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Research Paper

Multi-scale mechanical response of freeze-dried collagen scaffolds for tissue engineering applications

Giovanni S. Offeddu[a,b], *Jennifer C. Ashworth*[b], *Ruth E. Cameron*[b], *Michelle L. Oyen*[a,*]

[a]*Engineering Department, University of Cambridge, Trumpington Street, Cambridge CB2 1PZ, UK*
[b]*Cambridge Centre for Medical Materials, Department of Materials Science and Metallurgy, University of Cambridge, 27 Charles Babbage Road, Cambridge CB3 0FS, UK*

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ABSTRACT

Tissue engineering has grown in the past two decades as a promising solution to unresolved clinical problems such as osteoarthritis. The mechanical response of tissue engineering scaffolds is one of the factors determining their use in applications such as cartilage and bone repair. The relationship between the structural and intrinsic mechanical properties of the scaffolds was the object of this study, with the ultimate aim of understanding the stiffness of the substrate that adhered cells experience, and its link to the bulk mechanical properties. Freeze-dried type I collagen porous scaffolds made with varying slurry concentrations and pore sizes were tested in a viscoelastic framework by macroindentation. Membranes made up of stacks of pore walls were indented using colloidal probe atomic force microscopy. It was found that the bulk scaffold mechanical response varied with collagen concentration in the slurry consistent with previous studies on these materials. Hydration of the scaffolds resulted in a more compliant response, yet lesser viscoelastic relaxation. Indentation of the membranes suggested that the material making up the pore walls remains unchanged between conditions, so that the stiffness of the scaffolds at the scale of seeded cells is unchanged; rather, it is suggested that thicker pore walls or more of these result in the increased moduli for the greater slurry concentration conditions.

1. Introduction

The field of tissue engineering has been a rapidly evolving one in the past two decades due to its ability to address unresolved clinical problems such as osteoarthritis (Nukavaparu and Dorcemus, 2012; Ahmed and Hincke, 2014). The principle is to use a three-dimensional polymeric scaffold, seeded with appropriate cells and growth factors, to stimulate cellular differentiation and proliferation in order to regenerate the native tissue. Most of the research done in the field has traditionally focused on the biocompatibility and biofunctionality of the scaffold. The mechanical properties of the synthetic structure play a

*Correspondence to: Michelle Oyen, Engineering Department, University of Cambridge, Trumpington Street, Cambridge CB2 1PZ. Tel.: +44 1223 33 2680; fax: +44 1223 33 2662.
E-mail address: mlo29@cam.ac.uk (M.L. Oyen).

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significant role as well (Shapiro and Oyen, 2013). This is particularly important in the case of bone and cartilage tissue engineering, where the hydrated scaffold needs to bear load in a similar way to the original tissue. Simultaneously, at the nano and micro-scale the local mechanical response influences cell behavior, as evidence suggests that cells respond to substrate elasticity (Engler et al., 2006) and viscoelasticity (Cameron et al., 2011).

Freeze-drying is a well-established method for making porous materials of controllable architecture for use in regenerative medicine applications (O'Brien et al., 2004; Davidenko et al., 2010; Pawelec et al., 2014). The process works by freezing the interstitial fluid in a suspension or slurry of the material of use, and then reducing the environmental pressure imposed on the scaffold to allow the ice formed to sublime, yielding a highly porous structure. Thin films of material form at the edges of the ice crystals as these nucleate and grow, which then constitute the walls of the pores. The use of acetic acid as a solute addition results in dendritic formation of ice and therefore, interconnected porosity (Schoof et al., 2001). Pore size can be made to be isotropic throughout the structure, except for a layer with a characteristically smaller pore size that may form at the top of the sample due to convective rather than conductive cooling on the surface of the solution (Harley et al., 2007).

