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Correlation between porous texture and cell seeding efficiency of gas foaming and microfluidic foaming scaffolds

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ABSTRACT

In the design of scaffolds for tissue engineering applications, morphological parameters such as pore size, shape, and interconnectivity, as well as transport properties, should always be tailored in view of their clinical application. In this work, we demonstrate that a regular and ordered porous texture is fundamental to achieve an even cell distribution within the scaffold under perfusion seeding. To prove our hypothesis, two sets of alginate scaffolds were fabricated using two different technological approaches of the same method: gas-in-liquid foam templating. In the first one, foam was obtained by insufflating argon in a solution of alginate and a surfactant under stirring. In the second one, foam was generated inside a flow-focusing microfluidic device under highly controlled and reproducible conditions. As a result, in the former case the derived scaffold (GF) was characterized by polydispersed pores and interconnects, while in the latter (μFL), the porous structure was highly regular both with respect to the spatial arrangement of pores and interconnects and their monodispersity. Cell seeding within perfusion bioreactors of the two scaffolds revealed that cell population inside μFL scaffolds was quantitatively higher than in GF. Furthermore, seeding efficiency data for μFL samples were characterized by a lower standard deviation, indicating higher reproducibility among replicates. Finally, these results were validated by simulation of local flow velocity (CFD) inside the scaffolds proving that μFL was around one order of magnitude more permeable than GF.

1. Introduction

The 3D scaffold pore architecture is a critical design variable in the development of tissue engineering constructs, influencing not only the scaffold mechanical properties, but also determining its mass transport properties. A high permeability can produce superior diffusion within the scaffold, which would facilitate the inflow of nutrients and the disposal of degradation products and metabolic waste. Additionally, pore architecture influences the ability of cells to invade and colonize the scaffold.

Three main morphological parameters may be identified in order to characterize the fabricated porous biomaterials: porosity (*P%*), pore size distribution (*PSD*) and interconnect size distribution (*ISD*). Porosity represents the ratio between the void volume and the total volume of the scaffold, while *PSD* and *ISD* can be used as indicators of the spatial homogeneity of the porous texture of the materials and are generally strictly related to the fabrication method used.

Another important parameter is represented by pores interconnectivity, defined as the percentage of the void volume that is accessible from the exterior. Pores must be highly interconnected to facilitate cell migration and the flow of nutrients. If pore size or interconnectivity are too small, cell migration will be limited, resulting in the formation of a cellular capsule around the edges of the scaffold. This, in turn, can limit nutrients diffusion and waste removal, resulting in necrotic regions within the construct [1–3]. In addition, *PSD* and *ISD* play a determinant role in the obtainment of homogeneous cell distribution within the scaffold.

During *in vitro* cell culture under perfusion, the cell suspension flow is critical to develop an evenly populated scaffold. *In vivo*, such parameters are paramount for tissue ingrowth and construct vascularization. The flow path and rate are dominated by the size of interconnects and their orientation relative to the direction of flow. If interconnects are randomly oriented with respect to the direction of flow and the relative size distribution is broad, the flow within the scaffold will be characterized by regions of different permeability and, as a consequence, by an uneven cell distribution. The inability of cells to distribute uniformly throughout a tissue-engineered construct is extremely detrimental to the intended function of developing new isotropic tissues. In the recent

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past, it has been shown that an inhomogeneous distribution of cells upon seeding is associated with low rates of tissue formation [4], a less uniform tissue [5] and a different cell differentiation behavior [6]. Therefore, a low cell seeding efficiency and heterogeneous distribution of cells are the major drawbacks of scaffolds for tissue engineering applications characterized by either small pore/interconnectivity and broad pore/interconnect size distribution. This has stimulated research into the optimization of scaffold architectures and materials.

Among the plethora of different scaffold fabrication techniques that have been developed during the past years, those that guarantee regular and tailored morphological properties such as Additive Manufacturing (AM) technologies have become the fabrication techniques of choice [7–9]. An additional, relatively simple and inexpensive scaffold fabrication technique, so far explored to a limited extent, is based on emulsion and foam templating via microfluidics. This approach is based on the ability of generating an ordered "train" of closely packed, monodisperse droplets or bubbles inside a microfluidic chip. The ensuing scaffolds, being the replica of the emulsion/foam structure prior to gelation of the continuous phase, enjoy the same high degree of order and homogeneous porous texture. Recently, we have demonstrated that this approach is very versatile with respect to the possibility of independently tailoring the size of pores and the size and number of interconnects [10]. As a consequence, we believe that our microfluidic approach represents a valid and a complementary alternative for the production of porous *sponge-like* scaffolds to rapid prototyping techniques. So far, there are few reports on the application of scaffolds produced via microfluidics in tissue engineering [11–16] and none of them have explored the correlation between porous texture of such scaffolds and the ability of cells to infiltrate within them under perfusion seeding. The aim of this work is to demonstrate that microfluidic-based scaffolds are superior with respect to scaffolds produced according to conventional emulsion or foaming techniques. In this work we compare the influence of porous texture on cell seeding efficiency and spatial distribution of cells within two different scaffolds, one prepared through a conventional gas-in-liquid foaming technique [17–21] and the other through microfluidics foam templating.

