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TITLE: Development and Mechanical Characterisation of Self-Compressed Collagen Gels

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ABSTRACT

Collagen gels are considered a promising biomaterial for the manufacturing of tissue engineering scaffolds, however, their mechanical properties often need to be improved to enable them to provide enough mechanical support during the course of tissue regeneration process. In this paper, we present a simple self-compression technique for the improvement of the mechanical properties of collagen gels, identified by the fitting of bespoke biphasic finite element models. Radially-confined highly hydrated gels were allowed to self-compress for 18 hours, expelling fluid, and which were subsequently subjected to unconfined ramp-hold compression. Gels, initially of 0.2%, 0.3% and 0.4% (w/v) collagen and 13mm thickness, transformed to 2.9 ± 0.2%, 3.2 ± 0.3% and 3.6 ± 0.1% (w/w) collagen and 0.45 ± 0.06 mm, 0.69 ± 0.04 mm and 0.99 ± 0.07 mm thickness. Young's moduli of the compressed gels did not increase with increasing collagen fibril density, whilst zero-strain hydraulic permeability significantly decreased from 51 to 21 mm^4/Ns. The work demonstrates that biphasic theory, applied to unconfined compression, is a highly appropriate paradigm to mechanically characterise concentrated collagen gels and that confined compression of highly hydrated gels should be further investigated to enhance gel mechanical performance.

KEYWORDS:

Collagen gels, hydraulic permeability, biphasic theory

1. Introduction

Purified collagen may be processed to create a scaffold or matrix that can be seeded with cells and trigger tissue regeneration. Such gels may be considered biphasic, consisting of an insoluble network of fibrils and interstitial fluid. Currently, collagen gels are insufficiently stiff for tissue engineering applications due to their extremely low collagen proportion (0.2%-0.5% (w/v) collagen) and higher concentrations do not form gels. Several approaches have been developed to enhance them, including chemical (Zeeman et al., 1999), physical (Rich et al., 2014) and enzymatic (Orban et al., 2004) crosslinking, addition of cells (Saddiq et al., 2009) and glycosaminoglycans (GAGs) (Matsuda et al., 1990), and reinforcement with fibres of natural (Gentleman et al., 2003) or synthetic (Jeong et al., 2007) origin. However, many of these methods impart toxic effects on cells and impede optimal regeneration. Collagen hydrogels have been concentrated using unconfined plastic compaction (PC) in order to increase collagen fibril density (CFD) and therefore to increase mechanical properties (Brown et al., 2005). Whilst a 40-fold increase in CFD and a 3-fold increase in compressive modulus has been achieved (Neel et al., 2006), these gels were heterogeneous in CFD (Brown et al., 2005) potentially due to the fast compaction.

Biphasic modelling of confined compression has been successfully applied to characterise, and differentiate between, highly hydrated collagen gels (Busby et al., 2013). However, interdigitation of the gel into the porous platen and the permeability of the compressing platen itself may be factors in the experimental outcome. Unconfined compression between two impermeable platens, with known friction, potentially provides a more controlled boundary condition, but material properties must be extracted utilising inverse fitting of an axisymmetric biphasic model, hitherto not carried out.

The aims of this paper are twofold. Firstly, we present a simple technique for the slow compaction of collagen gels, increasing homogeneity and mechanical integrity. Secondly, we describe a novel approach for mechanically characterizing the gel by inversely fitting an axisymmetric biphasic model of unconfined compression to experimental data. We demonstrate that the method is sufficiently sensitive to identify mechanical differences which can be attributed to changes in CFD.

2. Materials and Methods

2.1 Experimental

Type I collagen solutions of 0.2%, 0.3% and 0.4% (w/v) were prepared from rat tail tendon according to the method of Elsdale and Bard (1972). 50 ml of collagen solution was poured into a 70mm diameter confining mould to rest for 2 hours at room temperature to become a gel. The mould fitted

snugly into a grooved plate into which a rubber O-ring was inserted. A plastic membrane covered the base of the tube preventing the gel adhering to the plate. After gelation, a solid cylinder, which fitted tightly into the tube was lowered until it was just in contact with the gel, and was kept in place using grub screws. The apparatus was turned upside-down and the plate and the plastic membrane were removed. The gel was detached from the walls of the cylinder using a scalpel. A nylon mesh layer (100 micron aperture), a filter paper and a porous plate (1mm pore diameter, 25 holes/cm^2, 6 mm thick) were placed on top, successively, and a second tube was bolted to it securing the porous plate in situ. The apparatus was turned upside-down again and the plug was removed (Figure 1). Under the influence of gravity, the gel started to self-compress with fluid expelled through the porous plate. The collagen gels self-compressed for 18 hours at 20^0C after which they were removed and immersed in PBS. This procedure was repeated for 0.2%, 0.3% and 0.4% (w/v) collagen gels.

