeneration. The properties of material surface e.g. topography, chemistry and mechanics have a great impact on cell behavior and need to be considered when designing new biomaterials. In addition to soluble interactions, solid-state interactions at the cell-material interface confer effects through physical distortion, chemical signaling, and cell adhesion points [2]. Cell adhesion is mediated by a variety of molecules, and especially the transmembrane heterodimers of the integrin family. In the ECM, integrins bind proteins such as fibronectin, vitronectin, collagen and laminin [1]. Examples of strong integrin-mediated cell-matrix junctions include hemidesmosomes (prominent attachment sites in basal epithelia, linking the cells to their underlying basement membrane) and focal adhesions, the ECM adhesion structures in adherent cells such as fibroblasts and epithelial cells [3]. For cell binding, activation of integrins drives a conformational change in the integrin dimer. ‘Outside-in’ signaling causes a switch to the active high-affinity form contributing to integrin clustering, thus exposing binding sites for proteins like talin and kindlin on the integrin β-chain inside the cell. Talin subsequently interacts with adaptor proteins such as vinculin connecting the integrins with intracellular actin filaments. ‘Inside-out’ signaling activation, on the other hand, is stimulated by intracellular signals leading to conformational change in the integrin dimer by increased ability of talin and kindlin to bind the intracellular β-chain [4–6]. Understanding the formation of focal adhesions and the anchoring mechanisms of the actin stress fibers to the cell membrane, is a key point in tissue engineering [3].

As scaffolds made from synthetic materials tend to lack ligands for cell binding, cell attachment to synthetic materials typically requires biofunctionalization. There are several ways to achieve this (for

review, please see Gibson et al 2011[7] and Krutty et al 2016[8]), including physical or chemical modification. Physical modification can for example be performed simply by coating the material with biomimetic components, such as proteins like collagen or laminin using adsorption or, as recently demonstrated by our team, Langmuir-Schaefer film deposition [9,10]. Typically, surface stability is higher by chemical modification than by physical adsorption. A commonly used chemical modification is plasma treatment with reactive gases to create new functional groups on polymer surfaces [11].

Nowadays, it is also widely acknowledged that biomaterial surface topography – e.g. size, geometric arrangement and shape of surface features – is essential in the induction of cell adhesion and subsequent guidance of cellular function and behavior [12]. Indeed, cells in living tissues also encounter nanoscale features in their ECM, in the form of pits, pores, protrusions and fibers, which affect their subcellular behaviors such as the organization of the cell adhesion molecule receptors [13,14]. Scaffold nanotopography can also regulate stem cell differentiation [15]. Furthermore, characteristics such as cell alignment, morphology, migration, and tissue organization are especially influenced by microscale topography [12,13,16].

1.2. Porous films and the breath figure method

When designing an artificially engineered substrate for tissue engineering, features such as biocompatibility, biodegradability, mechanical properties, three-dimensional (3D) architecture and manufacturing technology should be carefully considered. It is also essential for all tissue and cell types that the scaffold enables the free diffusion of nutrients and gases to the cells, and waste products away from the cells. For this purpose specifically, the presence of pores in the scaffold is often advantageous. The size and distribution of the pores is a critical parameter, as it determines whether cells are able to attach, proliferate and migrate on and into the scaffold. When cell migration into the scaffold is desired, interconnection of the pores affects the rate of migration, nutrient/waste diffusion, and the ability of the cells to interact with each other [17].

Porous materials can be prepared by several methods, including chemical vapor deposition, polymer phase separation, colloidal lithography, photolithography, electron beam lithography (EBL), electrospinning, soft lithography, microfluidics, structured hydrogels and polydimethylsiloxane (PDMS) molding, most of which require specific and expensive equipment [14–16,18,19].

In 1994, François et al observed for the first time the formation of films with essentially monodisperse pore size after evaporating star-shaped polystyrene or polystyrene-polyparaphenylene block copolymer solutions in carbon disulfide under a flow of moist air [20]. Factors such as the presence of humid conditions, type of solvent, polymer architecture and polymer molecular mass were already back then found to influence the spontaneous organization of the pores into periodic hexagonal arrays [20]. This method to produce porous honeycomb-like films under the influence of a humid air flow has been designated breath figure (BF) or water droplet templating method due to the water droplets in the air (or fog) acting as templates as they condense on the surface of the evaporating solution. The key steps of the method are illustrated in Figure 1. The polymer is typically dissolved in a volatile, water-immiscible organic solvent, and the solution cast under high relative humidity % (RH%). Water droplets condense and grow on the cold surface of the evaporating polymer solution and arrange into an ordered hexagonal lattice in order to reduce the free energy. In this self-assembly process, the polymer is positioned at the water droplet-solvent interface, and a porous pattern is formed after complete solvent and water evaporation. Compared to other methods such as lithography and micro-contact printing, the BF method has numerous advantages, which makes it one of the most widely employed methods for the fabrication of porous polymer films: i) it is a simple method that does not require a complex setup or multiple steps; ii) it is inexpensive since it does not require specific and costly equipment; iii) it utilizes a nontoxic and easily available templating medium (water); iv) it is fast and can quickly prepare numerous porous films with large surface area; v) it can produce highly ordered porous films employing a variety of polymers; and vi) it allows manipulation of pore size and shape through control of process parameters e.g. air humidity and polymer concentration [21,22].

Typically, the BF fabrication process generates films which are characterized by an evenly porous surface with a non-uniform pore distribution throughout the z-direction. In addition, pores are

normally larger at the top surface than at the bottom surface. In our previous research, we have reported some encouraging results in terms of substance permeability throughout honeycomb films [10,23]. Even so, the specific pore distribution profile throughout the BF films should be carefully considered, especially in scaffolds intended for tissue engineering, as the permeability can be difficult to predict, with effects on cellular interactions and on the material's biodegradability over time. For specific applications, small variations in the BF method can be employed in order to produce more symmetrical films (where the pores go through from top to bottom), as given by some examples in this review, with (expectedly) more predictable permeability profiles. In any case, studies accounting for the flow of substances through honeycomb films are still scarce and are thus increasingly needed.

The control of experimental variables influencing the formation of BF parameters is essential for the attainment of the highly ordered hexagonal array at the surface. For example, high RH% is considered essential to form the regular honeycomb pattern. Higher environmental humidity also contributes to increase pore size, due to the increasing number of water droplets accumulating at the surface of the polymer solution [21,24–26]. It is also generally accepted that water-immiscible solvents with low boiling point, high vapor pressure and good compatibility with the polymer are preferable for the formation of BF. Highly volatile solvents usually result in the formation of smaller pores due to fast solvent evaporation and short period of time for the growth of water droplets [21,26–28]. In consistency, high air speed also causes the solvent to evaporate more quickly, resulting in smaller pores, but excessive airflow can give the droplets insufficient time to grow and organize into a regular array [22,29]. Low polymer concentrations usually lead to larger pores, as long as the concentration is high enough to stabilize the water droplets and prevent their coalescence; however, at excessively high polymer concentrations, the viscosity of the polymer solution can compromise the arrangement of the water droplets, resulting in large, unevenly distributed or irregularly shaped pores [21,24,30]. Properties of the honeycomb films can also be considerably affected by the choice of polymer type, polymer architecture and molecular weight, as extensively shown in the literature [31–35]. Furthermore, different hierarchical structures may be obtained by introducing variations to the basic BF approach, for example by using the nozzle of a nebulizer to apply a high speed gas flow [21], by

spin-coating [36–38] or electrospinning [39,40] in a humid atmosphere, by dip-coating and drying in a humid environment [21,37], or by solvent casting on a water surface [41]. The fabrication of honeycomb films by a solution-immersion phase separation method without the need for air humidity or surfactants has also been reported [42].

In this review, we will focus on the use of honeycomb films prepared by the BF method, as a very simple tool to investigate the effect of topography on cell-biomaterial interactions, and to modulate cell fate. Several excellent review articles extensively discuss the physicochemical aspects leading to the formation of BFs, main techniques, influencing factors, chemical modification and functionalization, and their reading is recommended for a comprehensive analysis of such topics [21,22,32,43–45]. In the second part of this review, we will also present an overview of the BF approach as a means to create multiscale structures with special interest for drug delivery. Finally, the main conclusions of BF in tissue engineering and drug delivery will be drawn, in a critical section where we emphasize the current unmet needs and speculate on the future directions of this exciting field.

2. Use of honeycomb films as biomaterials

For the last two decades, the BF method has been widely utilized in materials science to prepare films with highly regular hexagonally arranged surface pores. The application range for these honeycomb films is, as expected, impressively diverse. For instance, they have shown to be promising materials for the fabrication of high sensitivity biosensors [46,47], as superhydrophobic and lipophobic surfaces for dust-free coatings [48,49], as membranes with highly uniform pore size acting as microsieves for size-selective separation of particles [50,51], and as templates for the fabrication of spherical micro lens arrays with hexagonal arrangement [52], and for soft lithography [53].

In tissue engineering, honeycomb films can provide unique materials as scaffolds for cell culture, capable of modulating cell behavior. Their defined surface topography can also be used as an exploratory tool to investigate intricate cell-biomaterial interaction mechanisms established when a specific cell type adheres to the porous surface. The pores can constitute storage and protection sites

for bioadhesive molecules, growth factors or may even form site-specific immobilization sites for drug delivery systems (DDSs). The pores and rims of the BF arrays can also contribute to entrap cells that are cultured on the material (increasing cell adherence) and mimic important microtopographical features of the native ECM, stimulating horizontal contact guidance and potentially the formation of 3D cultures. Finally, the presence of pores throughout the scaffold may facilitate the flow of nutrients, oxygen and waste products (albeit depending on the specific pore distribution in the matrix). Adequate substance flow is an extremely important requirement for cell survival and function in the cell culture medium and in case of transplantation. The multifunctional roles of honeycomb films as biomaterials are depicted in Figure 2.

2.1. As scaffolds for cell culture

2.1.1. Cardiac myocytes and endothelial cells

Shimomura and his team have made remarkable progress in establishing the influence of honeycomb-patterned substrates on the adhesion and behavior of numerous cell types, and several of the team's studies will be described throughout this review. In one early work [41], honeycomb films were fabricated by spreading the polymer solution on a water surface and by solvent evaporation under humid air, which resulted in perforated films with controlled surface morphology. When the films were placed over cell adhesive dishes, i.e. type I collagen-coated, cardiac myocytes isolated from rat embryos adhered both to the honeycomb mesh and to the more adherent underlying collagen layer. However, the increased exposure of the collagen layer in films with larger pore diameter (6 μm) caused an increase in cell adherence and spreading as compared to films with smaller pores (3 μm) or to the flat counterparts [41]. While this work did not clearly demonstrate the direct influence of surface topography on the behavior of cardiac myocytes, it confirmed the possibility of using pore size as a means to modulate the contact between cell surface and adherent proteins or components of the ECM and thereby control cell proliferation.

The high porosity of the hexagonally-packed films has also been shown to enable contact of cells cultured on opposite sides of the films, acting as 3D cell culture systems. Cardiomyocytes cultured on

both sides of perforated PLLA honeycomb films demonstrated cardiac contraction after 7 days, which occurred according to a synchronized rhythm, due to cell-cell contact being possible through the pores. In opposition, when the cells were seeded on flat films, cardiac contraction rhythm was random due to hindered vertical contact [54].

The effect of pore size on morphology, adhesion and cytoskeletal organization of cardiac myocytes was demonstrated in a subsequent study [55]. Cells seeded on polycaprolactone (PCL) films with subcellular (≈4 μm) and cellular (≈8 μm) pore size demonstrated both uniaxial elongated and multidirectional spreading, in similarity to the flat films. Cells also spread easily over the pores. Focal adhesion complexes were randomly located on the flat films, and actin filaments were clear and well-organized irrespective of culture time. In contrast, vinculin (focal adhesions) was located at the edge of the honeycomb films with subcellular and cellular pores, and cells with random and clear actin filaments coexisted after 3 days of culture. In contrast, the presence of large, overcellular pores (≈13 μm) caused the cells to extend their body along the rims and to have an elongated shape. In this case, both vinculin clusters and actin filaments were clear and well organized and located at the periphery of the pores, in agreement with the elongated shape of the cardiomyocytes [55].

Fibronectin, collagen and other ECM proteins are adhesion proteins that are frequently adsorbed to biomaterials in order to facilitate cell attachment. An increasing number of studies have shown that protein adsorption is governed by surface topography, which in turn is critical for the interaction of biomaterials with cells [56,57]. Studies on the adsorption of fibronectin to PCL honeycomb-patterned films showed that the regular array of micrometer-sized pores can increase fibronectin adsorption due to the larger surface area of the biomaterial when compared to the flat films [58–60]. The protein formed a non-uniform fibrillar monolayer and was located around the edges of the pores, inside and on the bottom of the pores [58]. Both rat cardiomyocytes and porcine aortic endothelial cells were shown to proliferate on the fibronectin-coated honeycomb films [58–60]. Focal adhesions of both cell types were distributed over the entire cellular surface and located on the rims of the BF arrays, suggesting a stable adherence to the biomaterial. On the flat films, focal contact points of the cardiomyocytes were randomly distributed over entire cell bodies [59], but the endothelial cells only

adhered to the flat films at cell peripheries [58,59]. In another work, porcine aortic endothelial cells similarly formed strong focal adhesions on honeycomb films, specifically at the edge of the pores and distributed over the entire projected cell area, which contrasted with the weak focal contacts created on flat substrates. Furthermore, cells adhered and spread better on surfaces with 5 μm pores than on surfaces with larger pores (9, 12 and 16 μm) [61]. In reality, films with smaller pores imply that a higher number of pores (as well as a larger length of pore edge) is available per unit surface area for the formation of focal adhesions. Importantly, studies on endothelial cells showed for the first time that the signal transduction pathways (implicated in cell adhesion, spreading and migration) following integrin-fibronectin binding are more effectively activated on honeycomb films than on the flat counterparts [58]. Finally, honeycomb films also promoted endothelial cell-specific function as assessed by the secretion of ECM proteins, such as collagen IV [61].

