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Structural determinants of hydration, mechanics and fluid flow in freeze-dried collagen scaffolds

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ABSTRACT

Freeze-dried scaffolds provide regeneration templates for a wide range of tissues, due to their flexibility in physical and biological properties. Control of structure is crucial for tuning such properties, and therefore scaffold functionality. However, the common approach of modeling these scaffolds as open-cell foams does not fully account for their structural complexity. Here, the validity of the open-cell model is examined across a range of physical characteristics, rigorously linking morphology to hydration and mechanical properties. Collagen scaffolds with systematic changes in relative density were characterized using Scanning Electron Microscopy, X-ray Micro-Computed Tomography and spherical indentation analyzed in a time-dependent poroelastic framework. Morphologically, all scaffolds were mid-way between the open- and closed-cell models, approaching the closed-cell model as relative density increased. Although pore size remained constant, transport pathway diameter decreased. Larger collagen fractions also produced greater volume swelling on hydration, although the change in pore diameter was constant, and relatively small at ~6%. Mechanically, the dry and hydrated scaffold moduli varied quadratically with relative density, as expected of open-cell materials. However, the increasing pore wall closure was found to determine the time-dependent nature of the hydrated scaffold response, with a decrease in permeability producing increasingly elastic rather than viscoelastic behavior. These results demonstrate that characterizing the deviation from the open-cell model is vital to gain a full understanding of scaffold biophysical properties, and provide a template for structural studies of other freeze-dried biomaterials.

Statement of Significance

Freeze-dried collagen sponges are three-dimensional microporous scaffolds that have been used for a number of exploratory tissue engineering applications. The characterization of the structure-properties relationships of these scaffolds is necessary to understand their biophysical behavior in vivo. In this work, the relationship between morphology and physical properties in the dry and hydrated states was investigated across a range of solid concentrations in the scaffolds. The quantitative results provided can aid the design of scaffolds with a target trade-off between mechanical properties and structural features important for their biological activity.
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1. Introduction

The fast-growing field of tissue engineering produces new developments every day [1], yet applications outside the laboratory are still often limited to the repair of planar tissues or organs that do not act predominantly as load-bearing structures [2]. Repair of large tissue damage, and eventually whole organs, requires three-dimensional scaffolds with the ability to determine the fate of the cells seeded within their structure. This can only be achieved through precise understanding and control of the scaffold's structure and physical responses, as well as its interaction with cells and biomolecules [3,4].

Collagen-based scaffolds have been the platform for a number of exploratory clinical trials that showed the true potential of tissue engineering for repairing significant tissue damage [5,6]. Making up almost a third of total body protein [7], collagen provides cells with a large number of natural cues for cell attachment [8]. By means of a freeze-drying process, collagen can be formed into

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a highly porous structure, with the pore space defined by the growth of ice crystals [9]. Recent innovations in the understanding of this process now allow pore size and anisotropy to be precisely controlled, in structures with porosity in excess of 99% [10–12]. The resulting versatility of the freeze-drying process has prompted further studies, in which microporous tissue engineering scaffolds were fabricated from a number of different polymers. These included gelatin [13] and its blend with hydroxyapatite [14], polylactic acid (PLA) [15], polycaprolactone (PCL) [16], and chitosan and alginate [17]. Methods for the characterization of key features of such microporous scaffolds, like the presence and characteristic size of transport pathways and the multi-scale mechanical response, have been developed [18,19]. Nevertheless, the relationships between solid arrangement and scaffold physical properties are still not well understood. In this study, a combination of morphological and physical characterization techniques is used to probe these relationships, focussing specifically on the response of freeze-dried collagen scaffolds according to their solid concentration. The structure of freeze-dried materials has often been approached using geometrical considerations, since their pore morphology has been observed to be similar to that of tetrakaidecahedra, showing an average of 14 faces per pore and nearly-tetrahedral vertices [20]. Equally, the materials have previously been reported to behave mechanically as open-cell foams, where the pore volume is delimited by struts only and no pore walls are present [21]. However, this model does not reflect the morphological properties of the scaffold, since pore walls have been clearly observed in multiple studies on these materials [19,22]. The potential implications of this clear deviation from the open-cell model have not yet been studied, but are likely to influence the mechanical behavior and transport characteristics of these materials, as well as affecting the surface area available for cell adhesion.