From each gel, thirteen 8 mm diameter samples were obtained using a hole punch, away from the periphery of the gel to avoid any variation in thickness near the edge. One sample was used for thickness determination and two samples were immersed in 4\% formaldehyde for 20 minutes and retained for surface imaging. The remaining ten samples were immediately mechanically tested.

The thickness of each sample was determined five times by penetrating the gel with a 1 mm diameter cylindrical rod, in displacement control at 0.1 mm/s (BOSE Electroforce® 3200, US) with a 250g load cell (Honeywell, US) measuring the force response, until the force dramatically increased denoting the base of the sample had been reached. Comparison of the force-displacement characteristics of the system, with and without the gel in situ, enabled the thickness of the gel to be ascertained (Figure 2).

To view the surfaces of the samples using SEM (TM-1000 Hitachi), gels were prepared by drying them in ethanol (70%, 90%, 95% and 100%) for 1 minute followed by gold sputtering (Bio Rad, SEM Coating System). The top and the bottom surfaces were observed under 5,000x and 10,000x magnification and pore size was estimated based on five images per surface taken from five different areas. Images were processed using ImageJ (Schneider et al., 2012).

Samples for mechanical testing were placed between two impermeable compression platens, covered with 1200 grit wet and dry paper to provide a no-slip boundary condition. The sample surface was located by lowering the upper platen until a force reading of 0.25g (~50Pa) was achieved using a 250g load cell (Honeywell, US). After 2 minutes, samples were subjected to 10% compression at 1 %/sec and held for 300 seconds. After mechanical testing, each sample was weighed, desiccated for 5 days at 37^0C and reweighed to determine CFD.

2.2 Finite Element modelling

Sample-specific quarter-cylinder finite element (FE) models were created using FEBio (Maas et al., 2012), parameterised by thickness and CFD. Each model consisted of 3,000 elements and loading exactly mimicked the experiment. An impermeable, non-slip boundary condition was imposed on the surfaces interfacing with the platens. Radial fluid expulsion was allowed. Elements were modelled as a biphasic material with an isotropic neo-Hookean solid matrix and an assumed Poisson's ratio of zero. Strain-dependent hydraulic permeability, k, was assumed to follow that of Lai and Mow (1980), i.e. $k = k_0 \exp(M\varepsilon)$, where ε is the strain of the solid phase, k_0 the zero-strain hydraulic permeability and M is a non-linear coefficient. Parameter optimisation was performed by matching the experimental axial force data in the hold phase (quartered) with that of the FE model by best-fitting E, k_0 and M, using a constrained Levenburg-Marquardt method within FEBio (Maas et al., 2012).

Sample differences in collagen proportion, thickness, peak stress and material parameters among groups were determined by analysis of variance (ANOVA) followed by Tukey' s HSD test, using Origin® 2015 (OriginLab, Graphing & Analysis).

3. Results

Gels, initially of 0.2%, 0.3% and 0.4% (w/v) collagen and 13 mm thickness, transformed into collagenous sheets of 2.9 ± 0.2%, 3.2 ± 0.3% and 3.6 ± 0.1% (w/w) collagen and of 0.45 ± 0.06 mm, 0.69 ±0.04 mm and 0.99 ± 0.07 mm thickness respectively (Figures 3 and 4); an approximate 10-fold increase in CFD. SEM revealed the average pore size on the bottom (fluid leaving) surface of the gels was equal to 0.88 ± 0.25 µm while pore size on the opposite surface was significantly smaller (0.68 ± 0.13 µm) ($p=0.05$) (Figure 5). The coefficients of determination, R^2, were greater than 0.90 in 26 out of 30 cases. Samples were removed from subsequent analysis if $R^2<0.70$, which occurred once due to a load cell error.

Young's moduli of 2.9% collagen samples (810 ± 344 Pa) was not larger than that of 3.2% collagen samples (755 ± 237 Pa), whilst 3.6% collagen samples (1,103 ± 217 Pa) were significantly stiffer than 3.2% gels (Figure 6a). Zero-strain hydraulic permeability, k_0, significantly decreased with increasing collagen content, from 51.4 ± 23.7 mm^4/Ns for 2.9% collagen samples to 31.8 ± 6.78 and 21.2 ± 2.31 mm^4/Ns for 3.2% and 3.6% collagen, respectively (Figure 6b). The non-linear permeability coefficient, M, being 11.9 ± 7.16, 2.40 ± 2.90 and 3.39 ± 2.07, for 2.9%, 3.2% and 3.6% collagen samples, respectively.

4. Discussion

Hyper-hydrated collagen hydrogels under slow self-compression transformed into structurally stable collagen sheets between 2.9 and 3.6% (w/w) collagen and assuming the density of fluid in the hydrogel to be equivalent to water w/w is directly comparable to w/v. In contrast to Brown and co-workers (2005), who applied a relatively high load for a short period of time, our gels self-compressed for 18 hours to ensure that the compaction process reached equilibrium. The small, albeit significant, difference in pore size between surfaces of the gels is testament to the fact that this slow compression gives rise to a more homogeneous gel than fast mechanical compression, which exhibited a dense layer at the fluid leaving surface (Brown et al., 2005).