The co-localization of adsorbed fibronectin and focal contact points along the rims of the film, suggested that pore size (i.e. distance between the rims around a pore) is determinant of the distance between adjacent focal points and/or density of the focal adhesions [59]. This adhesion behavior also makes it possible to guide the spreading of cardiac myocytes along a specific axis, as assessed by culturing heart cells from rat embryos over honeycomb films containing elongated pores created by mechanical stretching of the films [62]. Honeycomb films with elongated pores have similarly been shown effective in aligning bovine endothelial cells and smooth muscle cells, in a co-culture system where each cell type was cultured separately on one of the sides of the film [62].

2.1.2.Articular chondrocytes

In native cartilage, chondrocytes present a spherical morphology and are embedded in a dense ECM, containing collagen type II and proteoglycans. When cultured on a flat surface, however, chondrocytes frequently acquire an elongated fibroblast-like morphology and produce different proteins such as collagen type I, decorin and biglycan, which is suggestive of dedifferentiation and/or altered cell function [63]. Studies have shown that cell morphology and protein expression can be retained when using scaffolds with specific surface geometry and topography [64,65].

In this context, the surface topography of PLA honeycomb films (5 μm pore size) has been investigated as a factor influencing chondrocyte proliferation and properties [66]. Proliferation of chondrocytes from rabbit articular cartilage cultured on honeycomb films was lower than when the cells were seeded on flat films. However, the patterned surface caused the retention of the spherical morphology and the abundant production of ECM, whereas chondrocytes cultured on the flat films had a more flattened morphology and produced only small amounts of ECM. The retention of the spherical cell morphology on the honeycomb films was ascribed to the favorable three-dimensional surface structure of the biomaterial and to the formation of fewer focal adhesion points between the material and the chondrocytes when compared to the flat films. Cells cultured on both honeycomb and flat films produced significant amounts of glycosaminoglycans, independently of cell number; in other words, notwithstanding the lower number of cells present on the honeycomb films, glycosaminoglycans were still abundantly produced by the cells seeded on the patterned materials [66].

PCL honeycomb films with larger pores (10 μm) have similarly been shown to sustain the proliferation of rabbit articular chondrocytes and help to retain their spherical morphology [67]. The large pores can actually physically entrap the cells. Again, chondrocytes cultured on the flat PCL controls seemed to be more prone to adopt a flat morphology. Keratan sulphate (KS) is a glycosaminoglycan and an essential component of aggrecan, the major proteoglycan in the articular cartilage. After 7 days of culture, concentration of KS peaked and was significantly higher for chondrocytes cultured on the honeycomb films than on the flat controls, suggesting a superior retention of the molecule within the micrometer-sized pores and a higher capacity of the honeycomb films to induce neocartilage formation with functional ECM. DNA concentration, indicative of cell proliferation, was also higher for honeycomb films during this time period. In spite of these encouraging results, KS and DNA levels decreased on honeycomb substrates after 14 days, which was ascribed to the incomplete digestion of the samples and low access to the internal regions of the pores, rather than to a decrease in cell viability. During the 14-day study, gene expression of type II collagen was upregulated on both flat and honeycomb films, in consistency with matrix synthesis. However,

the downregulation of aggrecan and the expression of collagen I (indicative of dedifferentiation) on both samples were unexpected [67], and strongly suggest that further studies are necessary in order to safely conclude about the importance of surface porosity on the *in vitro* behavior of chondrocytes and applicability of such films in cartilage tissue engineering.

2.1.3. Osteoblasts

Bone tissue engineering is an advanced approach for bone repair, and an important solution to the problem of tissue shortage. For this reason, the field of biomaterials for bone regeneration is nowadays immense and multiple biomaterials with diverse properties have been designed and evaluated as scaffolds for growth and proliferation of osteoblasts [68]. Adhesion of osteoblasts onto the surface of such biomaterials depends on protein adsorption and formation of focal adhesion complexes, and is followed by proliferation, cell migration and finally matrix synthesis and bone-like mineral formation [69]. Noticeably, the initial phase of the osteoblast-biomaterial interaction is highly dependent on the specific physicochemical surface properties of the material and its surface topography [69–72]. Among these properties, scaffold porosity is considered one of the most important, as the pores guide the cells to proliferate into the desired 3D shape of the defect and promote vascularization of the tissue, apart from being essential for an adequate diffusion of nutrients and oxygen which is vital for cell survival and proliferation [72,73]. In addition, surface topography can have an important effect on the adsorption of biological molecules such as adhesion proteins, which can dramatically influence the initial attachment of osteoblasts to the material surface [71,73]. Chaudhuri et al [25] demonstrated for the first time that PLA and PLGA honeycomb films of subcellular pore size could enable attachment and proliferation of the anchorage-dependent osteoblast-like MG-63 cells. The porous structure did not affect the normal phenotype of the cells and additionally seemed to favor cell attachment via penetration of cell fibrils through the pores [25]. Subsequent studies [24] showed the complete preservation of cell viability of osteoblast-like MG-63 cells when cultured on honeycomb PLGA films (pore size 4-5 μm) for 1, 3 and 7 days, as compared to the tissue culture-treated polystyrene controls (TCPS). The study showed the importance of scaffold production conditions i.e. solvent, PLGA monomer ratio and humidity on scaffold structure.

However, when investigating honeycomb films with similar morphologies, the copolymer composition (PLGA 50:50; PLGA 75:25: PLGA 85:15) did not affect cell attachment or proliferation over the 7-day period of the experiment. The cells spread and flattened over the pores, and the filopodia of the leading edge of the cells were found to hook inside the pores, demonstrating that the porous structure can be used to facilitate cell attachment. These findings highlight that the regular surface topography of honeycomb films can have a favorable effect on cell-biomaterial interactions, particularly at the initial stages of cell culture [24].

Remarkably, PCL honeycomb films with different average pore diameter were all found to promote adhesion of preosteoblastic MC3T3-E1 cells, and to facilitate spreading and proliferation with better efficiency than the flat films, as confirmed by the higher cell numbers, higher number of focal adhesions and enhanced integrin expression [74]. The results were considerably better on honeycomb films with smaller pores (3.5 and 6 μm) than on surfaces containing larger pores (10 μm) (Figure 3). Furthermore, the regular surface porosity of the honeycomb films increased serum protein adsorption, which followed a linear relationship with the surface area, i.e. honeycomb films with smaller pores led to the highest protein adsorption. Alkaline phosphatase (ALP) activity and calcium content, two indicators of osteogenesis, as well as the expression of bone-specific differentiation markers were also encouragingly favored by the honeycomb porosity and particularly by the presence of smaller pores (3.5 μm). When films were stretched into groove-like structures, cell proliferation was not affected, but the new topography induced cell alignment, reduced cell area and increased cell elongation [74], in agreement with the previous studies on cardiac myocytes cultured on stretched PCL honeycomb films [62].

Primary rat osteoblasts were shown to grow and proliferate on both flat and honeycomb PCL films, but the former leading to more spread and round cells than the porous surfaces [75]. After 35 days of culture, all materials demonstrated ALP activity. However, mineralized matrix deposition was higher on the honeycomb films than on the flat films, especially on the samples with smaller pore size (3-4 μm). In some areas, the topography of the honeycomb films was found to guide matrix deposition to the rims, which may be related to the prior deposition of ECM proteins on these structures that

thereby acted as mineralization points. Smaller pore-sized samples and flat films enabled the formation of a higher number of focal adhesion structures per unit cell area than the samples with pores larger than 5 μm. The presence of cytoplasmic protrusions that extended into the pores was pointed out as a means to create an extracellular space capable of inducing the deposition of mineralized matrix [75].

These studies suggest a strong relevance of the honeycomb surface topography and pore size in bone tissue regeneration. Duan et al [76] took a step forward by taking advantage of the high air humidity to modify the surface chemistry of the honeycomb films. The authors showed that under high humidity, the polar atoms of the amino acid-substituted polyphosphazene including P and N migrated to the surface due to the higher deposition of water droplets, resulting in scaffolds with increased surface hydrophilicity. Such honeycomb films, also characterized by high surface roughness, demonstrated improved protein adsorption capacity, and better adhesion and proliferation properties for the mouse calvaria-derived osteoblast cell line MC3T3-E1, than the flat films. The surface topography additionally guided cell morphology, and the high presence of surface phosphorous elements enhanced the deposition and transformation processes of biomineralization, suggesting that these films could be efficient bioactive materials for bone regeneration. In fact, the biodegradable phosphorous-rich polyphosphazene honeycomb films could significantly stimulate osteogenic differentiation of the osteoblast cells, as assessed by the ALP activity, calcium content, von Kossa stain (calcium deposition) and collagen content [76].

In a very interesting recent article, Zhao et al [77] went on to demonstrate that the combination of positive charges and honeycomb topography can have a very encouraging effect on the behavior of mouse osteoblastic MC3T3-E1 cells. The authors prepared symmetric poly(L-lactide) (PLLA)-based dendritic L-lysine copolymer (PLLA-d) having PLLA block as the core and lysine dendrons at the two ends, which endowed the copolymer with positive charges. It was theorized that the lysine dendrons were involved in the stabilization of the condensing water droplets during the fabrication of the honeycomb films, which thereby resulted in hydrophilic, positively charged lysine dendron-rich pore walls (pore size ~2 μm). Furthermore, it was discussed that these surface properties could contribute

to form electrostatic interactions between the biomaterial surface and fibronectin (causing high protein adsorption), as well as between the biomaterial and the negatively charged cell membranes. When compared to the flat controls or to honeycomb films based on unmodified PLLA, the results were significant: the honeycomb PLLA-d showed the highest hydrophilicity, the best cell attachment, the best cell spreading area, the highest proliferation and the highest presence of filopodia, in consistency with a strong adhesion. Bone-specific gene markers were also significantly upregulated when the cells were cultured on these cationic honeycomb films. Notwithstanding the above, positive results were also obtained for the honeycomb films based on unmodified PLLA, and on the flat films based on the dendritic copolymer, although in less expressive numbers, which suggests that the individual features can also have a positive outcome, as observed in the previously discussed studies. The least promising sample was the flat unmodified PLLA control [77].

Lately, the properties of honeycomb films have been enhanced through the preparation of composite materials. One example of this is provided by Wu et al [78], who prepared poly(L-lactide) honeycomb films containing nano-hydroxyapatite for bone repair. Once again, porous honeycomb films showed better results in terms of MC3T3-E1 cell adhesion, proliferation and differentiation than the flat films, while the best results were observed for honeycomb films containing nano-hydroxyapatite – an inorganic mineral with similar chemical composition to the mineral phase of human bone. The presence of the inorganic component had a measurable influence on the homogeneity of pore formation and increased surface hydrophilicity. In turn, the encouraging results concerning the increased number of focal adhesions, cell number, spreading area and cell differentiation (as measured by ALT activity and calcification) were thought to be related with the release of Ca and P ions from the mineral [78].

A particularly interesting concept concerns the use of honeycomb films as coating materials for macroporous calcium phosphate cement (CPC) [79]. Due to its resemblance with the composition of natural bone, CPC has been pointed out as a promising material for bone repair, but macroporous scaffolds are required for enhanced permeability, which can weaken their mechanical properties. In this context, macroporous CPC scaffolds have been coated with photo-crosslinked poly(L-lactide)

triactrylate, where the coating layer was formed under moist airflow. The resulting honeycomb-patterned surface covered the macropore walls, valleys and ridges, and the layer caused an increase in the compressing strength of the CPC scaffolds, as well as in their surface area, water adsorption and protein (fibronectin) adsorption capacity. Furthermore, the presence of the coating layer effectively slowed down the degradation rate of the uncoated scaffold, but the honeycomb pores favored the ion penetration in relation to the solid-coated scaffolds (i.e. prepared without the humid airflow). In a very encouraging way, MC3T3-E1 cell adhesion, spreading, proliferation and differentiation were enhanced by the honeycomb surface in a similar fashion to the previously reported results for photo-crosslinked PCL honeycomb films [80]. The reasons for the success of CPC composites seem to be three-fold: firstly, the slow release of Ca and P ions is recognizably an important factor for bone cell growth, as pointed out above; secondly, the superior fibronectin adsorption capacity (created by the higher surface area and by the hydrophobic nature of the polymeric coating) could have improved the interactions between the biomaterial surface and integrin receptors, influencing the formation of focal adhesions; and finally, the topography itself created by the honeycomb pattern could have created a favorable microenvironment for cell response [79].

