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Ice-templating of anisotropic structures with high permeability

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ABSTRACT

Nutrient diffusion and cellular infiltration are important factors for tissue engineering scaffolds. Maximizing both, by optimizing permeability and scaffold architecture, is important to achieve functional recovery. The relationship between scaffold permeability and structure was explored in anisotropic scaffolds from a human collagen I based recombinant peptide (RCP). Using ice-templating, scaffold pore size was controlled (80–600 μm) via the freezing protocol and solution composition. The transverse pore size, at each location in the scaffold, was related to the freezing front velocity, via a power law, independent of the freezing protocol. Additives which interact with ice growth, in this case 1 wt% ethanol, altered ice crystallization and increased the pore size. Variations in composition which did not affect the freezing, such as 40 wt% hydroxyapatite (HA), did not change the scaffold structure, demonstrating the versatility of the technique. By controlling the pore size, scaffold permeability could be tuned from 0.17×10^{-8} to 7.1×10^{-8} m^2, parallel to the aligned pores; this is several orders of magnitude greater than literature values for isotropic scaffolds: 10^{-9}–10^{-12} m^2. In addition, permeability was shown to affect the migration of osteoblast-like cells, suggesting that by making permeability a design parameter, tissue engineering scaffolds can promote better tissue integration.

1. Introduction

Scaffolds for tissue engineering applications are required to possess open and interconnected pore structures. Nutrient perfusion has been shown in many cases to be the key factor limiting scaffold effectiveness, and leading to necrotic areas within the scaffold center [1]. Therefore, increasing attention is being paid to the way in which scaffold pore structure is described. While porosity and pore sizes are most often reported, it has been shown that other measures, such as the size of interconnects, also has a direct effect on scaffold biocompatibility [2]. These structural features cannot be described by a simple pore size measurement, prompting research into new ways of describing scaffold architecture, such as percolation diameters and permeability [3,4].

As a parameter which relates directly to the perfusion of blood and nutrients, scaffold permeability is dependent on porosity, pore size, interconnectivity, and mechanical loading [5–7]. More importantly, permeability has been shown to affect biological behavior [8]. Increasing fluid mobility from 2.93×10^7 to 15.37×10^7 $m^4\ N^{-1}\ s^{-1}$, by varying the size of the pore interconnects, increased the chondrogenic differentiation of bone marrow stromal cells, while decreasing chondrogenic markers in mature chondrocytes [8]. Studies in vivo have also shown a relationship between the permeability and the healing in native bone tissue [2,9,10]. Conductance below a threshold of 1.5×10^{-3} $m^3\ s^{-1}\ Pa^{-1}$ resulted in a greater probability of non-union with native tissue, and thus a poor healing response [9].

Not only is interconnectivity an important factor for scaffolds, but many native tissues also possess inherent anisotropy. Bone from the femoral neck, for example, can have up to twice the permeability parallel to the femoral neck axes as opposed to perpendicular to the axes [9]. One of the most versatile systems for creating interconnected scaffolds with tunable pore size and architecture is ice-templating [11,12]. During ice-templating, an aqueous solution is frozen, allowing ice to nucleate and grow. Solutes and solid particles in the solution are excluded from the ice crystals and concentrated at the interstitial spaces. When the ice is sublimed away, the scaffold walls are an inverse of the ice structure. Thus, it is important to understand and control ice crystal nucleation and growth, which can be affected by many parameters, including the freezing protocol and the composition of the solution [12,13].

While collagen type I, the major component of the extra cellular matrix (ECM) in bone, is often used for tissue engineering constructs, concerns exist regarding the immunogenicity and batch-to-batch variation of an animal derived protein [14]. An alternative are recombinant polymers, which retain the native biocompatibility of collagen, but have a defined structure and composition, without immunogenic concerns. Like collagen scaffolds, recombinant peptides can be shaped into porous, interconnected scaffolds, with tunable properties, and they have been shown to support mesenchymal stem cell proliferation and differentiation [15]. In addition, recombinant peptide scaffolds can

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incorporate other biological cues into the scaffold structure, such as hydroxyapatite, a calcium phosphate mineral which has been shown to improve bone tissue engineering [16,17]. However, introduction of another component into the ice-templating solution could shift the permeability and pore size. In this study, utilizing ice-templating, linear scaffolds from a collagen I based recombinant peptide (RCP) were constructed, both with and without 40 wt% of biomimetically nucleated hydroxyapatite. A further modification of the solution was the addition of ethanol, which has been shown to interact with ice growth, and therefore affect scaffold structure [18].

The sum of research to date has demonstrated that anisotropic structures are required to mimic biological tissues and enhance integration, especially in musculoskeletal tissues. However, the need for porous scaffolds with high permeability is not limited to regenerative medicine, but occurs in many advanced engineering applications such as flow separation systems and catalysts [19,20]. In this study, ice-templated, anisotropic scaffolds were produced with a previously unreported range of pore sizes, from 80 to 600 μm, by controlling the freezing protocol and scaffold composition. A relationship between the permeability and porosity in the anisotropic scaffolds was described, allowing permeability to be easily tuned by altering the pore size. After determining the biocompatibility of the linear RCP scaffolds with osteoblast-like cells, the implications of the permeability in the field of tissue engineering were highlighted by studying cell migration through the scaffolds. This study delves into the physics behind scaffold formation through ice-templating and how permeability can be used as a design parameter to improve a porous scaffold's performance, either in the clinic or other engineering applications.

