JOURNAL OF THE MECHANICAL BEHAVIOR OF BIOMEDICAL MATERIALS 42 (2015) 19–25

Available online at www.sciencedirect.com
ScienceDirect
www.elsevier.com/locate/jmbbm

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

ARTICLE INFO

Article history:
Received 26 June 2014
Received in revised form
28 October 2014
Accepted 30 October 2014
Available online 11 November 2014

Keywords:
Scaffolds mechanics
Type I collagen
Indentation
Viscoelastic
Colloidal probe AFM

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).

http://dx.doi.org/10.1016/j.jmbbm.2014.10.015

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

varying the indentation depth between 0.1 and 0.5 mm. Strains were calculated using (Johnson 1985):

$$\varepsilon = 0.2\sqrt{\frac{h}{R}} \quad (1)$$

where h is the indentation depth and R the indenter radius.

Thin membranes were manually excised from the scaffolds using microtweezers and deposited onto a copper grid with an array of circular wells with a radius of 35 μm (Agar Scientific, UK). Hydration through a drop of distilled water made the membranes stick flat on the grid, with a lateral size up to two millimeters. Samples were then tested after allowing sufficient time to dry. Colloidal probe AFM on a Veeco Dimension 3100 machine (Bruker, USA) was performed using a 5 μm diameter borosilicate glass spherical tip (sQube, Germany) to indent the membranes over the center of the wells. Sixteen indents per condition were carried out. The Young's modulus E was calculated at an indentation depth h of 100 nm using the solution developed by Scott et al. for spherical indentation of free standing circular elastomer films in the plate regime (Scott et al., 2004). This is given as the sum of two contributions arising from the penetration of the indenter into the membrane and the deflection of the membrane itself:

$$E = E_{\text{penetration}} + E_{\text{deflection}} = \sqrt[3]{\frac{9P^2(1-\nu^2)^2}{16Rh^3}} + \frac{3Pa^2(1-\nu^2)}{4\pi ht^3} \quad (2)$$

P is the load recorded, a the well radius, t the thickness and ν the Poisson's ratio of the material. The assumption was made that the membrane acts as clamped around the circular well, due to the small area of indentation compared to the size of the whole membrane.

A comparison between the methods used to investigate the mechanical response of the scaffolds at the macro and nano-scale is depicted in Fig. 1.

3. Results and discussion

3.1. Pore size

Fig. 2 shows a representative micrograph for each condition. On qualitative observation, the porosity was observed to be interconnected in all samples: pore walls, with thickness below 100 nm, did not enclose the pores completely. The average pore size was measured to be 80±13 μm for 0.5% w/V, 96±19 μm for 1% w/V in silicone molds and 70±6 μm for 1% w/V in stainless steel molds, where the range is given by the standard deviation. Similarly to what has been reported previously (Tierney et al., 2009), the average pore size increased with increasing concentration of collagen in the slurry between the two conditions produced in silicone molds. The stainless steel mold samples had a slightly smaller and more uniform pore size. This is most probably due to the different heat transfer experienced by these samples: the thermal conductivity of stainless steel is greater than that of silicone, therefore the samples experienced a faster cooling rate, expected to result in a smaller pore size (O'Brien et al., 2004).

3.2. Viscoelastic response of bulk scaffolds

The top 50–100 μm of each scaffold was observed to have smaller pore size compared with the bulk (Fig. 3a). The effect of this surface layer was considered by varying indentation strain to vary the amount of bulk material included, but was found to have no effect on the instantaneous modulus (Fig. 3b). It was found that the values measured are comparable for the indentation depths considered despite the smaller strain being at an indentation depth in the range of the layer thickness. From composite mechanics calculations, Harley and co-workers deduced that the top layer does not affect the compressive modulus of the scaffolds (Harley et al., 2007), and the results presented here seem to confirm this.

The results obtained from the study of varying slurry concentration and porosity are reported in Fig. 4 for both the dry and hydrated states.

A number of observations can be made: the difference between the dry and hydrated states is seen first in that the moduli in the former are always greater. This is most probably due to the flow of interstitial fluid and its viscous drag, as well as the plasticizing effect of the fluid on the collagen fibrils. The latter was previously observed to make these more compliant (van der Rijt et al., 2006), and the two phenomena result in a more compliant bulk response in the hydrated state.