In light of these recent investigations, it becomes clear that the behavior of osteoblasts can be significantly affected by both surface topography and surface chemistry. Studies described in this section are representative of the multiple roles that honeycomb films can have in terms of the promotion of bone cell adhesion and growth. Careful biomaterial design can lead to biomimetic materials that can resemble the basement membranes, leading to optimal results in bone tissue engineering.

2.1.4. Hepatocytes

Hepatocytes have been extensively investigated as potential cell sources in liver tissue engineering but the expansion and maintenance of these cells *in vitro* has been proven very difficult, as human hepatocytes can easily suffer significant dedifferentiation and loss of gene expression and function [81,82]. Different strategies have therefore been investigated, such as finding alternative and reliable

cell sources (e.g. pluripotent stem cells), and modulating and optimizing hepatocyte polarity, multicellular organization and properties of the ECM *in vitro* [81]. The 3D multicellular organization of hepatocytes into spheroids, for instance, can exhibit higher similarity to real tissues than cell monolayers [83]. In addition, substrates with micro and nanometer scale features have been increasingly studied as a means to achieve precise control of the 3D organization of hepatocytes [82–84]. Honeycomb films have already demonstrated their potential as 3D cell culture systems for hepatocytes. In fact, culturing the liver cells on both sides of perforated PLLA honeycomb films has shown to result in double layers with a thickness of 20 µm on each side of the honeycomb film, resembling the tissue structure of the liver *in vivo*. The absence of tissue formation for non-porous controls confirms the importance of the pores in preserving vertical cell contact [54]. The pore size of the hexagonal array can also affect the adhesion and spreading of hepatocytes. Subcellular-sized pores (ca. 10 µm) have been shown to favor the spreading of primary rat hepatocytes (in spite of some cell aggregation), while cellular-sized pores (ca. 20 µm) restricted cell mobility and spreading [85]. Another work using primary rat hepatocytes demonstrated that flat surfaces facilitated cell spreading compared to the honeycomb films [86]. While the former enabled the formation of a flat cell monolayer, the latter did not prevent cell adhesion but restricted cell spreading. Cells cultured on the micropatterned surface additionally appeared as hepatocyte spheroids of approximately 100 µm, which is known to favor the hepatic function. In fact, the spheroids revealed a higher level of liver specific function than the flat cell monolayers [86]. Albumin-production, a marker for hepatic metabolic function, seems to be affected not only by the surface topography of honeycomb films but also by the size of pores [87]. Specifically, mature rat hepatocytes were more active when cultured on honeycomb films with an average pore size of 16 µm (as compared to flat films or honeycomb films with smaller pores), and small hepatocytes were similarly active on flat and 16 µm-pore sized films, but less metabolically active on films with smaller pores [87]. The yield and survival of hepatocytes cultured on flat films decreased with increasing culture time, while the opposite trend was found for the micropatterned films; this positive effect of the honeycomb films has been associated with the porous nature of the scaffold that enables the diffusion of nutrients and cellular products from the media and through the film, thus stimulating cell survival [87].

In short, micropatterned porous films seem to be able to control the attachment, proliferation and function of hepatic cells. However, in-depth knowledge concerning cell-biomaterial and cell-cell interactions is still scarce when it comes to honeycomb films and hepatocytes. Further research should be carried out in order to establish the importance of this particular surface topography on cell morphology, adhesion mechanisms, and hepatocyte function and gene expression.

2.1.5. Fibroblasts and keratinocytes

Skin tissue engineering is an interdisciplinary and expanding field in regenerative medicine that aims at developing new skin substitutes for the treatment of acute and chronic skin wounds, as well as *in vitro* skin models for test systems [88,89]. Skin tissue engineering typically starts with the isolation of primary keratinocytes and fibroblasts from the patient, which are then expanded *in vitro* and finally seeded in an appropriate scaffold [90]. In this context, the success of the process and treatment is significantly dependent on the properties of the biomaterial, which should obey a number of important requirements such as not inducing any adverse reactions after transplantation and providing an adequate biomimetic environment *in vivo*. Natural components of the ECM (e.g. collagen and fibronectin) are thus common choices, although synthetic polymers have also been gaining increasing attention [90]. In addition, evidence suggests that several factors account for keratinocyte and fibroblast fate in skin graft materials, including the chemical nature of the scaffold, its wettability and topographical features [91–95].

Thin films exhibiting a highly regular arrangement of pores can not only provide unique study models to evaluate the influence of surface topography and porosity on skin cells, but also adequate biomaterials for skin replacement. Honeycomb PLA films, for instance, have been shown to be suitable scaffolds for NIH 3T3 mouse embryonic fibroblast cells, enabling cell adherence and proliferation [96]. However, cell growth seems to be dependent on pore size (3-4 μm being better than 7-8 μm and flat controls over a 72-h period), being further promoted by PLA surfaces with higher hydrophilicity created by plasma treatment [97].

In pioneering research, honeycomb-like TiO_2 films were fabricated by BF using a single organometallic small-molecule compound (titanium *n*-butoxide or TBT) as the building block [98]. Pore size was successfully controlled by changing parameters such as TBT concentration and the airflow rate. The results showed that the porous surfaces enabled the attachment of growth of NIH T3T fibroblasts but, as shown in the previous case for PLA films [97], cell proliferation increased with decreasing pore size (4.6 > 7.7 > 10.7 μm). Noteworthy was the fact that all porous TiO_2 films were better materials for cell culture than the glass controls, and that the honeycomb film with the smallest pores was superior to the flat-bottomed polystyrene (PS) cell-culture wells [98]. Similarly, honeycomb films prepared from polypyrrole-containing block copolymers promoted the adherence and proliferation of L929 mouse fibroblasts, albeit in a pore size-dependent manner, i.e. smaller pores (<1 μm) enhancing cell attachment when compared to films containing larger pores (> 1 μm) [99].

Self-organizing porous films can be also interesting options for periodontal tissue regeneration, as demonstrated by Ishihata et al [100]. In fact, fibroblast-like cells derived from the periodontal ligament of human molar teeth have been shown to use the pillars of the PCL honeycomb structure as support structures for the attachment of the extending pseudopodia, whereas the lumen of the porous scaffolds is populated after several days by the cells which eventually grow in multilayers [100]. In a similar way, porous PCL films with pore sizes ranging between 3 and 20 μm have also demonstrated to enable adhesion and proliferation of human fibroblasts and keratinocytes, although in different degrees according to the specific pore size of the film [101]. Cell-substrate adhesion and growth of keratinocytes was significantly higher on films containing smaller pores (3 and 5 μm) than on films containing larger pores (10, 15 and 20 μm) or even on the flat counterparts, whereas fibroblasts followed the same trend although with less prominent differences. The fact that keratinocytes were so susceptible to surface topography was attributed to these cells requiring direct adjacent cell-cell contact for proper cell growth and function, which can be impaired by the large pores. Furthermore, films with small pores also have a greater surface area and a greater length of pore rims (edge) per surface area to which cells can adhere. The porous nature of the surface was also shown to somewhat limit lateral migration and spreading of both cell types. Pore size was important in transmembrane cell

migration with the small pores of 3 and 5 μm preventing the migration of keratinocytes across the biomaterial, and only the smallest pores (3 μm) preventing the migration of fibroblasts due to the capacity of these elongated cells to extend and penetrate through pores. This means that the smaller pore-sized films constitute more adequate barriers for the potential design of a bilayered human skin equivalent or dermal equivalent, since they can prevent two cell types co-cultured on opposite sides of the film from mixing [101].

Zhao and his team demonstrated that the presence of honeycomb-patterned surfaces on 3D scaffolds can have a positive outcome on cultured fibroblasts [102]. In this study, porous chitosan sponges were prepared and immersed into a PLLA chloroform solution to obtain honeycomb-patterned composites after solvent evaporation. The chitosan/PLLA patterned composites showed better mechanical properties and hierarchical porous structure than the plain chitosan sponges, used as controls. When mouse 3T3 fibroblast suspensions were cultured on the honeycomb composites, the authors observed a high seeding efficiency, apparently due to the cells being entrapped within the 3D network, in a way that exceeded the efficiency of plain chitosan sponges. The improved properties of the honeycomb-patterned composites were also patent in terms of the secretion of ECM proteins, cell viability and proliferation index, which were consistently higher than for the controls [102].

Encouraging results were similarly obtained by Du et al [29], who produced, for the first time, honeycomb films following the self-assembly of diphenylalanine (FF) peptide building blocks by the BF method. Human embryo skin fibroblasts cultured on the FF honeycomb films spread more extensively than on FF organogels, used as controls. Furthermore, cells were elongated and formed cytoplasmic projections into the pores, and developed stress fibers and focal adhesions suggesting stable cell-biomaterial attachment; conversely, only a limited number of cells were observed for the controls, and they maintained a globular morphology, adhering poorly to the underlying surface [29].

Kawano et al. showed for the first time that the elasticity of honeycomb films could expressively affect the adhesion of mouse fibroblasts [103]. In this insightful study, the authors showed that when the substrate is softer, the pores around cell edges become deformed (elongated) because of the

traction forces created by the forming focal adhesions. In opposition, more rigid substrates – created by photo-cross-linking – increased the traction force needed for cytoskeleton contraction, which was essential for cell spreading, as shown by the more favorable results obtained for harder honeycomb films, when the topography and chemical composition were the same. This effect of matrix stiffness on cell adhesion is not new, and has been increasingly investigated for the development of biomimetic substrates for various tissue engineering applications [104,105]. Work by Kawano [103] demonstrates that the honeycomb films can be interesting substrates not only to model the importance of stiffness in fibroblast adhesion, but also in the behavior of other cell types.

2.1.6. Mast cells

Mast cells originate from pluri-/multipotent bone marrow hematopoietic stem cells and they have been implicated in both innate and acquired immunity. These cells are typically released from the bone marrow into the circulatory system in an immature form, and they only undergo differentiation in the vascularized organs and tissues [106,107]. Having this principle in mind, Choi et al [106] theorized that changes in mast cell proliferation and morphology could be triggered by the microenvironment in these tissues, and hence they used honeycomb-like films of different pore size to put the theory to test. Indeed, the authors showed that both morphology and proliferation rates of a proliferative mouse non-tumor cell line of mast cells (NCL-2) were affected by the surface topography and pore size of the polystyrene micropatterned films. After 7 days, the total number of cells was higher on the honeycomb films than on the flat counterparts. Cells seeded on overcellular pores (10 μm) were retained inside the pores, where they subsequently divided. Conversely, subcellular pores (3 and 5 μm) were too small for cell penetration, causing some cells to divide and form multinuclear cells along the rims of the films, which thereafter were capable of entering the pores, as depicted in Figure 4 [106].

Mast cells are also a source of proinflammatory mediators, such as proteoglycans, proteases and biogenic amines (e.g. histamine) that are pre-formed or synthesized *de novo* in cytoplasmic granules and rapidly released in response to environmental triggers [107,108]. In recent work [108], it was

demonstrated that the extracellular environment, namely the surface topography of honeycomb films, can modulate the release of mediators from mouse mast cells. Tumor necrosis factor-α (TNF- α), leukotriene B4 (LTB-4) and substance P were always down-regulated by the porous surface, regardless of the size of pores (range 3-10 μm), when compared to the flat films. On the other hand, histamine release did not significantly differ among cells cultured on the flat or on the honeycomb films. The results seemed to suggest that the surface topography could have a pronounced effect over the release of *de novo* synthesized molecules; in addition, the use of honeycomb films was pointed out as a potential therapeutic strategy for mast cell disorders via the inhibition of mast cell activation [108]. Clearly, these studies confirm that honeycomb films can offer unique platforms for studying the effect of microenvironment on mast cell response. Further research is however required in order to establish the complex mechanisms leading to cell proliferation, and to the synthesis and release of mediators by mast cells cultured on the porous surfaces.

2.1.7. Stem cells

Stem cells are undifferentiated cells with the capability to self-renew and differentiate into specialized cells in response to appropriate signals, and as such they hold high potential for tissue engineering applications, including cell therapy and disease modeling. Cultured stem cells can be categorized as pluripotent, multipotent or unipotent. Human pluripotent stem cells (hPSCs), which can differentiate into all cell types in a body, can be obtained from early-stage, preimplantation embryos [109] or induced from adult somatic cells [110]. Tissue-specific stem cells can be multipotent, such as mesenchymal stem cells (MSCs), giving rise to several different cell types, or unipotent, such as epidermal stem cells generating only keratinocytes. In their natural environment, stem cell fate is controlled by intrinsic factors and by cellular environments, called niches. In culture conditions, in addition to biochemical stimuli, also biophysical signals such as topography, mechanical forces and stiffness of the matrix can influence the fate of stem cells [15,18,111,112].

In culture, surface topography can be used to either maintain self-renewal or to guide differentiation towards a desired direction without the use of potentially harmful chemical substances [113]. One

example concerns hPSC cultured in serum-free media, where basic fibroblast growth factor (bFGF) is typically required to maintain expression of OCT-4, a marker gene for pluripotency/stemness. Work by Kong et al. [114] showed that OCT-4 expression could be maintained in human embryonic stem cells (hESCs) without bFGF supplementation when the cells were cultured on nanotopographical surfaces with hexagonal or especially honeycomb configurations prepared by imprint lithography. The topographical configurations reduced the number and maturity of focal adhesions in hESCs affecting integrin-based focal adhesion signaling and subsequently OCT-4 expression. Disruption of integrin-based focal adhesions also appeared to increase cell–cell contact formation, which has been shown to be essential for maintenance of hESC survival and self-renewal [114].