The mechanical response of bulk freeze-dried scaffolds has been previously reported to depend on their density, with the modulus increasing with the concentration of solid in the materials (Harley et al., 2007). However, the reason behind the greater stiffness of the scaffolds with larger solid concentration remains unknown: the arrangement of solid within the porous structure is not well understood, and if caused by a densification of the material in the pore walls might lead to significant differences in the stiffness of the substrate experienced by the adhered cells. This work therefore, aimed to characterize the mechanical properties of freeze-dried type I collagen scaffolds both at the macro and nano-scale, where structural and intrinsic material properties, respectively, determine the response.

The scaffolds were made with varying solid concentration in the slurry and possessing different pore sizes for the same solid concentration. The macromechanical response of the bulk scaffolds was investigated by indentation in both the dry and hydrated conditions. The former is important for the handling of the materials and the latter when the scaffold is placed in the body and filled through its porous structure by interstitial fluid in the same way as the target native tissue. The presence of the fluid is expected to have an effect on the response due to its frictional drag upon the application of a load (Hu et al., 2010). At the same time, evidence of time-dependent behavior of these scaffolds has been presented (Elias and Spector, 2012). A viscoelastic framework of analysis was therefore used for the analysis of the results, which does not take into consideration the porous structure of the scaffolds and their complex poroviscoelastic behavior, yet provides information about their stiffness and extent of time-dependent deformation. The extent of the intrinsic response of the material making up the pore walls of the different conditions examined was characterized by colloidal probe Atomic Force Microscopy (AFM) on thin membranes extracted directly from the scaffolds.

2. Materials and methods

2.1. *Scaffold fabrication*

Insoluble fibrillar type I collagen from bovine Achilles tendon (Sigma-Aldrich, UK) was hydrated overnight with 0.05 M acetic acid (Alfa Aesar, UK) in two suspensions of concentrations 1% w/V and 0.5% w/V. After homogenization and centrifugation to remove air bubbles, the suspensions were poured into molds made of silicone (both concentrations) or stainless steel (SS, 1% w/V suspension only). Freeze-drying was carried out with a VirTis adVantage benchtop freeze-drier (BioPharma Process Systems, UK) using a cooling rate of 0.5 °C/min down to −20 °C. The temperature was held for two hours to ensure freezing was complete, at which point the ice was sublimed under a vacuum of 80 mTorr at a temperature of 0 °C, maintained for 20 h. The collagen sponges thus obtained were cross-linked for 2 h using a solution of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS), with ethanol-water (95% V/V) as solvent. EDC and NHS were used in the molar ratio 5:2:1 relative to the collagen carboxylic acid groups (EDC:NHS:COOH) as described by Olde Damink et al. (1996). The scaffolds were then washed five times in distilled water for five minutes each, before freeze-drying once again using the same process as described above.

2.2. *SEM characterization*

Micrographs of the scaffolds were acquired using an EVO LS15 Scanning Electron Microscope (SEM, Carl Zeiss, Germany) at an accelerating voltage of 15 kV. Pore size was measured using the linear intercept method (ASTM., 2014) on twelve SEM micrographs for each condition so to reach a count in great excess of two hundred intercepts for statistical representation. The free software ImageJ (NIH, USA) was used for the analysis.

2.3. *Mechanical testing*

The time-dependent mechanical response of the bulk scaffolds was determined by means of displacement-control indentation on an Instron 5544 universal testing machine (Instron, USA). A stainless steel spherical indenter with a diameter of 1.2 mm was used for this purpose at an indentation depth of 0.5 mm. The ramp-hold profile involved a ramp time of ten seconds and hold of 300 s, within which a force plateau was reached. Tests were performed at room temperature at four sites on each of four samples per condition, for a total of 16 tests per condition. The scaffolds were next tested in the hydrated state after being submerged in distilled water overnight. Algorithms based on exponential curve fitting of the relaxation section of the load-time response were used for the analysis, which yield values of three mechanical parameters (instantaneous modulus E_0, equilibrium modulus E_{inf}, viscoelastic ratio E_{inf}/E_0) for the materials tested (Oyen, 2005, 2006).

The induced strain ε was varied to investigate a possible effect of the small pore-sized top layer on the results. One sample per condition was indented at four different locations