The viscoelastic ratio, which varies inversely with the degree of viscoelastic relaxation, was found to be independent of concentration of collagen in the slurry and pore size. A

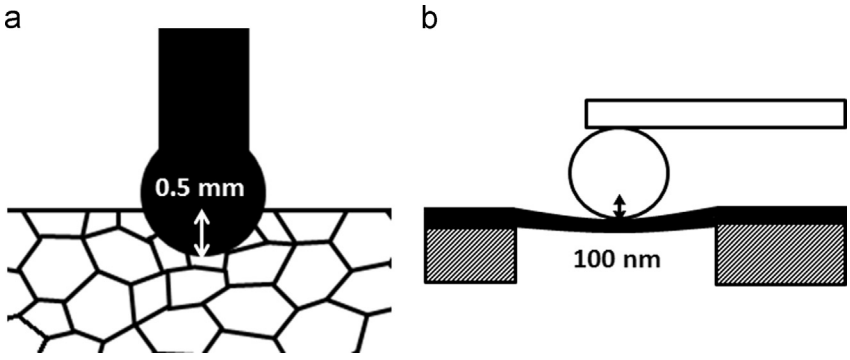

Fig. 1 – Comparison of indentation depth between (a) spherical macroindentation of bulk scaffolds and (b)colloidal probe AFM of thin membranes.

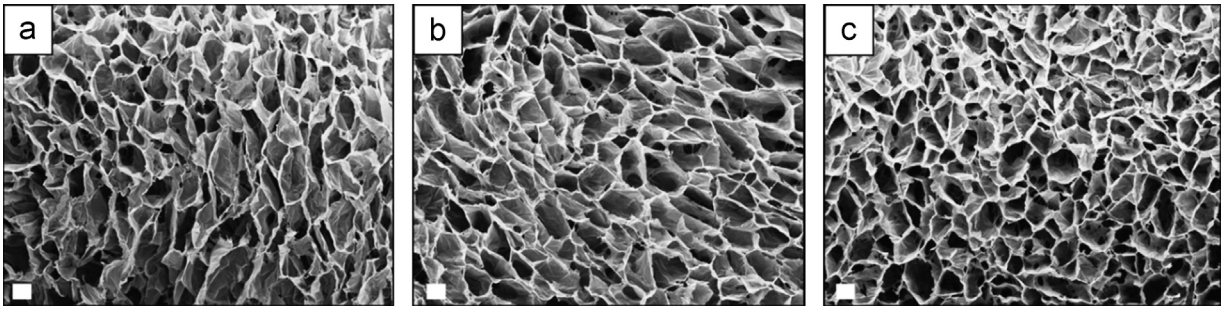

Fig. 2 – Freeze-dried collagen scaffolds, vertical cross-section, (a) 0.5% w/V, (b) 1% w/V in silicone molds, and (c) 1% w/V in stainless steel molds. The scale bar is 100 μm.