In recent years, the potential of honeycomb-patterned films prepared by the BF method in modulation of stem cell differentiation has also attracted attention. Human MSCs cultured on honeycomb films have shown to follow specific differentiation pathways depending on the size of the pores and rims [115,116]. Specifically, films with subcellular pore size (1.6 μm and 3.2 μm) and small rim width (0.4 and 0.6 μm) led to low cell count and low cell area, due to the low surface area available for the cells to adhere, which in turn caused the cells to adopt a polygonal cell shape (spheroids). The expression of osteopontin, an osteogenic protein marker, indicated that hMSCs underwent osteospecific differentiation. Conversely, when average pore size was similar to cell size (~5 μm), cells adhered and spread significantly, and became elongated [115,116]. The myogenic protein MyoD1 was also expressed suggesting that the cells underwent myospecific differentiation. However, this differentiation pathway seems to depend on the presence of thin rims (0.8 μm), as similar pore-sized films with larger rim size (1.6 μm) failed to express the myogenic marker [115].

The presence of large, overcellular, pores implies that a significant number of cells is captured by the pores, whereas the remaining cells stretch over the pores, forming strong attachments to the rims [117]. Studies on rabbit bone marrow-derived MSCs (rMSCs) recently showed that the presence of large pores can restrict to some extent the spreading area of most cells, while still favoring the typically elongated spindle-like morphology of rMSCs [117]. In a very interesting addition to the current state-of-the-art, Liu et al coated the PCL films with bioactive molecules via layer-by-layer

(LbL) assembly, and elegantly showed that early cell adhesion, as well as proliferation and spatial distribution of cells (inside vs over the pores), depend not only on surface topography, but also on the identity of the coating polyelectrolytes. In general, rMSCs could spread better over gelatin-containing multilayer films, but remained predominantly rounded and trapped inside the pores when the surfaces contained chitosan [117]. The pioneer approach of combining the BF method with the LbL assembly, created an effective means to study cell response of rMSCs in a complex environment, where several cues – e.g. topography and surface chemistry – were modulated to regulate cell behavior. We further anticipate that the simultaneous modulation of the different physicochemical properties in such systems will result in precise control of stem cell differentiation down a specific lineage without the need for biochemical stimuli, which has to date not been investigated by these or other authors.

Adipose-derived stem cells (ASCs) are regarded as a very promising tool in regenerative medicine, since they are easily available and possess multilineage differentiation potential. Nanopatterned PLA films investigated as culture substrates for human ASCs demonstrated to have a higher fibronectin adsorption capacity when compared to the flat counterparts [118]. In spite of the lower cell numbers observed for flat and nanopatterned PLA films compared to the TCPS control – the optimized substrate for cell attachment and growth –, cell growth rates were equivalent between the three substrates. Cells cultured on the PLA substrates were also more elongated and contained fewer focal adhesion clusters compared to the TCPS control. However, both flat and porous PLA films showed higher expression of lineage-specific genes. Furthermore, the nanopatterned surface proved to remarkably induce myogenic differentiation, whereas genes involved in the cardiomyogenic, chondrogenic and adipogenic pathways were also upregulated compared to the flat surfaces (Figure 5). The authors hypothesized that the observed differences of gene expression could be related with the superior protein adsorption capacity of the patterned films [118].

Work by Tsuruma et al. elegantly demonstrated how the pore size can dramatically affect the differentiation of mouse neural stem cells (NSCs) [119]. Cells cultured for 4 days on flat PCL films differentiated into mature neural cells, but the differentiation potential was largely reduced when NSCs were cultured on honeycomb films with an average pore size of 3 μm. In fact, under these

conditions, most cells remained undifferentiated. What is interesting is that the differentiation potential into mature neurons was regained at larger porosities, increasing steadily as average pore size increased in the sequence 5 < 8 < 10 < 15 μm. Consistently, none of the cells cultured for 4 days on films with large pores revealed to be NSCs, and only a small percentage corresponded to immature neurons. The low differentiation observed for honeycomb films with pore size of 3 μm was related with the entrapment of NSCs within the small pores after cell seeding, which may have compromised cell adhesion and the intercellular signaling of differentiation. Larger pores were thus found more adequate for neural differentiation and neurite extension [119].

2.1.8. Other cell types

In an earlier study, rat mesenteric-stromal vascular cells (mSVCs) were successfully differentiated into mesenteric-visceral adipocytes (mVACs) with the intention of establishing a culture system for studies related with the pathophysiology of the metabolic syndrome [120]. However, the differentiation and function of the adipocytes dramatically decreased over time, compromising long-term studies [120,121]. Honeycomb films demonstrated a remarkable effect in maintaining differentiated mVACs in long-term culture, namely when compared with the commercial culture dish and with the flat films, prepared from the same polymer. Furthermore, while adiponectin secretion (indicative of mVAC function) decreased over time when mSVCs were cultured on the conventional and flat films, the numbers were substantially higher when mSVCs were cultured on the micropatterned surfaces, suggesting that surface topography had an important effect over cell fate. The significance of pore diameter was further demonstrated in this study, as larger pores (20 μm) provided the best results in terms of cell function, followed by the 10 μm- and 5 μm-pore-sized films [121].

In tissue engineering, high porosity in scaffolds is essential to ensure adequate diffusion of nutrients to cells, as well as diffusion of waste products and scaffold degradation products away from the tissue [17]. In retinal regeneration, this is a particularly relevant issue, as the diffusion of ions and molecular species between the choroid blood vessels and the outer retina is of the utmost importance to ensure

tissue survival and function after transplantation [10]. Recently, we tackled the need for semi-permeable films as supportive materials for retinal pigment epithelial cells (RPE) through the preparation of honeycomb 96/4 L-lactide/D-lactide copolymer (PLDLA) films [23]. The porosity was controlled by changing RH%, and the permeability of honeycomb films was assessed for the first time with the clear observation of a relationship between porosity and permeability. Furthermore, the research showed that surface topography and the hydrophobic nature of the copolymer positively influenced the adhesion of collagen, where the pores constituting 'storage' points for the ECM protein (Figure 6) [23]. Work described above by Yamamoto showed comparable absorption of fibronectin onto PCL honeycomb-patterned films [58], and in both cases the protein adsorption patterned was expected to influence the distribution of focal adhesion points of cultured cells. In a very interesting outcome, hESC-derived RPE cells – characteristically anchorage-dependent – adhered and proliferated on the collagen-coated films in spite of the high surface porosity, and demonstrated by the expression of RPE-specific markers [23].

In subsequent work, we carried out sequential Langmuir-Schaefer (LS) deposition of collagen type I and collagen type IV onto the PLDLA honeycomb films in order to create biodegradable, semi-permeable materials, with similar properties to the native Bruch's membrane, the natural RPE support 'scaffold' in the eye [10]. The deposition of two highly organized collagen layers onto honeycomb films completely covered the pores and created a biomimetic microenvironment for the hESC-RPE monolayer whilst not significantly affecting the diffusion of ions through the material [10]. The interconnected porous nature of the honeycomb films and the presence of the LS layers additionally prevented the cells from migrating to the opposite side of the scaffold. In spite of the slow degradation of the double collagen layer, the honeycomb structure was mostly preserved after 8 weeks in cell culture, with hESC-RPE demonstrating the typical RPE morphology, good expression of specific protein markers, and good phagocytic capacity toward photoreceptor outer segments [10].

In anticancer therapeutics, the cytotoxicity of anticancer agents is traditionally evaluated using 2D matrices. However, as discussed before for other tissues, such simple systems hardly reflect the *in vivo* microenvironment of tumors. The fact that 2D models are not capable of accurately mimicking

the features of tumors makes them poor predictors of the drug response in humans [122]. For this reason, it has been increasingly discussed that new engineered 3D culture models are needed to accurately replicate the microenvironmental cues found *in vivo* [37,122]. In this context, porous PLGA-based BF films have been developed as 3D model systems mimicking the microenvironment of breast cancer cells [37]. Studies using the human breast adenocarcinoma cell line MCF-7 showed that 3D tissue growth was promoted by seeding the cells on BF substrates, while glass substrates and non-porous PLGA controls restricted cell growth to a thin single cell layer (Figure 7). Additionally, cells grew favorably into well-defined ductal and lobular structures when cultured on the porous substrates, but much less so when the 2D glass substrates were used. Substrates containing larger pores also caused the cells to grow into significantly larger aggregates. The formed lobulo-alveolar acini and ductal outgrowths showed a preserved epithelial phenotype including cell-cell adhesions and the normal apico-basal polarity. The associated upregulation of genes implicated in mammary differentiation was also considerably improved by the 3D structures, particularly when the pores were larger. Importantly, the tight cell-cell contact and cell-matrix interactions in the 3D tissues made them significantly less susceptible to doxorubicin treatment than the 2D cultures created on the flat glass control, emphasizing the importance of biomimetic 3D models in drug screening [37]. These encouraging results, together with the simplicity of the BF method, anticipate that we will see, in the years to come, significant progress in the use of honeycomb films as substrates for 3D cultures.

Studies on commercial cancer cell lines can provide an indication about the biocompatibility of scaffolds and their bioadhesive properties. GFP-U87 glioblastoma cells showed good morphology and proliferation after being cultured for 24 hours on poly(ethylene glycol)-*b*-poly(lactic acid) (PEG-PLA) films containing micrometer-sized pores; in addition, cell numbers were also significantly higher than those observed when cells were cultured on flat films [123], which again highlighted the potential use of the patterned films as a matrix for cell growth. Using an innovative approach, Li et al [124] produced honeycomb-patterned films by the BF method where, for the first time, the condensing water droplets were used not only as templates but also as initiators to trigger the polymerization of the a monomer in solution into polyalkylcyanoacrylate. The biocompatibility of the newly formed

films was assessed by culturing HeLa cells (a human cervical cancer cell line) on the flat and porous polymeric materials for 48 hours, revealing once more that the porous surface could have a favorable effect in terms of cell spreading and activity when compared to the flat films [124]. Enhanced growth and proliferation was similarly observed when ordered honeycomb-structured films prepared from newly synthesized triblock dendritic poly(L-lysine)-*b*-poly(L-lactide)-*b*-dendritic poly(L-lysine) were used as the substrate for HeLa cells. It was conjectured that the promising results obtained for the porous scaffold – better than for the flat films – could be due to a superior supply of nutrients and to the greatly enlarged surface area [125]. Elsewhere, the adhesion of HeLa cells could be tuned by changing the distribution of a zwitterionic polymer on the porous film surface; in fact, both highly adhesive and antifouling surfaces could be created by simply employing distinct methods to graft the polymer to the honeycomb surface [126].

As pointed out before, cells encounter in their natural ECM environment a number of nanoscale features which directly influence their fate [13,14]. Notably, a wide number of studies have shown the importance of nanoscale features – including size, pattern and symmetry – on relevant cell types for tissue engineering [127]. In this review, we have already shown that nanopatterned surfaces prepared by BF can be used to induce stem cell differentiation down a specific lineage [118]. Recent work by Chen and colleagues describes a completely novel approach to create hierarchically structured surfaces by *in situ* growth of polyphosphazene nanoparticles (PNP) on the honeycomb surface of polystyrene films [128]. Growth of the nanoparticles on the porous surface was induced by step-by-step immersion of the films in an ethanol solution of phosphonitrilic chloride trimer and 4,4-dihydroxydiphenylsulfone, where trimethylamine acted as the acid-binding agent. The nanoparticles completely covered the outer surface of the films as well as the inner surface of the pores. In addition, the so-prepared phosphorous-containing surfaces significantly stimulated the attachment and spreading of HeLa cells, particularly when compared to the films without the polyphosphazene coating, which could be both related with the phosphorous content and the nano hierarchical structure [128]. More recently, Fragal et al developed a method based on spin-coating at high humidity to fabricate 2D nanoporous structures, where the size and distribution of the pores could be easily

tailored by changing the spin speed [38]. Few studies so far have demonstrated the easy manipulation of nanometer-scale feature size by BF, and this work offered a straightforward approach to investigate the optimal nanotopographical features for cell growth. Remarkably, kidney (Vero) epithelial-ATCC CLL-81 could sense small porosity differences, as shown by the more uniform morphology and higher cell viability of cells grown on substrates with average pore size of 346 nm than of cells cultured on substrates containing 256 nm or 1.22 μm pores, or even on flat controls. This work is a good example of the advantages that can be achieved by combining the BF method with other technologies, specifically in what concerns the precise control of pore structures and substrate thickness. In effect, this combination of methods allows the production of very thin (nanometer-sized) films within seconds, while the polymer deposition itself can be made onto a variety of substrates creating unique substrates with interest for a number of tissue engineering applications [38].