2. Materials and methods

2.1. Materials

Alginate (molecular weights 100 and 33 kg/mol) was a kind gift from FMC Biopolymer (Philadelphia, PA). N-ethyl-N′-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), 2-[N-morpholino]ethanesulfonic acid buffer (MES), cetyl trimethyl ammonium bromide (CTABr), ethanol, $SnCl_2$, and $CaCl_2$ were purchased from Sigma-Aldrich-Fluka (St. Louis, MO) and unless specified used without further purification. Argon was used as the gas phase.

2.2. Fabrication of microfluidic chips

The microfluidic chips were fabricated by a CNC milling machine (MSG402, ErgWind, Poland) from a polycarbonate (PC) sheet 5 mm in thickness. The dimension of the chip channels are as follows: height = 150 μm, orifice width = 100 μm, width of the outlet channel = 200 μm. Chips were sonicated in isopropanol for 30 min and then sealed by hot pressing at 130 °C (P = 0.5 MPa) for ca. 30 min. After sealing, the microchannels surface was modified to render it hydrophilic, following a procedure describe in the literature [22]. The reaction mixture used to modify the surface of PC consisted of an ethanolic solution of $SnCl_2$ (20% *w*/w), which was injected through the microchannels for 2 h at a flow rate of 10 mL h^{-1}. During this time, the device was kept at a constant temperature of 60 °C. The chip was then cleaned with deionized water for 20 min prior to use.

2.3. Scaffold fabrication

Scaffolds were produced by conventional gas foaming or microfluidic foaming (hereinafter denoted as GF or μFL, respectively). In both cases the composition of the aqueous phase consisted of a blend of alginates of different molecular weight (100 and 30 kg/mol used in a 4:1.5 ratio) and Tyloxapol (a nonionic liquid polymer of the alkyl aryl polyether alcohol type) was used as surfactant at a concentration of 2% and 0.2% *w*/*v* in the case of GF and μFL scaffolds, respectively. The detailed description of the apparatus and experimental procedure used in gas-in-liquid foaming have been described previously [20,21]. As far as the preparation of μFL scaffolds is concerned, [19] the experimental procedure followed consisted of regulating P_g and Q_l until a stable and close-packed 2D bubble pattern in the outlet channel was obtained. All of the scaffolds were produced with a dispersed phase fraction between 60 and 80%. When an acceptable amount of foam (≈1.5 mL) was collected inside a 3 mL glass vial, it was frozen with liquid nitrogen vapors and subsequently freeze-dried. The lyophilized solid foams were then soaked in a 2 M $CaCl_2$ solution for 24 h. The solid foams were then transferred in a 0.1 M $CaCl_2$ solution in MES buffer (0.2 M, pH = 4.5) and cross-linked with EDC/NHS. The following molar ratios were used: alginate/EDC = 3:1 and EDC/NHS = 5:1. The cross-linked scaffolds were dialyzed twice against a 1 M NaCl solution and then against distilled water until reaching a conductivity of 2–3 μS/cm followed by freeze-drying.

2.4. SEM analysis

Scaffolds microstructures were investigated by scanning electron microscopy (SEM) (LEO 1450VP) operating at 5 kV. Prior to observation, fractured samples were mounted on aluminum stubs using adhesive carbon tape. Specimens were observed without any metallization under a LEO 1450VP microscope (Carl Zeiss Microscopy, Jena, Germany) operating at 5 kV.

2.5. Micro-computed tomography (μCT) analysis

Micro-computed tomography is a non-destructive technique that generates a stack of 2D cross-sectional images of a sample using an X-ray source. Through the manipulation of these 2D images, it is possible to create a 3D reconstruction of the sample and, as a consequence, to perform 3D analyses such as Pores size distribution (*PSD*), Interconnects size distribution (*ISD*), wall thickness (WT) and percentage porosity (*P*%) [14]. Acquisition was performed on freeze-dried scaffolds using Xradia MicroXCT-400 with the following parameters: 40 kV voltage, 10 W power, no filter material, 0.18° rotation step in an angle interval of 184°. Voxel size was the same for all samples ($2 \times 2 \times 2$ μm^3). A parallelepiped volume of interest (VOI) was selected and processed resulting in a 3D binary image with physical dimension of $1.2 \times 1.2 \times 0.6$ mm. Scanning was performed at atmospheric pressure and room temperature (25 °C). Image analysis of μCT data was carried out as previously described [14].

2.6. FTIR spectroscopy

IR analysis of scaffold fragments was performed on a Nicolet 6700 FT-IR spectrophotometer (Thermo Electron Corporation, Waltham, MA) equipped with a Golden Gate accessory (Specac, Kent, UK) to perform measurements in Attenuated Total Reflection (ATR) mode. The sequence of spectra was acquired in absorbance mode in the 800–2000 cm^{-1} range, by executing 200 scans at 4 cm^{-1} resolution.

Hydrogel specimens were oven-dried at 80 °C for 4 h prior to analysis. Spectra were processed using Happ-Genzel apodization, ATR correction, smoothing and baseline correction algorithms. Deconvolution of spectra was carried out by means of PeakFit 4.12. The second and fourth