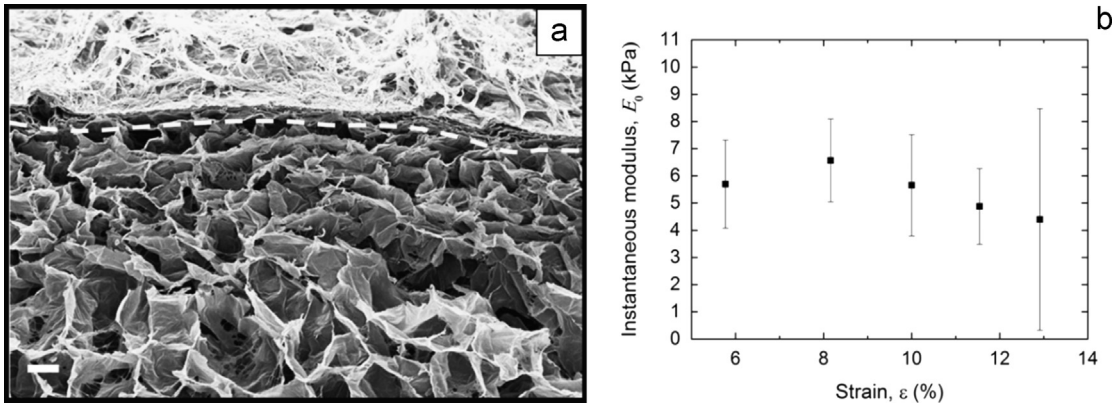

Fig. 3 – (a) Top layer SEM micrograph of a freeze-dried 0.5% w/V collagen slurry sample. The dashed line shows the extent of the anomalous porosity. The scale bar is 100 μm. (b) Instantaneous modulus of a hydrated sample as measured by spherical indentation as a function of strain. The error bars express the standard deviation.

previous study on freeze-dried collagen scaffolds reported that the degree of relaxation increases with increasing solid concentration in the scaffold (Elias and Spector, 2012). The large variability observed in the results might have masked this trend. However, it is clear that the extent of relaxation is actually smaller for the hydrated scaffolds, *i.e.* the viscoelastic ratio is larger, and this could be the result of the presence of the interstitial fluid and the additional pressure it exerts on the collagen structure, hindering its buckling with time. This interesting and perhaps counter-intuitive result has also been observed in other hydrated materials, including hydrogels (Galli et al., 2009).

A dependence on slurry composition of the elastic stiffness of the scaffolds can be observed both for the dry and hydrated samples, whereby the greater concentration of collagen in the slurry results in larger moduli of the freeze-dried scaffolds. The average modulus of the samples made in stainless steel molds was lesser than that of the ones made in silicone molds with the same concentration in the slurry. The large variability displayed by the former might result in this dissimilarity.

The mechanical response of these materials has been previously shown to resemble that of open-cell foams, in which pores with no walls are delimited by struts of material at their edges (Gibson, 2005). From basic cellular materials mechanics, the stiffness of open-cell porous scaffolds is expected to be directly proportional to the square of their relative density (Gibson and Ashby, 1997):

$$E \propto \left(\frac{\rho_*}{\rho_S}\right)^2 \tag{3}$$

where ρ_* and ρ_S are the scaffold density and that of the solid material which composes it, respectively. It follows that doubling the concentration by volume of collagen in the scaffold would increase four-fold its stiffness. The relationship was observed within error for the materials made in silicone molds, suggesting that the concentration of collagen in the freeze-dried scaffolds possibly varies linearly with that in the slurries.

It remains to be understood how the larger stiffness of the greater concentration scaffolds results from the different arrangement of collagen within the same volume. When l is the average length of the pores struts, and t their average thickness, then (Gibson and Ashby, 1997):

$$\frac{\rho_*}{\rho_S} \propto \left(\frac{t}{l}\right)^2 \tag{4}$$

The larger pore size with increasing solid concentration in the scaffold implies that l becomes larger. An increase in the average thickness of the struts could then result in the larger moduli measured for the greater concentration scaffolds. However, the materials investigated here are not ideal open-cell solids, as struts were not observed, and it is rather the walls partially enclosing the pores that define their edges. These are expected to contribute to the mechanical response depending on the amount of solid present in them (Gibson and Ashby, 1997). It is therefore possible that a larger quantity of