In the conventional cell culture systems, cells that grow into sheets are typically detached from the substrates following harsh treatments such as by using proteolytic enzymes. In the last two decades, we have witnessed remarkable progress in cell sheet engineering, revealing new methods for the easy detachment of cell sheets using non-invasive triggers. In the most common example, cells that are cultured on a temperature-responsive surface can be easily detached by a temperature decrease, which thereby contributes to preserve both cell-cell interactions and ECM proteins [129]. The potential use of honeycomb films in cell sheet engineering has been demonstrated by Chen et al [130]. A smart temperature-responsive honeycomb surface was created by grafting *N*-isopropylacrylamide onto the surface of porous polystyrene films. The thermoresponsive materials were shown to be biocompatible, not compromising the viability of cultured HeLa cells. When the temperature was raised above the lower critical solution temperature (LCST) of the polymer, cells attached to the hydrophobic surface due to the collapse of the polymer chains, and proliferated until the intended confluence. After that, decreasing the temperature below the LCST caused the cells to detach due to the expansion of the polymer, and creation of a hydration layer on the honeycomb surface [130].

2.2. In drug delivery

Advanced biomaterials for tissue engineering are no longer bioinert like the first generation of biomaterials, which elicited a minimal response from the host tissues. Quite the contrary, decades of research brought us to the stage where scientists indeed aim to stimulate specific cellular responses leading to the regeneration of the tissue. The honeycomb topography has been widely shown to elicit specific interactions with cell surface integrins and to influence cell proliferation, migration and differentiation, as discussed in the previous sections. Another promising possibility that has so far been poorly investigated is the design of honeycomb films that not only have the capacity to control cell fate due their intrinsic surface topography, but that additionally constitute drug depots enabling the controlled release of active therapeutics to the surrounding media or to the cells cultured on the material. The high interconnected porosity of BF films endows them with a high loading capacity, even for low solubility drugs, which can be nicely dispersed in hydrophobic polymer matrices. Moreover, studies have demonstrated that the encapsulation within the porous films contributes to reduce the 'burst release', i.e. ensuring therapeutically relevant concentrations of the active ingredient over long periods with minimum systemic toxicity [131].

Ponnusamy and co-workers were the first to correlate time-dependent BF morphology changes to drug release characteristics [36]. Drug-loaded porous films were prepared in a series of studies by a combination of spin-coating and BF processes, where the drug and the polymer were co-dissolved, and the solvent was let to evaporate under humid environment [36,132,133]. Thin (20 μm) PLGA and PEG/PLGA honeycomb films released ibuprofen and salicylic acid faster than the corresponding non-porous equivalents. Further, salicylic acid was released faster than ibuprofen in all cases, due to the superior water solubility of salicylic acid. The degradation patterns of honeycomb films showed a surface flattening where the porous layer on the surface is degraded first, followed by the gradual degradation of the underlying layers, leading to pore enlargement and ultimately to film break-up. In spite of this specific degradation pattern, it was assumed that the primary driving mechanism causing drug release was diffusion, due to the low molecular weight of the drugs and to the low thickness and high surface area of the films [36]. In very interesting work, the same team proceeded to demonstrate that drug-loaded honeycomb films could have high potential as coating materials for glaucoma

drainage devices (GDD) in the treatment of intraocular pressure [133]. The study showed that honeycomb films could constitute drug depots in implantable systems, thus increasing the chances for treatment success. From a material design point of view, it is particularly appealing to develop multiple layer systems, where each layer can elute a drug over the course of treatment. Ponnusamy et al demonstrated the versatility of honeycomb films in terms of the possibility of forming multiple layers arranged in a 'sandwich' architecture from which the antifibrotic drugs mitomycin C and 5-fluorouracil could be slowly eluted over 28 days, effectively inhibiting fibroblast proliferation – one of the main postoperative complications related with GDD implants. *In vitro* studies also showed that the presence of a drug-free honeycomb film as topmost layer successfully reduced the initial burst release, contributing to the observed long-term release times, progressing along with matrix erosion [133]. *In vivo*, the drug delivery system effectively decreased the postoperative fibrosis in the rabbit model, with no significant side effects being observed [134]. In a different version, Dai et al designed and developed a silicon GDD, which was coated with cyclosporine A (CsA)-loaded honeycomb PLGA films [135]. In this study as well, the porous coating enabled a slow, near-linear release of the antifibrotic CsA over several days, successfully causing fibroblast inhibition and decreasing scar tissue formation *in vivo*.

In other cases, however, the BF porosity has been shown to increase the drug release rate, as observed for porous fiber scaffolds prepared by electrospinning [136,137]. The faster (burst) release of β-tricalcium phosphate (TCP) from the electrospun fibers was ascribed not only to the higher level of TCP desorption from the surface, but also to the enhanced diffusion of medium through the fibers, which moreover accounts for the faster scaffold degradation observed in this study. When the scaffolds were used as substrates for human adipose derived stem cells (hASC), the porous fibers caused the highest levels of osteogenic differentiation and cell-mediated calcium accretion [136]. In spite of this exciting achievement, additional experiments would be able to clarify whether this outcome is due to the drug release kinetics itself, or due to effects related with the micro- and nanotopographical features of the materials (or possibly due to both). For example, earlier work showed that the topographical properties of similarly prepared porous electrospun fibers causes them

to adsorb ~80% more proteins than the non-porous fibers, resulting in enhanced cell attachment and survival, and improving the cell binding strength [40].

One of the main concerns related with the use of biomedical coatings is the prevention of bacterial contamination. Poly(methyl methacrylate) (PMMA) and polyurethane (PU) honeycomb films have been investigated as novel coatings for biomedical devices [138]. Isatin thiosemicarbazones (ITSCs), recognized antibacterial agents, were incorporated in the films by mixing the polymer solution with aliquots of the drug solution, followed by spin-coating. In an interesting outcome, the presence of the ITSC was found to promote the formation of the structured honeycomb surface, suggesting that important polymer-drug interactions take place during film formation. In fact, this is the first study demonstrating that the presence of small organic molecules (other than surfactants) can affect the properties of the formed films. Further, *in vitro* experiments showed a gradual release of ITSC from PMMA films, in a pH-dependent way. The results seem nonetheless to be polymer- and porosity-dependent, as no release was observed during the 24-hour dissolution study for the less porous PU films [138].

Fenofibrate can be a powerful drug against glioblastoma, particularly if intracranially delivered at a slow rate following brain tumor resection [132]. Having this in mind, Grabacka and colleagues developed double-layered porous PLGA films, where the drug was incorporated in the bottom layer prepared by solvent casting under humid airflow, and the top layer – a sealing, drug-free layer – was spin-coated under humid atmosphere. These double-layer wafers enabled a slow and constant release of fenofibrate, which actively inhibited the growth of glioblastoma cells even in a more meaningful way than the single dose of the drug administered in high concentrations. The drug-loaded porous wafers thus demonstrated exciting potential as anticancer systems for the intracranial delivery of the hydrophobic fenofibrate, which is typically poorly available in brain tumor tissue when orally administrated [132].

The high loading and slow release capacity of BF porous films has also been demonstrated by Velayudhan and colleagues who proposed the use of porous polyurethane membranes as substrates for

cell-based assays [131]. Cell-based assays are often used for toxicity and efficacy screening of multiple compounds via analysis of the effects on e.g. cell growth, proliferation and metabolism, and are considered a powerful alternative to animal studies. Even so, studies are typically carried out in multi-well plates involving numerous steps, which makes the methods time-consuming and complex. As such, it has been increasingly pointed out that suitable alternatives are needed to fulfil the increasingly demanding needs of pharmaceutical and cosmetic industries in terms of substance screening [139]. In the new cell-based assay developed by Velayudhan et al, embedded model dyes commonly used for live/dead cell staining could be slowly eluted from the honeycomb films, staining the cells directly above the membrane, and hence providing relevant information about their biological activity following exposure to test chemicals. The honeycomb-based assay is particularly advantageous as it allows multiple substances to be tested simultaneously on the same tissue, with minimum amounts of assay reagents required for a measurable response, while the method itself is faster and simpler than the most conventional cell-based assays [131].

In addition, the wide distribution of pores within BF films makes them ideal reservoirs for DDSs. Polymer microspheres [140,141] and nanoparticles [142,143], for instance, can be easily dispersed inside the pores of honeycomb films due to gravity, electrostatic interactions or by simple physical entrapment. In effect, the presence of such secondary DDSs implies not only an increase in the overall loading capacity of the system, but can also help us to modulate the drug release, and even to achieve a controlled release in response to external stimuli such as pH, temperature, ionic strength and light [144,145]. In one recent example, pH- and temperature-responsive nanogels have been electrostatically anchored to functional porous films for controlled protein delivery [144]. Homopoly(styrene) was blended with poly(styrene)-*b*-poly(acrylic acid) block copolymer (PS + PS_{19}-*b*-PAA_{10}) to fabricate negatively charged honeycomb films, or alternatively with amino-terminated poly(styrene) (PS + PS_{45}-NH_2) to create positively charged surfaces. As illustrated in Figure 8, the BF method enabled the formation of a regular array, where the charged groups selectively covered the inner surface of the pores, causing the site-specific immobilization of oppositely charged nanogels through electrostatic interactions. Furthermore, the controlled release capacity of the immobilized

nanogels was confirmed after the encapsulated protein was released following a temperature increase that triggered the collapse of the nanogel structure [144]. This study, along with others [76,77], demonstrated that functional pores can be selectively created when the hydrophilic (or charged) groups migrate toward the inner part of the holes as the water vapors fall onto the evaporating polymer solution. The same principle of pore functionalization has been employed for selective immobilization of thermoresponsive microgels, which spontaneously assemble at the water-organic interface during BF formation [146]. Elsewhere, β-cyclodextrin (β-CD)-functionalized pores have been created using of a blend of styrene and styrene modified with β-CD, and by spontaneous migration of the β-CD units towards the interface [147]. CDs are well-known 'hosts' for 'guest' drug molecules. Here, the efficiency of host-guest interactions was confirmed by the formation of high affinity complexes with adamantine, a molecule known to fit almost perfectly into the β-CD cavity. Additional advantages of the system were demonstrated, namely: i) the increased versatility created by the anchoring of adamantine end-terminated poly(acrylic acid), thereby enabling the electrostatic interactions with polycations, and ii) the complete reversibility of the host-guest interactions [147], which is an essential feature to ensure the release of the captured drugs. More recently, the preparation of smart films based on β-CD-functionalized amphiphilic block copolymers has also been described [145]. In this work, porous films based on polystyrene-*b*-poly(*N*-isopropylacrylamide-*co*-acrylate-*β*-CD) were prepared by a combination of self-assembly and BF methods, exhibiting temperature-responsive behavior as a result of the presence of the temperature-responsive poly(*N*-isopropylacrylamide) (pNIPAAm). As before, the humid environment during solvent evaporation ensured the migration of the hydrophilic moieties into the pores – specifically β-CD units –, which further contributed to stabilize the condensed water droplets and enabled the formation of the honeycomb topography. Also very interesting was the demonstration that pore features and surface hydrophilicity could be carefully controlled by simply changing the β-CD content in the hydrophilic segments of the copolymers. When the temperature was increased above the LCST of pNIPAAm, the polymer chains collapsed, causing the pores to open and increasing the overall hydrophilicity of the

surface. As expected, this effect was also responsible for the temperature-responsive release of loaded doxorubicin [145].

Using a somewhat different approach, Arora et al [148] developed a groundbreaking method for the immobilization of liposomes to the pores of the self-assembled honeycomb films. In short, the authors introduced an aerosol creating polymer-containing water droplets responsible for the formation of the BF over a spin-coated polystyrene film. After evaporation of both water and solvent, the polymer in the aerosol (hydrophobically-modified chitosan) was found deposited specifically in the pores of the BF array, creating a unique microenvironment for spatially directed immobilization of liposomes. In fact, added liposomes adhered exclusively to the pores, due to the insertion of chitosan's long alkyl-chains into the lipid bilayers, which caused the immobilization of the lipid vesicles [148].

In 2009, a groundbreaking modification of the BF method – designated *reverse* BF method – was introduced by Xiong et al [149], who showed that polymeric microsphere patterns could be prepared by casting the polymer solution onto a glass substrate in an organic nonsolvent vapor atmosphere. In this work, poly(styrene-*block*-butadiene) microspheres were created rather than a porous film, when the surface tension of the polymer solution was at least 1.5 mM/m higher than that of the condensation liquid (non-solvent), and also provided that the polymer solution had the appropriate viscosity. It was there hypothesized that the lower surface tension of the non-solvent (typically methanol or ethanol) make it easier to spread on the substrate, while reversely the higher surface tension of the polymer solution would be important to induce the formation of microdroplets and thereafter microspheres upon complete solvent evaporation [149,150]. Since then, a small number of studies have demonstrated the potential of the reverse BF method to prepare microspheres [151–153], and even asymmetrical particles [153]. An illustration of the method provided by Gong et al [153] is reproduced here in Figure 9. The fact that the reverse BF method is so recent, however, explains that only a few researchers have attempted to explore the microstructures in the biomedical field. In effect, the first study approaching the biocompatibility of reverse BF was published in 2017, where Duarte and colleagues point out the relevance of the method for tissue engineering and drug delivery [154]. In this work, poly(lactic acid) and a starch-poly(lactic acid) blend were used to prepare patterned

surfaces, where specific conditions led to microsphere formation. *In vitro* studies on a human osteogenic cell line meaningfully showed that both microspheres and honeycomb films promoted cell attachment and spreading, without detrimental effects on cell viability [154]. In the meantime, a number of other variants of the BF method have emerged reporting the successful preparation of microspheres, e.g. by static BF under water vapors using specific monomer concentrations of a linear supramolecular polymer [155], by casting polymer solutions prepared in the presence of a small amount of water followed by solvent evaporation at high RH% [156,157], and by spin-coating the polymer solution under a nitrogen humid flow [158]. A novel approach also disclosed the potential of BF in introducing the typical honeycomb porosity into microspheres prepared by non-solvent assisted electrospraying [159]. In spite of the clear progress in this field, the full potential of these microparticulate systems in drug delivery and /or tissue engineering is yet to be unveiled, as the literature is still sparse regarding appropriate *in vitro* and *in vivo* data. Furthermore, studies are still needed to reveal how the presence of the drug can affect the self-assembly process of the microspheres by reverse BF; in fact, studies on honeycomb films have already shown that interactions between polymer and drug can influence the properties of the films that are formed [138]. Optimization of production conditions will certainly be required, in a case-specific manner, to establish the best methods for drug loading and to achieve the desired drug release kinetics.