2. Materials and methods

2.1. Scaffold production

Unless noted, all reagents came from Sigma Aldrich. Two types of scaffolds were produced: non-mineralized and mineralized with 40 wt% hydroxyapatite (HA). For non-mineralized scaffolds, a 7.5 wt% solution of human collagen I based recombinant peptide (RCP, Cellnest™, Fujifilm) was prepared and degassed prior to ice-templating. Mineralized scaffolds were produced via a biomimetic nucleation process, which results in small amorphous hydroxyapatite (HA) particles bound to the RCP chain [21]. A solution of 20 wt% RCP was prepared with phosphoric acid (H_3PO_4) and added drop-wise, over 30 min, to a calcium hydroxide suspension, prepared by hydrating 1.72 g CaOH in 18.3 ml water. The calcium and phosphate content was calculated to have a final molar ratio of 1.67 calcium to phosphate. After all components were mixed, the pH was adjusted to 7, using 1 M HCl, and the solution was left stirring for 2 h to complete mineralization.

Prior to freezing, 1 wt% ethanol was added to the RCP solutions, unless specified, to increase the final pore size of the scaffolds. The mixture was transferred to an aluminum mold, with Teflon around the sides. The RCP solution was gelled for 20 min at 10 °C. The bath temperature was adjusted to −30 °C and held for 10 s, allowing ice to nucleate at the mold base. The temperature was then immediately adjusted to an initial bath temperature, Fig. 1, either −4 or −8 °C, and thereafter cooled with a constant freezing slope until the entire sample had frozen. The freezing slope was varied to determine the transverse pore size: 2, 1, 0.5, 0.1, 0.03 °C min^{-1}. After freezing, scaffolds were immediately lyophilized in a Zirbus freeze drier for 24 h at −15 °C, below 8 Pa. Secondary drying was completed for 10 h at 25 °C and scaffolds were stored at room temperature prior to use.

2.2. Thermal data

During ice-templating, the temperature was recorded in the RCP solution via thermocouples at 7 points, 5 mm from the edge of the mold to avoid distorted cooling rates due to edge effects. One thermocouple was placed in contact with the base of the mold. The remaining six thermocouple microprobes (IT-1E, Physitemp (USA), bead size 0.3 mm) were inserted into glass capillaries, with an outer diameter of 1 mm, which were held in place via a cap over the mold. The temperature was recorded every 0.5 s throughout freezing. Thermal profiles from each point in the solution were evaluated to assess the time spent around the equilibrium temperature, Fig. 1. The freezing front velocity was computed by finding the slope of the height in the slurry versus the time at which the temperature at each point in the slurry had dropped below −0.4 °C, indicating that no further crystallization was occurring.

2.3. Scanning electron microscopy

Prior to imaging with secondary electron microscopy, samples were sputter coated with gold. Micrographs were taken using a Jeol JSM-6335F Field Emission Scanning Electron Microscope at 5 kV. For pore size analysis, at least three micrographs from each scaffold were taken, at the top and base (10 and 4 mm from the dense region respectively). Using Image J software, individual pores were outlined and measured; the pore size reported is the average Feret's diameter of at least 40 pores.

Cells on scaffolds were imaged after fixing the sample in 3.7% paraformaldehyde for 15 min, and subsequently drying in graded ethanol dilutions. Scaffolds were incubated for 45 min in each dilution, at room temperature: 70%, 80%, 90%, and 100% ethanol. Finally, the scaffolds were washed for 10 min in hexamethyldisilazane (HMDS) and allowed to dry overnight in a fume hood. Sections were cut from the scaffolds with a razor blade and sputter coated, as before.

2.4. Micro-computed tomography

Sections of dry scaffolds, 3 mm diameter, 10 mm high, were imaged via micro-computed tomography (μCT). A Skyscan 1072 (Bruker), operating at 35 keV, 5 W, was used for all scans and reconstructions were performed using Skyskan software. 3D views of 2 × 2 × 2 mm sections were created in Image J. The resolution ranged from 3.11–4 μm $pixel^{-1}$. To determine the available interconnected volume for an object of set diameter (100 μm), a directional shrink-wrap function was applied to the scaffold reconstruction using CTan (Skyskan) [3]. Percolation diameter was also determined using the directional shrink-wrap function, as discussed by Ashworth et al. [3]. The scans were also used to compute the percent porosity:

$$\text{Percent porosity} = \left(1 - \frac{\text{volume}_{\text{scaffold}}}{\text{volume}_{\text{total}}}\right) * 100\%$$

2.5. Permeability

Permeability measurements were performed with phosphate buffered saline (PBS) at room temperature. All measurements are the average of at least three individual scaffolds. Samples of 5 mm diameter and 10 mm height were cut in the longitudinal direction (parallel to the pores) and in the transverse direction (perpendicular to the pores). Prior to permeability measurements, scaffolds were cross-linked using hexamethylene diisocyanate (HMDIC), which allows scaffolds to swell in PBS, ensuring that measurements were not significantly affected by leakage around the sample. Scaffolds were hydrated in 96.5 vol% ethanol with 0.025 g HMDIC per gram RCP scaffold in a sealed container. The volume of cross-linking solution was kept constant at 20 times the mass of the RCP. The scaffolds were then rotated at room temperature at 250 rpm for 24 h. Unreacted cross-linker was removed by washing three times in 100% ethanol, 10 min each. Excess ethanol was removed and the sample was dried overnight at 60 °C in an oven.

Immediately before testing, scaffolds were hydrated in PBS and any air bubbles were removed with a vacuum. The scaffold was