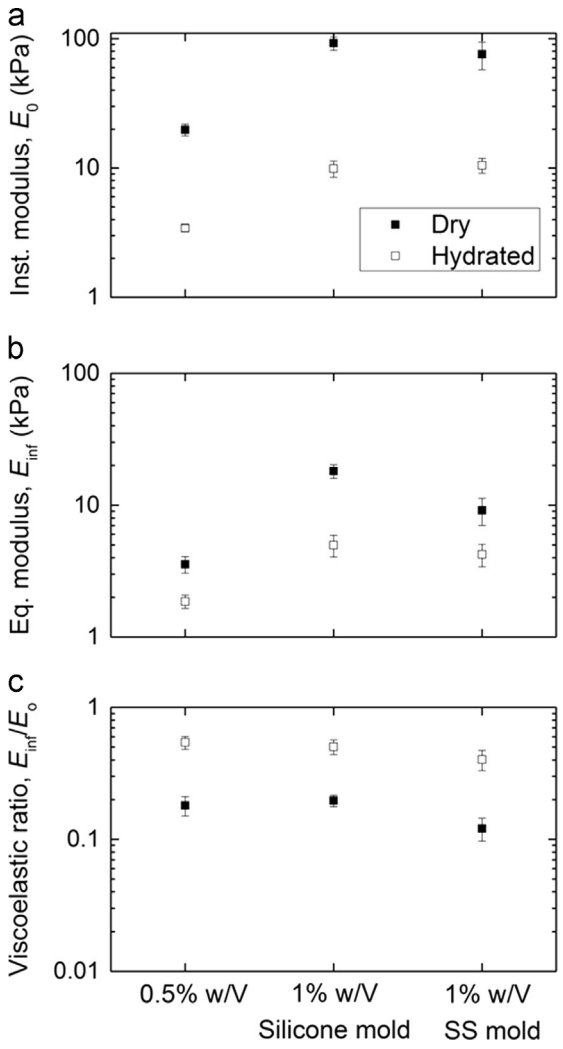

Fig. 4 – Viscoelastic parameters of dry and hydrated collagen scaffolds: (a) instantaneous modulus, (b) equilibrium modulus, (c) viscoelastic ratio. The error bars express the standard error of the mean.

solid in the faces, either by means of densification of the material or by thicker cell walls, led to the greater stiffness of the larger concentration scaffolds. The average number of pore walls present per pore is also expected to affect the stiffness of the constructs as the material's behavior shifts towards that of a closed-cell foam, where pores are completely enclosed by walls: Roberts and Garboczi have computed models of partially open-cell foams and found that an increase in the number of pore walls leads to greater moduli (Roberts and Garboczi, 2001). Both phenomena, the larger solid amount in the pore walls and greater number of pore walls, could therefore result in the densification of the porous material and the resultant increased modulus, and may happen concurrently.

3.3. *Elastic response of membranes*

The overall thickness of the extracted membranes at the point of indentation could not be measured, but was recorded to be in the range of 4–10 μm in all direct observations by SEM, which showed that they consisted of stacks of pore walls collapsed on to the grid (Fig. 5).

All load–displacement profiles for AFM indentation showed a linear relationship (Fig. 6a) indicative of a plate regime, *i.e.* one for which the stretching of the membrane is negligible due to small deflections, and the response is purely elastic. Komaragiri and coworkers found that for a thickness to well span ratio greater than 0.075, corresponding to a membrane thickness of 1.3 μm or larger in this case, a non-linear response will not be observed irrespective of load and indentation depth imposed (Komaragiri et al., 2005).

The load recorded upon indentation at 100 nm was averaged per condition and found to be 35.88±2.26 nN for 0.5% w/V, 33.76±2.31 nN for 1% w/V in silicone molds, and 37.17±2.49 nN for 1% w/V in stainless steel molds. The comparable load measured for the different conditions suggests that the mechanical properties of the material composing the scaffolds are not altered as a consequence of varying solid concentration or pore size. Therefore, that densification of the material is not the cause of the trend observed for the bulk scaffolds results.

The average load recorded per condition was used in conjunction with Eq. (2) to calculate the Young's modulus of the membranes as a function of their unknown thickness within the range observed (Fig. 6b). The modulus measured ranged between 400 kPa and 700 kPa, depending on the thickness. In the calculation, the contribution of the indenter penetration into the membrane, independent of membrane thickness, becomes larger than that due to bending of the membrane for thicknesses greater than 4 μm, eventually dominating the response. The membrane thicknesses