In the years to come, significant advances will probably be seen in the design of honeycomb films as unique, bio-inspired, drug-eluting scaffolds. Understandably, the biomaterial design possibilities are extremely vast if one considers all the flexibility of choice in terms of e.g. scaffold structure, composition and architecture, drug entrapment and release mechanism, in addition to the biomimicry features in the framework of the specific application (e.g. soft tissue regeneration, stem cell differentiation, bone and cartilage engineering, etc). One challenging aspect that has been difficult to tackle is the design of DDSs enabling a precise control over scaffold degradation and drug release rate, in such a way that meets the exact needs of the growing tissue [160]. Similarly, the complex topography and high surface area of honeycomb films will expectedly create a particular degradation profile with implications in drug release kinetics and cell fate. Controlled drug release may be

additionally achieved by combining honeycomb films with secondary DDSs or when employing the reverse BF method to fabricate polymeric microspheres, as described in the examples above. All these features and systems are worth special consideration in future research.

3. Conclusions and future perspectives

For many years, tissue engineers have been trying to establish the intricate cell-microenvironment relationships in order to control cell behavior *in vitro*. Several studies have shown that the BF method is unique in its simplicity to fabricate honeycomb-patterned 3D films with interconnected pores. The BF method allows us to fabricate micro- and nanoscale topographies that we can use to study cell type-specific interactions at the cell-scaffold interface and between cells. Environmental sensing by cells can be mediated via several different mechanisms, of which focal adhesion-mediated mechanosensing is among the most characterized. The magnitude of this effect is shown in this review to be significantly dependent on other surface properties such as pore size, hydrophilicity and mechanical stiffness, as well as on the cell type. Depending on their origin and differentiation stage, cells are tuned to respond to extracellular signals provided by the microenvironment in different manners. Subsequent translation into biochemical signals will modulate cell functions. The honeycomb topography can additionally endow the biomaterial with a biomimetic character, resembling the microenvironment of the native tissues, the so-called cell niche, often formed itself by micro- and nano-sized structures. This helps to explain the enhanced proliferation of specific cell types, such as osteoblasts, keratinocytes and mast cells on BF films when compared to the flat controls. At the macromolecular level, the surface topography dictates the formation and distribution of focal adhesions and actin filaments in the initial stages of adhesion, and subsequent proliferation. In addition, studies described here show that the specific pore diameter and rim width of the material can influence cell behavior in a number of ways, e.g. by affecting the physical entrapment of cells within the pores, or the cell hooking through extending filopodia. Quite often, large pores hamper the cell-cell contact and limit the lateral migration and spreading required for example for RPE monolayer maturation, while smaller pores, although providing a larger surface area for cell adhesion, prevent vertical migration across the film, which is important e.g. for osteoblast proliferation in bone tissue

engineering. Studies described here also clearly show that the distance between neighboring focal adhesions is usually dependent on the distance between the rims around the pores. In other words, when the distance between adjacent rims is smaller, the formed focal adhesion complexes are also smaller (and their density higher), resulting in a stronger anchorage. Furthermore, the fact that the tension in the anchoring points is weaker on films with smaller pores may facilitate the extension of the cytoplasm forming filopodia.

Pore distribution can have a pronounced effect on cell morphology. One exciting example of this is the elongation of cells and aligned spreading when cells are cultured on stretched honeycomb films. Evidently, these surfaces can constitute promising substrates for the growth of tissues characterized by an elongated morphology and alignment, such as smooth muscle cells and cardiac myocytes.

Noteworthy is also the exceptional capacity of honeycomb films to adsorb proteins in a site-specific manner, and to store them inside the pores. The selective adsorption of ECM proteins such as fibronectin onto the rims and pores of honeycomb films has frequently been associated with the local formation of focal adhesions, thereby generating cell attachment, and promoting cell spreading and function. Future research contemplating the extension of protein immobilization possibilities through chemical functionalization – as already reported by a small number of studies [161–163] – may offer renewed possibilities in terms of the control of cellular behavior. The Langmuir-Schaefer technologies and LbL assembly hold huge promise for the design of ECM-based multilayer coatings, adding chemical variability onto the honeycomb films and enabling the design of bioactive scaffolds for tissue engineering. In a similar fashion, multiple studies have shown that the hydrophilicity of the surface of honeycomb films can be modulated in different ways to improve cell adhesion and growth.

The importance of surface topography on stem cell differentiation has been consistently confirmed over the last years. Honeycomb films are versatile materials capable of offering the necessary topographical cues for cells to differentiate towards a specific lineage. For instance, specific pore and rim size may dictate whether hMSCs follow the osteoblastic or the myogenic differentiation pathway. A few studies also offer clues concerning the differentiation of NSCs and ASCs using honeycomb

films with specific nano- or micro-topographical features. Although quite relevant, the information is, however, still quite limited, and the precise signaling pathways leading to the observed topography-associated differentiation are still mostly unknown. What emerges is that pore diameter and rim width are determinant of the available area for the formation of focal adhesions, which consecutively affects cell adhesion and morphology. In recent years, however, it has also become evident that mechanosensing via cell-scaffold interactions is important for cell fate determination and functional cell maturation. In addition to surface topography, stem cells are especially sensitive to ECM stiffness and, in general, reproducing the stiffness of the native tissue guides stem cell differentiation into corresponding tissue lineages. Stem cells respond to matrix stiffness by a crosstalk of integrins, cytoskeleton and signal transduction machinery. For example, MSCs on scaffolds with different elasticity, have been shown to present variations in cell spreading area, actin cytoskeleton, levels of inner nuclear membrane protein lamin-A expression and focal adhesion assembly, with subsequent effects on stem cell differentiation [164]. The in-depth knowledge of the effects of surface topography on stem cell differentiation and the ability to modify stiffness without surface chemical changes [103] will make honeycomb films a simple and accessible tool for cell culture laboratories to obtain lineage-specific cells without the need for biochemical or biological agents. On the other hand, BF scaffolds may reveal to be particularly useful substrates in the study of stem cell mechanotransduction, growth and differentiation [165].

Currently, the majority of studies have concentrated on culturing cells on the surface of BF scaffolds, where smaller pore sizes of under 20 μm are typically favorable. From another perspective, the versatility of parameters controlled in the BF method is incomparable in allowing the production of different 3D film architectures with also larger pore sizes. Realization of the full potential of BF in generation of 3D scaffolds with properties attracting cell migration within the scaffolds is still in its infancy and awaits further studies. Perforated honeycomb films – where the pores go through from top to bottom – can also be favorable materials for site-specific cell culture, by enabling cells to attach to exposed underlying adhesive surfaces. On the other hand, we can simply create co-culture systems

by seeding two different cell types on opposite film surfaces, where vertical cell-cell contact is maintained through the pores.

Composite materials typically offer enhanced properties compared to the individual components. In this context, a number of studies have shown that the combination of the porous substrates with other materials can result in a biomaterial with improved properties for the intended application, such as for bone repair. In fact, honeycomb films have been shown to improve cell adhesion to the biomaterial – as a result of the improved adsorption of ECM proteins, the topographical cues or even due to better cell entrapment within the pores –, as well as the mechanical properties or substance flow through the material.

In this review, we have also shown examples demonstrating the potential of honeycomb films as DDSs, and we have highlighted their high loading capacity, their ability to host low solubility drugs and their capacity to modulate the drug release. In this respect, further studies are needed to investigate the influence of the presence of drugs during self-assembly in the BF method, and how the drug release is affected by the complex 3D structure of the scaffold. A unique feature of the BF method is that it enables the formation of functional pores through migration of hydrophilic and/or charged groups onto the material surface; this is a particularly appealing approach to modulate cell adhesion and to immobilize drug-loaded carriers such as microgels or cyclodextrins inside the pores. Such drug-loaded systems will certainly be considered in future research, particularly in terms of their use for tissue engineering and regenerative medicine purposes. In tissue engineering, selective immobilization of carriers can be particularly beneficial for the local release of biomolecules (e.g. growth factors, cytokines) to cultured cells. In regenerative medicine, drug-eluting cell carriers hold huge potential, as replacement of the damaged tissue can occur simultaneously with the elution of e.g. immunosuppressant drugs, antibiotics or growth factors. One important message to keep is that the interconnected pores of honeycomb films offer a unique environment to host other micro or nanosystems providing protection to fragile biopharmaceuticals such as proteins and nucleic acids. In effect, the localization of such carriers inside the pores causes the drug cargo to be nicely protected and inaccessible to circulating proteins and macrophages [148]. Very recently, the first studies

emerged reporting the production of microspheres by the so-called reverse BF. Although clearly still in its infancy, this field will likely grow significantly in a near future, as the microspheres hold promise as DDSs for a variety of applications. Overall, it is clear that the use of BF substrates in composites and as storage biomaterials for bioactive molecules is far from being comprehensively explored.

Finally, the most important question remains unanswered: what are the exact mechanisms by which the surface features of honeycomb films influence cell fate? So far, most studies merely tried to establish the most adequate pore size for cell adhesion and growth, while only a few of those provide data concerning cell function (e.g. in terms of gene expression and protein synthesis). Numerous researchers have been investigating the effects of different surface topographies on cell-cell and cell-biomaterial interactions, and this topic gained a new interest in recent years. While this general knowledge can offer clues to answer the proposed question, the particular nature of honeycomb films in terms of their self-assembly character (affecting surface chemistry), the organized porosity array and their 3D architecture, require extensive and specific research. More profound studies on the molecular events taking place at the honeycomb film-cell interface are needed to deepen our understanding on the dynamics and consequences of these interactions. A key point is also that the literature is still insufficient to allow comparison of data between research groups for the same cell type and utilizing comparable materials, which highlights the need for further studies. Mechanisms leading to protein adsorption, integrin binding, formation of actin microfilaments and subsequent cell spreading and cytoskeletal reorganization should be thoroughly investigated, along with the associated signaling cascades regulating protein production. Methods used previously to shed light on these issues, and also applicable here, include super-resolution microscopy to address the nanoscale architecture and spatial distribution of proteins within cell–matrix adhesions, biophysical methods to study mechanical forces on scaffold-integrin-cytoskeleton linkages, and quantitative mass spectrometry to refine the identity and regulation of the integrin adhesome [166]. When the question is answered, it will become possible to tailor the structure and composition of honeycomb films for

enhanced cell survival, growth, migration and differentiation, having in mind a variety of tissue engineering applications.