The compressive moduli of these self-compressed gels were around 3-fold greater than those of highly hydrated gels found by (Knapp et al., 1997) but similar to those obtained by (Busby et al., 2013). The significant loss of permeability with increasing CFD demonstrates the appropriateness of the model and fitting algorithm, since compaction of collagen gels would reduce porosity, the size and shape of the pores, and increase the tortuosity of the flow path.

A major novelty of this work is the application of inverse techniques to determine biphasic model parameters using unconfined compression. Previous modelling of soft tissue deformation has predominantly utilised confined compression, which is numerically simpler, but experimentally more complex, with interdigitation, platen permeability and platen-side wall friction all being potential sources of error. Unconfined compression is experimentally simpler, however the interface between the compression platen and the tissue needs to be well-defined mechanically. We chose to use 1200 grit abrasive paper, with an approximate particle size of 3.8 μm, as we believe this provided sufficient interface friction to assume a no-slip boundary condition for the model. The alternative of a friction-free interface is experimentally demanding (Farrell, 2013) and also unsubstantiated.

The strength of the model lies in its simplicity since only three parameters required fitting: Young's modulus, zero-strain hydraulic permeability and non-linear permeability coefficient. We chose isotropic neo-Hookean constitutive material behaviour to model the solid phase, due to the potential for large, localised deformations at boundaries. Poisson's ratio effects of porous media should be considered in terms of apparent and true values (Farrell, 2013). By assuming a true Poisson's ratio of zero, we are defining that, at equilibrium, there is no radial expansion of the gel. We would expect this to be the case due to the very low solid fraction of the solid phase.

We acknowledge that other hyperelastic models, with additional parameters, may yield a better fit to the data. Moreover, a poroviscoelastic model may also be more physically appropriate as it may account for the intrinsic viscoelasticity of the collagen fibres, as well as the flow of fluid around them. Again, more complex relationships characterising strain-dependent permeability than the one

chosen may also yield improved fits. Such augmentation may result in improved data fitting, but it would also slow down the inverse determination of the parameters, and increase the potential for non-global solutions to the inverse problem and non-unique parameter sets. Despite its simplicity, since $R^2 > 0.9$ in 26 out of 30 cases, the adopted model accounted for over 90% of the experimental variability.

Correctly characterising the constitutive behaviour of scaffolds is of utmost importance for mechanobiological studies. Viscoelasticity theory, whilst it may be usefully utilised for comparative purposes, does not deliver the information that biphasic analysis provides, such as localised stress, strain, fluid pressure and fluid velocity within the scaffold.

5. Conclusions

A novel axisymmetric FE model, with well-defined boundary conditions, successfully characterised self-compressed collagen gels, which demonstrated increased collagen density and decreased permeability. The study forms an excellent basis from which to devise methodologies of controlled plastic compression further and details a methodology for their mechanical characterisation.

6. Acknowledgements

We are grateful to Mrs. Katie Henderson for assistance with the collagen hydrogel preparation, to Mr. Davide Erbogasto for helping with the SEM, and to Mr. Stephen Murray for building the experimental apparatus for the collagen gel concentration.

7. Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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9. Vitae

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CAPTION OF FIGURE 1:

Experimental setup for collagen gel concentration. Part A (acrylic tube) and Part B (plastic tube) were bolted together. The rubber O-ring and the nylon mesh layer prevented fluid leakage and direct contact of the gel with the filter paper, respectively. The filter paper allowed fluid, but not collagen fibrils, to leave the tissue.

CAPTION OF FIGURE 2:

Exemplar penetration-force profiles with (sample indentation) and without (control) sample in situ.

CAPTION OF FIGURE 3:

Collagen gel that has undergone confined self-compression for 18 hours. The collagen sheet originates from a 0.4% (w/v) collagen hydrogel of 13mm thickness that after 18 hours of compression under the force of gravity transformed into a relatively thin and stable collagen sheet of 3.6% (w/w) collagen and of 0.99mm thickness.

CAPTION OF FIGURE 4:

Final Collagen proportion (% w/w) (a) and thickness (b) of self-compressed collagen gels. Error bars indicate the standard deviation. * and *** indicate $p<0.05$ and $p<0.001$, respectively.

CAPTION OF FIGURE 5:

Scanning electron microscopy of the bottom fluid leaving surface (a) and the top surface (b) of a self-compressed gel of 3.2% (w/w) collagen. Bar scale: 20um.

CAPTION OF FIGURE 6:

Young's modulus (a) and zero-strain hydraulic permeability (b) of self-compressed collagen gels. Error bars indicate the standard deviation for each group. * and *** indicate $p<0.05$ and $p<0.001$, respectively.