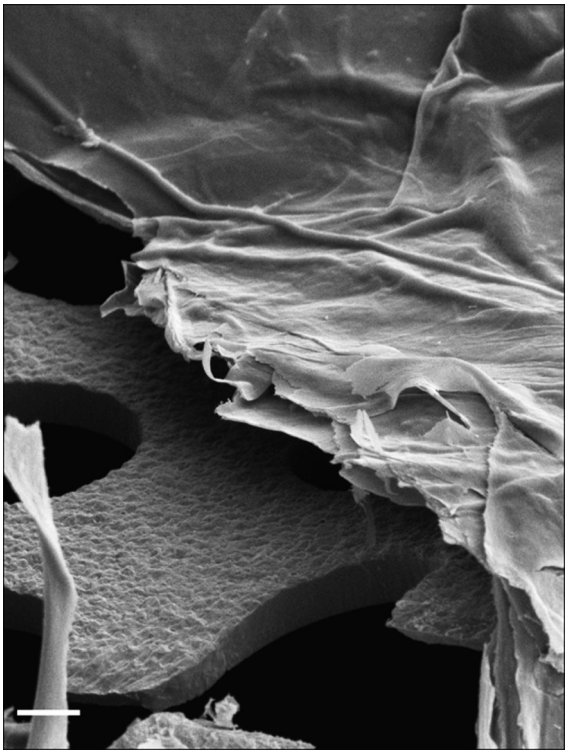

Fig. 5 – 1% w/V in silicone molds membrane deposited on a copper grid. A stack of single pore walls can be seen lying parallel to the substrate. Scale bar is 10 μm.

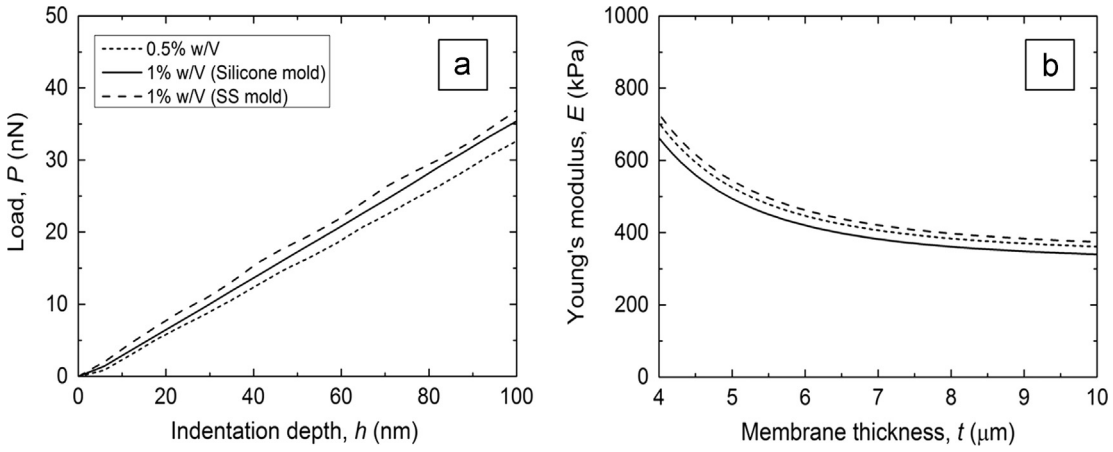

Fig. 6 – (a) Example load responses against indentation depth and (b) membranes elastic response as a function of thickness for each condition investigated.

observed in the present study lie in that range, much larger than the indentation depth, so that the unknown exact value of the thickness at the point of indentation becomes less relevant. The stiffness of these materials at the scale of single cells is potentially important in their intended use as tissue engineering scaffolds. If the intrinsic material properties are indeed not dependent on solid concentration in the scaffolds and pore size, the cells seeded in these structures would experience a mechanical response only dependent on the material composing the scaffold.

4. Conclusions

The multi-scale mechanical response of freeze-dried collagen porous scaffolds was explored: the bulk scaffolds were more compliant in the hydrated state, where interstitial fluid flow and permeability of the porous structure, as well as the decreased stiffness of collagen, are expected to affect the mechanical properties. However, their degree of viscoelastic relaxation was lesser in this state, possibly due to the pressure exerted by the fluid that hinders time-dependent buckling of the collagen. The stiffness of the bulk scaffolds increased quadratically with increasing collagen concentration in the slurry, suggesting the solid concentration in the scaffolds possibly varies linearly with that in the slurry and the material behaves as an open-cell porous solid. The increase in stiffness with slurry concentration is either due to a greater amount of solid in the pore walls or to a larger number of these. Colloidal probe AFM of membranes made of stacks of pore walls showed that the elastic response of the latter is comparable for the conditions examined within the membrane thicknesses observed, suggesting that the material composing the scaffolds is unchanged between conditions. Therefore, densification of the material is not expected as a mechanism for the increased amount of solid in the pore walls, and seeded cells would experience a mechanical response only dependent on the intrinsic properties of the material composing the scaffold. Further work will be required to ascertain the arrangement of collagen as a function of increasing concentration, ultimately building an understanding of the design and control of the scaffolds structure and consequent mechanical properties.