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Breath figures in tissue engineering and drug delivery: state-of-the-art and future perspectives

FIGURES

Figure 1

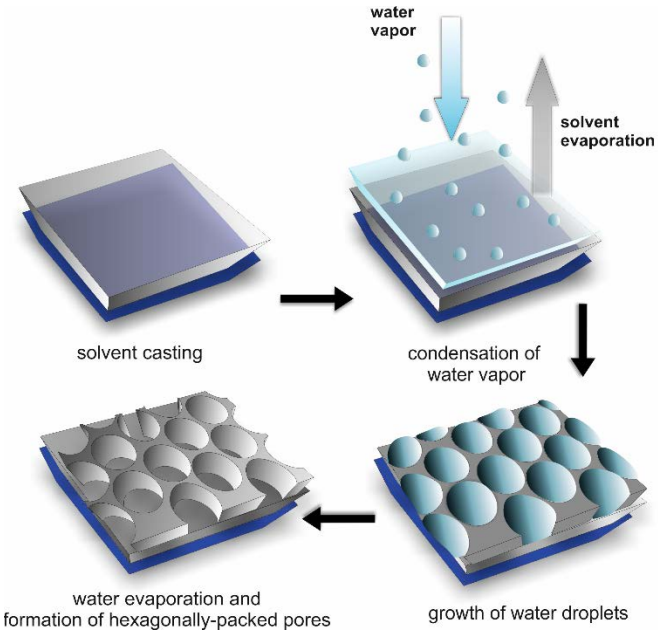
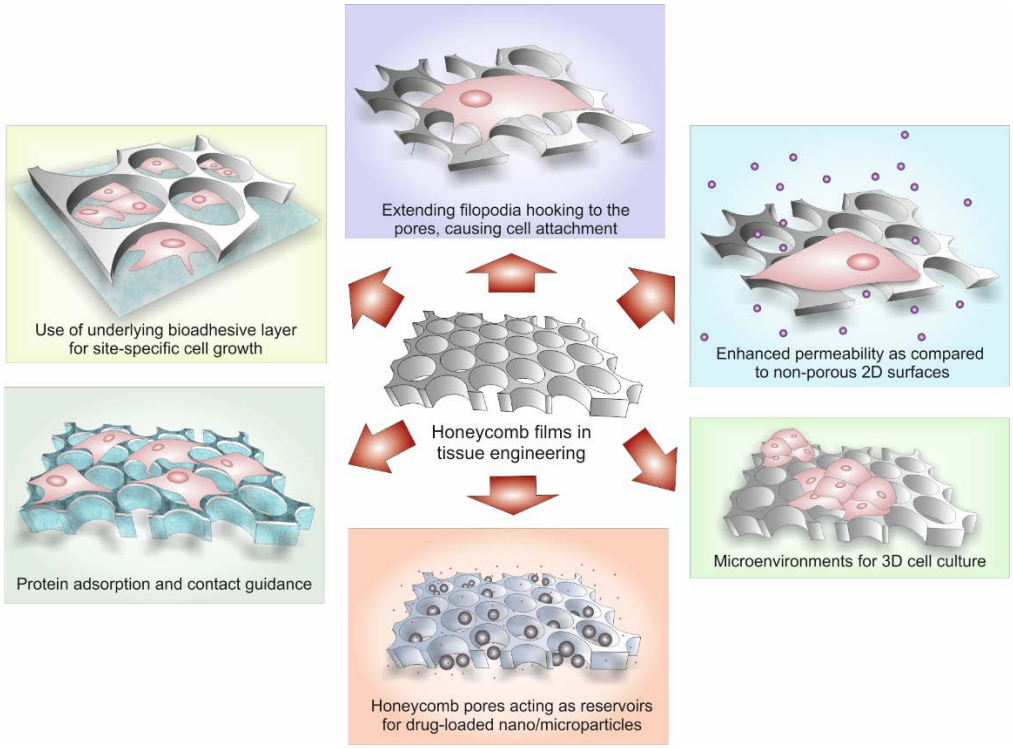


Figure 2



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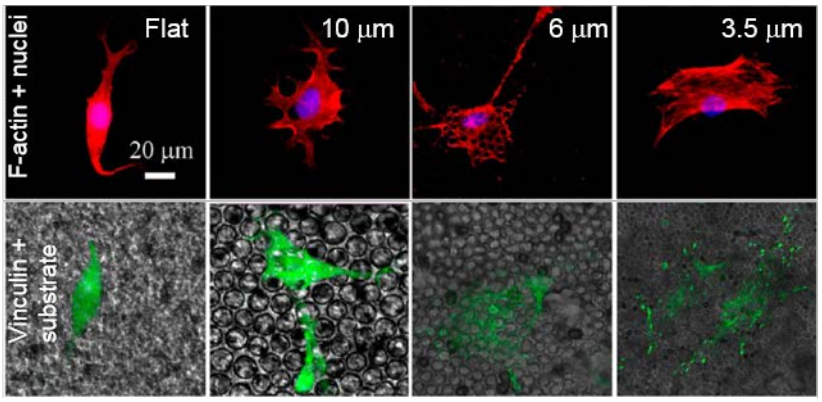

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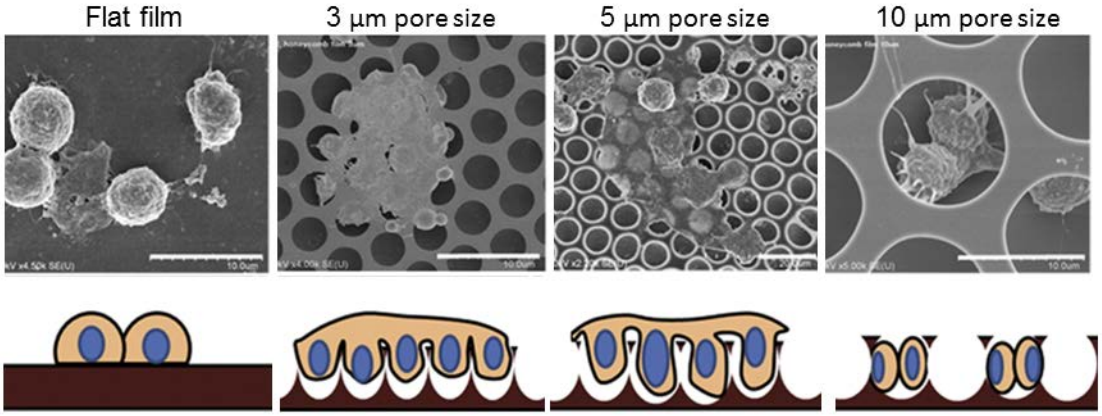

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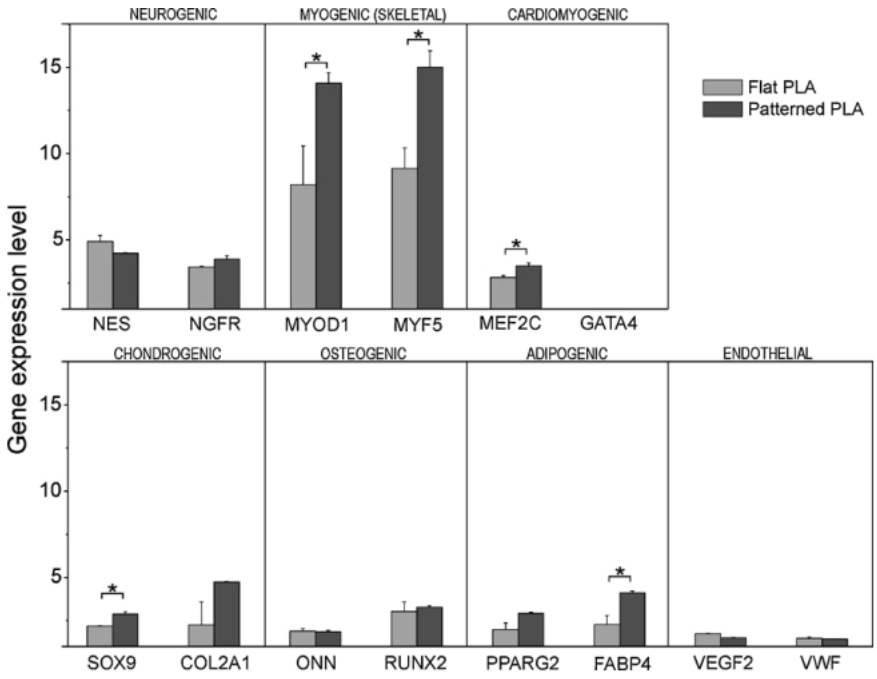

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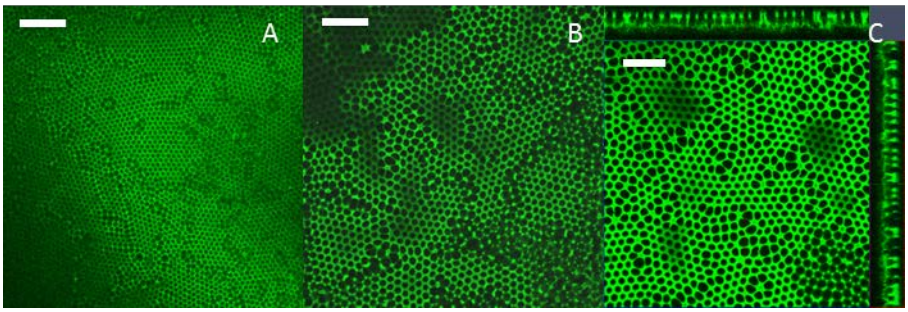

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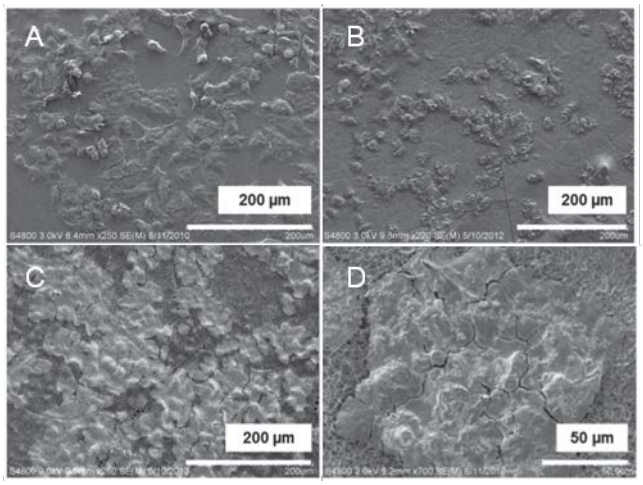

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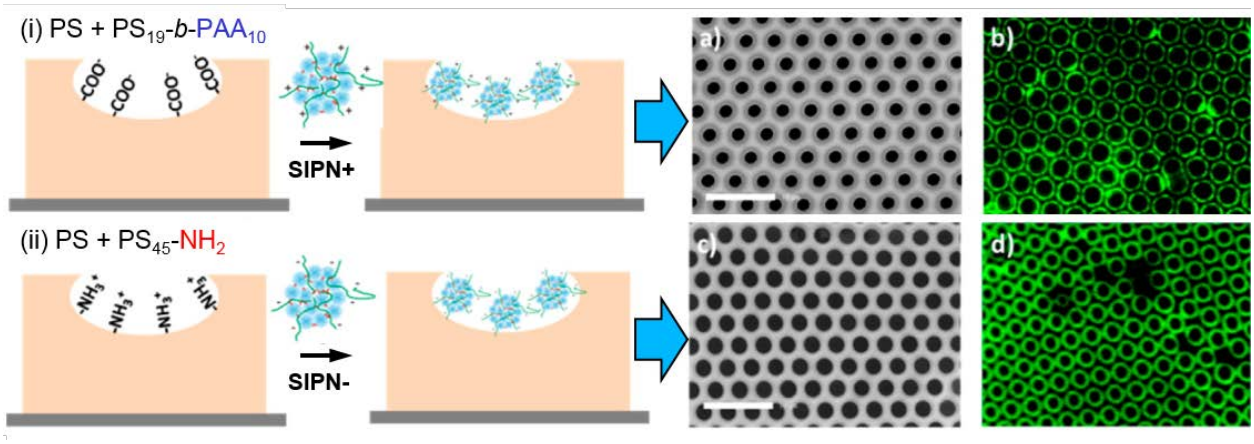

Figure 9

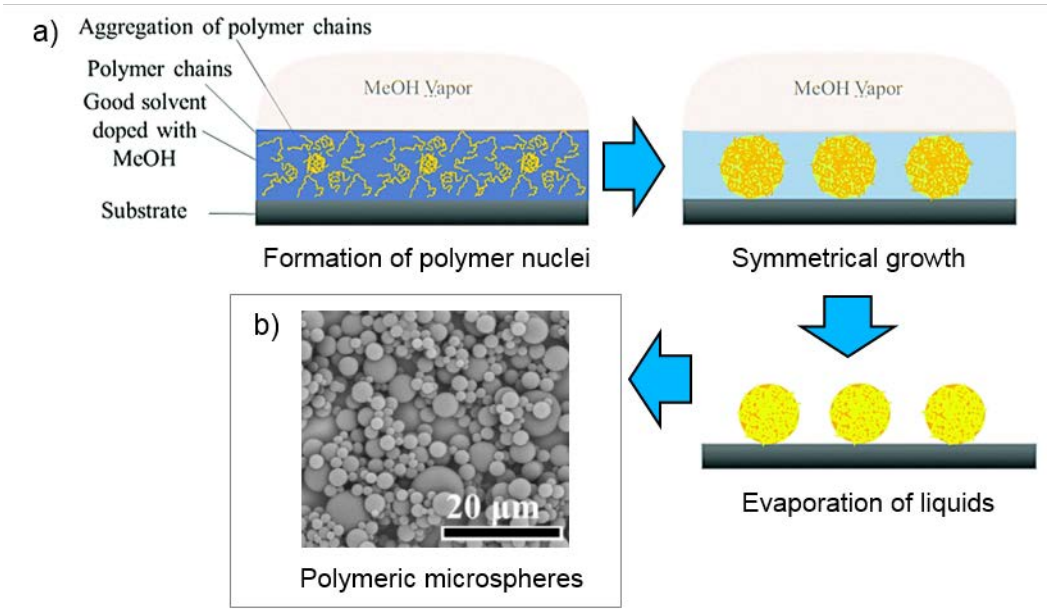

Figure captions

Figure 1. Production of honeycomb films by the breath figure method. The evaporative cooling of the solvent initiates water droplet formation on the surface of the casted polymer solution. The water droplets grow as they arrange in ordered arrays, thereafter sinking down into the polymer solution. Finally, the evaporation of both solvent and water leads to the formation of the highly porous structure.

Figure 2. Different roles and advantages of honeycomb topography in tissue engineering.

Figure 3. Preosteoblastic MC3T3-E1 cells cultured on flat and honeycomb films of distinct pore size. Green: vinculin; Red: Actin; Blue: nuclei. Adapted and reproduced from reference [74]. Copyright (2012) with permission from American Chemical Society.