In a preliminary study by the authors, it was shown that while the mechanical response of these scaffolds varies with solid concentration at the macroscale, it remains constant at the scale of a single cell [19]. As the material making up the structure remains unchanged and is not otherwise densified with increasing concentration, it must therefore occupy more volume in the form of either thicker struts or an increasing number of walls per pore. The aim of the current study is therefore to use rigorous morphological characterization methods to understand these changes in more detail, and to correlate them with the observed physical properties of each scaffold. The morphology is first investigated in the dry state, for ease of characterization by Scanning Electron Microscopy (SEM) and X-ray Micro-Computed Tomography (Micro-CT). As the materials are designed to function in the highly hydrated environment of the body, particular attention is then given to the effect of hydration on both morphology and mechanical properties. This includes the movement of fluid through the structure and its effect on the time-dependent deformation of the materials. In this way, the relevance of the open-cell model is assessed for predicting and explaining the functional properties required of a freeze-dried tissue engineering scaffold.

2. Experimental methods

2.1. Scaffold fabrication

Insoluble fibrillar type I collagen from bovine Achilles tendon (Sigma-Aldrich, UK) was used for scaffold fabrication using a freeze-drying technique, as previously described [19]. Briefly, collagen was suspended in 0.05 M acetic acid (Alfa-Aesar, UK), at a concentration controlled between 0.5% w/V and 1.5% w/V in increments of 0.25% w/V, and blended to form a homogeneous slurry. Freeze-drying of the slurry took place in silicone molds at approximately 5 mm filling height. The freezing step was carried out at −20 °C, following a cooling rate of 0.5 °C/min from room temperature, and the drying step took place at 0°C under a vacuum of 80 mTorr. The scaffolds were then cross-linked with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC, Sigma-Aldrich) and N-hydroxysuccinimide (NHS, Sigma-Aldrich) in ethanol-water (95% V/V), in the molar ratio 5:2:1 relative to the collagen carboxylic acid groups (EDC:NHS:COOH). The dry scaffold density, ρ^*, was measured as the weight of the samples over their volume and used in conjunction with that of collagen (ρ_S = 1.3 g/cm^3 [21]) to calculate their relative density $\left(\frac{\rho^*}{\rho_S}\right)$. The observed relationships between scaffold properties and slurry concentration are described in terms of this relative density. However, since relative density was calculated to change upon hydration (see Section 2.3), comparisons between the dry and hydrated states are described in terms of slurry concentration itself.

2.2. Morphological characterization

The morphology of the freeze-dried scaffolds was investigated by SEM in the dry state and Micro-CT in the dry and hydrated states. SEM micrographs, acquired with an EVO LS15 machine (Carl Zeiss, Germany) at an acceleration voltage of 8 kV, were used to measure the thickness of the pore walls, t_w, and struts, t_s, as a function of solid concentration in the scaffolds (Fig. 1). The expected strut diameter, $t_{s,open}$, for a tetrakaidecahedral open-cell scaffold was also calculated as a function of scaffold relative density and pore size, D_p [23]:

$$t_{s,open} = 2\sqrt{\frac{\frac{\rho^*}{\rho_S}}{1.06}}\frac{D_p}{2.78} \quad (1)$$

Measurements of D_p were made from 3D Micro-CT visualization. Samples were scanned using a Skyscan 1172 system (Bruker, BE) at 25 kV and 137 μA, with a pixel size of 4 μm. Exposure time was set at 750 ms, averaged over 2 frames, with a rotation step of 0.2°. Pore size D_p was also measured for the hydrated state, by staining samples approximately 10 mm × 10 mm × 2 mm for 48 h in a solution of 0.3% phosphotungstic acid (Sigma-Aldrich, UK) in 70% ethanol. Submerged samples were degassed under vacuum to ensure complete penetration of the staining solution. Stained samples were washed in 70% ethanol and in deionized water, before scanning in fresh deionized water at 40 kV and 250 μA with a 0.5 mm Al filter. Exposure time was increased to 1500 ms, with all other scan settings kept constant. The shadow projection images were reconstructed into 3D datasets using the Skyscan software NRecon, and binarized using the Trainable Segmentation plugin in the ImageJ software distribution FIJI. Image noise was removed using the FIJI Despeckle function in 2D, followed by a 2 × 2 × 2 median filter in 3D. Pore size D_p was calculated from 2D slices of area 8 mm^2, sampled at 100 μm spacing in three perpendicular planes. Outliers up to 2 pixels in size were removed to allow ellipse fitting using the automated Watershed and Analyse Particles functions in the FIJI software. Pore size was defined as the diameter of the circle of equivalent area to each ellipse. The average pore size for a given Micro-CT volume was calculated as the mean value over all three planes.

Micro-CT analysis was also used for numerical analysis of the pore space transport pathways through the structure, by calculation of percolation diameter as described previously [18]. Briefly, the distance accessible in a specified direction, L, to a spherical object of diameter d may be described using the following relationship:

$$L \propto (d - d_c)^{-0.88} \quad (2)$$

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