Acknowledgments

The authors are grateful to the Nano Doctoral Training Centre (NanoDTC), University of Cambridge, and the EPSRC who supported this work through the EP/G037221/1 grant. They would also like to thank Rachel Oliver and Anne Bahnweg for their advice on the work undertaken.

REFERENCES

Ahmed, T.A., Hincke, M.T., 2014. Mesenchymal stem cell-based tissue engineering strategies for repair of articular cartilage. Histol. Histopathol. 29 (6), 669.

Cameron, A.R., et al., 2011. The influence of substrate creep on mesenchymal stem cell behaviour and phenotype. Biomaterials 32, 5979.

Davidenko, N., et al., 2010. Collagen-hyaluronic acid scaffolds for adipose tissue engineering. Acta Biomater. 6, 3957.

Elias, P.Z., Spector, M., 2012. Viscoelastic characterization of rat cerebral cortex and type I collagen scaffolds for central nervous system tissue engineering. J. Mech. Behav. Biomed. Mater. 12, 63.

Engler, A.J., et al., 2006. Matrix elasticity directs ste cell lineage specification. Cell 126, 677.

Galli, M., et al., 2009. Viscoelastic and poroelastic mechanical characterization of hydrated gels. J. Mater. Res. 24, 973.

Gibson, L.J., 2005. Biomechanics of cellular solids. J. Biomech. 38, 377.

Gibson, L.J., Ashby, M.F., 1997. Cellular Solids: Structure and Properties, second ed. Cambridge University Press, Cambridge, UK.

Harley, B.A., et al., 2007. Mechanical characterization of collagen-glycosaminoglycan scaffolds. Acta Biomater. 3, 463.

Hu, Y., et al., 2010. Using indentation to characterize the poroelasticity of gels. Appl. Phys. Lett. 96, 121904.

Johnson, K.L., 1985. Contact Mechanics. Cambridge University Press.

Komaragiri, U., et al., 2005. The mechanical response of freestanding circular elastic films under point and pressure loads. J. Appl. Mech. 2, 203.

Nukavaparu, S., Dorcemus, D., 2012. Osteochondral tissue engineering: current strategies and challenges. Biotechnol. Adv. 31, 706.

ASTM Standaqrd E112. (Accessed 2014).

O'Brien, F.J., et al., 2004. Influence of freezing rate on pore structure in freeze-dried collagen-GAG scaffolds. Biomaterials 25.

Olde Damink, L.H., et al., 1996. Cross-linking of dermal sheep collagen using a water-soluble carbodiimide. Biomaterials 17, 765.

Oyen, M.L., 2005. Spherical indentation creep following ramp loading. J. Mater. Res. 20, 2094.

M.L. Oyen, Analytical techniques for indentation of viscoelastic materials Philos. Mag. (2006), 86: 5625.

Pawelec, K.M., et al., 2014. Understanding anisotropy and architecture in ice-templated biopolymer scaffolds. Mater. Sci. Eng. C 37, 141.

Roberts, A.P., Garboczi, E.J., 2001. Elastic moduli of model random three-dimensional closed-cell cellular solidsActa Mater. 49, 189.

Schoof, H., et al., 2001. Control of pore structure and size in freeze-dried collagen sponges. J. Biomed. Mater. Res. 58, 352.

Scott, O.N., et al., 2004. Indentation of freestanding circular elastomer films using spherical indenters. Acta Mater. 52, 4877.

Shapiro, J.M., Oyen, M.L., 2013. Hydrogel composite materials for tissue engineering scaffolds. J. Min., Met. Materials Soc. 65, 505.

Tierney, C.M., et al., 2009. The effects of collagen concentration and crosslink density on the biological, structural and mechanical properties of collagen-GAG scaffolds for bone tissue engineering. J. Mech. Behav. Biomed. Mater. 2, 202.

van der Rijt, J.A.J., et al., 2006. Micromechanical testing of individual collagen fibrils. Micromol. Biosci. 6, 697.