Figure 4. Effect of pore size on the cell division of NCL-2 mast cells. The upper panel corresponds to SEM images (scale bar = 10 μm) and the lower panel to schematic diagrams of the cells adhering to the porous films. Adapted and reprinted from ref. [106]. Copyright (2014) with permission from Elsevier.

Figure 5. Effect of flat and honeycomb PLA surfaces on the spontaneous multi-lineage gene expression of adipose-derived stem cells (normalized to the TCPS control). NES: nestin; NGFR: nerve growth factor receptor; MYOD1: myogenic differentiation 1; MYF5: myogenic factor 5; GATA4: GATA binding protein 4; MEF2C: myocyte enhancer factor 2C; SOX9: sex determining region Y-box 9; COL2A1: collagen, typeII, alpha 1; ONN: osteonectin; RUNX2: runt related transcription factor 2; PPARG2: peroxisome proliferator-activated receptor gamma 2; FABP4: fatty acid binding protein 4; VWF: von Willebrand factor; VEGFR2: vascular endothelial growth factor receptor 2 (*p<0.05). Reproduced from reference [118]. Copyright (2011) with permission from Elsevier.

Figure 6. Adsorption pattern of collagen type IV (green) onto the rims and pores of PLDLA films with different average porosity, namely 3 μm (A), 4 μm (B) and 5 μm (C). The insets in C denote the vertical

confocal sections, showing the presence of collagen along the pore walls. Scale bar: 20 μm. Reprinted from reference [23]. Copyright (2016) with permission from Wiley Periodicals Inc.

Figure 7. Scanning electron microscopy (SEM) of MCF-7 cells. A and B: cells cultured on flat (2D) glass and PLGA films, respectively. C and D: Low and high magnifications of cells cultured on the spin-coated porous films, demonstrating the formation of a 3D multilayer tissue. Adapted and reprinted from reference [37]. Copyright (2013) with permission from Wiley-VCH Verlag GmbH & Co.

Figure 8. Illustration of the immobilization of FITC-labeled cationic (SIPN+) and anionic (SIPN-) semi-interpenetrated nanogels respectively within the anionic (i) and cationic (ii) pores of PS films. On the right side, scanning electron micrographs (SEM) of the films are shown (a), along with the fluorescence microscopy images (b) confirming the site-specific immobilization. Adapted and reprinted with permission from de Léon et al [144]. Copyright (2016) American Chemical Society.

Figure 9. a) Scheme of the mechanism leading to the formation of polymer microspheres by BF in MeOH vapor. b) SEM micrograph of PS microspheres prepared by reverse BF in THF. Adapted and reprinted with permission of the PCCP Owner Societies from Gong et al [153]. Copyright (2016), Royal Society of Chemistry.

Table 1. Summary of the main findings concerning the effect of the honeycomb topography on different cell types.

Polymer used in film preparation/ surfactant	Cell type	Properties of honeycomb film yielding the best outcome	Advantages or effect of honeycomb structure	References
PCL/ amphiphilic copolymer [a]	Rat cardiomyocytes	Honeycomb films with 13 μm pores better than substrates with smaller pores	• Porous films (13 μm) better than flat films • The honeycomb topography can control cell spreading and organization of actin filaments • Large pores cause cells to elongate in a similar way to the native fibrous-like cardiac tissue	[55]
PCL/amphiphilic copolymer [a]	Rat cardiomyocytes	Honeycomb films with 5 μm pores	• Porous films (5 μm) better than flat substrates • Focal adhesions governed by the pattern of fibronectin adsorption (around pore edges) • Focal adhesions distributed over the entire cell surface, suggesting strong attachment	[59]
PCL/amphiphilic copolymer [a]	Rat cardiomyocytes	Stretched honeycomb films (pore size 5 x 20 μm)	• Guided cell alignment and formation of fibrous tissue • Co-cultured cells formed separate monolayers on each side of the stretched film, aligned along the direction of the long axis of the micrometer-sized pores	[62]
PCL/amphiphilic copolymer [a]	Porcine aortic endothelial cells	Honeycomb films with 5 μm pores	• Porous films better than flat substrates • Increased site-selective adsorption of fibronectin and significantly increased number of focal adhesion points	[58,60]

PCL/amphiphilic copolymer [a]	Porcine aortic endothelial cells	Honeycomb films with 5 μm pores better than films containing larger pores	• Porous films (5 μm) better than flat substrates • Numerous focal adhesions over the entire projected cell area distributed along the rims • Increased cell proliferation and production of ECM proteins	[61]
PLA/DOPE or PLA/amphiphilic copolymer [a]	Rabbit articular chondrocytes	Honeycomb films with 5 μm pores	• Porous films (5 μm) functionally better than flat substrates • Lower cell numbers observed on the honeycomb films as compared to the flat substrates, but efficient retention of the normal spherical morphology of chondrocytes • Increased levels of ECM production	[66]
PDLLA and PDLLGA	Osteoblast-like MG-63 cells	Flat films better than honeycomb films with ~3 μm pores in terms of cell attachment and proliferation	• Cell attachment aided by hooking of extending fibrils to the pores (3 μm)	[25]
PLGA	Osteoblast-like MG-63 cells	Honeycomb films with 4-5 μm pores	• Porous films (4-5 μm) better than smooth TCPS controls • Increased cell numbers on honeycomb substrates • Pores acted as cell anchorage points, improving cell-biomaterial interactions	[24]

PCL/amphiphilic copolymer [a]	Preosteoblastic MC3T3-E1 cells	Honeycomb films with 3.5 μm pores better than films containing larger pores	• Porous films (3.5 μm) better than flat substrates • Improved cell adhesion and spreading • Enhanced protein adsorption • Increased ALP activity and calcium content • Improved mRNA expression of bone-specific differentiation markers • Cell elongation and alignment when cultured on stretched films, particularly when the pores were smaller (3.5 μm)	[74]
PCL/amphiphilic copolymer [a]	Primary rat osteoblasts	Honeycomb films with 3-4 μm pores better than films containing larger pores	• Porous films (3-4 μm) better than flat films • Increased bone formation • Guidance of matrix deposition • Pores acting as depots for the mineralized matrix • Increased relative abundance of small focal adhesion complexes on the surfaces with smaller pores	[75]
PLLA	Hepatocytes	Honeycomb films with ~6 μm pores	• Porous films (6 μm) better than flat films • Three-dimensional co-culture system causing the formation of a layered hepatocyte structure, resembling the liver tissue	[54]
Amphiphilic copolymer [a]	Rat hepatocytes	Honeycomb films with ~4 μm pores	• Porous films (4 μm) functionally better than flat films • Cell arrangement into spherical morphology	[86]

			• Strong adhesion but limited spreading (lower than that observed on flat substrates) • Improved hepatic function	
PCL/amphiphilic copolymer [a]	Rat hepatocytes	Honeycomb films with 16 μm- pores superior to films with smaller pores (6 and 12 μm) for mature hepatocytes	• Porous films (16 μm) better than flat films • Strong adhesion but limited spreading (lower that that observed on flat substrates) • Higher albumin production by mature hepatocytes • Higher albumin production (but comparable to flat films) by small hepatocytes	[87]
PLA/DOPE (and other surfactants)	3T3 fibroblasts	Honeycomb films with 3 μm pores prepared with DOPE better than when using other surfactants	• Enabled cell growth	[96]
PLA	3T3 fibroblasts	Honeycomb films with pore sizes 3-4 μm better than with 7-8 μm pores	• Porous films (3-4 μm) better than flat controls • Plasma treatment in combination with a honeycomb structure enhanced cell growth compared to flat controls	[97]
TiO_2	3T3 fibroblasts	Cell proliferation increased with decreasing pore size (4.6 > 7.7 > 10.7 μm)	• Cell adhesion and spreading were better on TiO2 films than on glass, and the film with the smallest pores was superior to flat-bottomed polystyrene cell-culture wells	[98]

Amphiphilic copolymer [a]	3T3 fibroblasts	Hard, UV-crosslinked, honeycomb films with 5 μm pores	• Porosity (5 μm) increased cell proliferation compared to flat or non-crosslinked films	[103]
PCL*	Human dermal fibroblasts	All honeycomb films (3-20 μm) supported cell attachment, films with small pores (3 μm and 5 μm) slightly better than films with bigger pores (10-20 μm) in terms of cell viability	• Films with small pores (3 μm and 5 μm) similar to flat controls • Cell growth dependent on pore size, better on 3 μm and 5 μm pores than on 10-20 μm pores • Transmembrane migration controlled by pore size, only 3 μm pores prevented cell migration	[101]
PCL[b]	Human epidermal keratinocytes	Honeycomb films with small pores (3 μm) better than with bigger pores (5-20 μm)	• Increased proliferation and/or survival on porous (3μm) films compared to flat films • Rims acting as hooking points for cell projections • Transmembrane migration controlled by pore size, pores over 5 μm allowed migration	[101]
Polystyrene/Amphiphilic copolymer [a]	Mouse non-tumor NCL-2 mast cells	Cell attachment and proliferation highest on honeycomb films with 10 μm pores	• Honeycomb films (3-10 μm) better than flat films in terms of cell proliferation • Entrapment of cells within overcellular (10 μm) pores • Observation of some multinucleated cells on films with 3- and 5-μm pores • Down-regulation of TNF-α, LTB-4 and substance P	[106,108]
Polystyrene/ Amphiphilic	Human MSCs	Honeycomb films with sub-cellular pores and rims causing osteo-	• Microtopography-induced morphology changes	[115]

copolymer [a], Polystyrene/DOPE		specific differentiation; films with pore size similar to cell size and rims smaller than cell size inducing myospecific differentiation	• Induction of cell differentiation by the honeycomb topography	
PCL/Amphiphilic copolymer [a]	Mouse NSCs	Differentiation suppressed on films containing small pores (3 μm); differentiation into mature neurons by films containing large pores (15 μm)	• Topography-induced modulation of differentiation • Small pores causing trapping of NSCs	[119]
PLA/DOPE	Human ASCs	Honeycomb films with pore size of ~250 nm	• Reduced cell attachment in relation to TCPS controls but significantly increased expression of lineage-specific genes e.g. involved in the myogenic, cardiomyogenic and chondrogenic pathways • Improved fibronectin adsorption compared to flat films • Pattern of protein adsorption dependent on surface topography	[118]
PCL/Amphiphilic copolymer [a]	Rat mVACs	Honeycomb films with a pore size of 20 μm	• Porous films (20 μm) better than commercial dishes and flat controls for long-term culture • Maintenance of high secretion levels of adiponectin during the 40-day culture period	[121]

			• Increased mRNA levels of both adiponectin and C/EBPa	
PLGA	Human breast adenocarcinoma MCF-7	Honeycomb films (pore size 5-30 μm) better than flat controls	• Topography-induced 3D growth of tumor with the development of mammary structures • Enhanced expression of mammary differentiation genes • Increased resistance of tissue to chemotherapeutic drugs, indicating similarities with the tissue *in vivo*	[37]
PEG-PLA	GFP-U87 glioblastoma cells	Honeycomb films containing micrometer-sized pores	• Cell numbers on films containing pores were higher than on flat films	[123]
Polystyrene with and without PNP nanoparticles on the honeycomb surface	HeLa cells	Honeycomb films containing micrometer-sized pores and nanoparticle coating promoted cell attachment and growth better than films without nanoparticles	• Cell spreading was better on nanoparticle decorated films with filopodia extended into pores	[128]
Polystyrene with pNIPAAm on surface	HeLa cells	Honeycomb structure promotes cell attachment better than flat surface	• Cell attachment on honeycomb films with NIPAAm (at 37 °C) similar to flat cell culture dish and better than films without NIPAAm or NIPAAm hydrogels • Thermoresponsive NIPAAm allows harvest of cells without enzymes	[130]

Polystyrene	kidney (Vero) epithelial-ATCC CLL-81	Films with average pore size of 346 nm better than films containing 256 nm or 1.22 µm pores	• Porous films better than flat controls • More uniform morphology and higher cell viability of cells especially on films with average pore size of 346	[3a8]
PLDLA/DOPE	hESC-RPE	Films with 5 µm pores	• Porous (5 µm) films supported cell attachment, proliferation and maturation and provided good permeability essential for subretinal applications	[23]

[a] A co-polymer of N-dodecylacrylamide and ω-carboxyhexylacrylamide (Cap)
[b] Non-disclosed use of surfactant or co-polymer

Abbreviations: PCL, polycaprolactone; PLA, Poly(lactic acid); DOPE, dioleoylphosphatidylethanolamine; PDLLA, Poly(D,L-lactide); PDLLGA, Poly(D,L-lactide-co-glycolide); PLGA, Poly(lactide-co-glycolide); PLLA, Poly(L-lactic acid); PEG-PLA, Poly(ethylene glycol)-b-poly(lactic acid); ECM, extracellular matrix; TNF-α, Tumor necrosis factor-alpha; LTB-4, Leukotriene B4; MSC, mesenchymal stem cells; NSC, neural stem cells, ASC, adipose stem cells; mVAC, mesenteric-visceral adipocytes; PNP, polyphosphazene; NIPAAm, Poly-(N-isopropylacrylamide); ESC-RPE, embryonic stem cell –derived retinal pigment epithelial cells