



Hydraulic permeability of multilayered collagen gel scaffolds under plastic compression-induced unidirectional fluid flow

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ABSTRACT

Under conditions of free fluid flow, highly hydrated fibrillar collagen gels expel fluid and undergo gravity driven consolidation (self-compression; SC). This process can be accelerated by the application of a compressive stress (plastic compression; PC) in order to generate dense collagen scaffolds for tissue engineering. To define the microstructural evolution of collagen gels under PC, this study applied a two-layer micromechanical model that was previously developed to measure hydraulic permeability (k) under SC. Radially confined PC resulted in unidirectional fluid flow through the gel and the formation of a dense lamella at the fluid expulsion boundary which was confirmed by confocal microscopy of collagen immunoreactivity. Gel mass loss due to PC and subsequent SC were measured and applied to Darcy's law to calculate the thickness of the lamella and hydrated layer, as well as their relative permeabilities. Increasing PC level resulted in a significant increase in mass loss fraction and lamellar thickness, while the thickness of the hydrated layer dramatically decreased. Permeability of lamella also decreased from 1.8×10^{-15} to $1.0 \times 10^{-15} \text{ m}^2$ in response to an increase in PC level. Ongoing SC, following PC, resulted in a uniform decrease in mass loss and k with increasing PC level and as a function SC time. Experimental k data were in close agreement with those estimated by the Happel model. Calculation of average k values for various two-layer microstructures indicated that they each approached 10^{-15} – 10^{-14} m^2 at equilibrium. In summary, the two-layer micromechanical model can be used to define the microstructure and permeability of multi-layered biomimetic scaffolds generated by PC.

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1. Introduction

Control of the three-dimensional (3-D) fibrillar microstructure of collagen gel scaffolds is required for tissue engineering and bio-compatible delivery systems [1–4]. However, in vitro reconstituted, highly hydrated collagen gels are characterized by a dominant volume fraction of unbound fluid [5,6]. Therefore, the hydraulic permeability (k) of these porous structures can significantly influence the mechanical properties (biphasic or poroviscoelastic theory) [7–9] and mass transfer characteristics (convection and diffusion of oxygen, nutrients and other macromolecules) [10,11] of constructs. A similar role of k has been reported for the control of interstitial fluid flow in tissues such as cartilage and bone [8,12,13].

Several experimental methods have been developed to assess the k (conductivity) of tissues and scaffold structures. For example, incorporation of the sedimentation velocity of physically entangled macromolecules in an aqueous solution during ultracentrifugation

into Darcy's law has been used to measure the k of the sediment [10,14,15]. However, this approach is limited by the dilute concentration of macromolecules in aqueous solutions, scattered sedimentation velocity, reswelling of the sedimented polymer, and unaccounted buoyant and frictional forces [15,16]. Alternatively, the average k of hydrated scaffolds and tissues has been measured by incorporation of experimental creep deformation data of constructs, under confined compression, into a model for one-dimensional creep [17–19]. However, accurate evaluation of creep deformation in highly hydrated scaffolds is difficult due to their unstable physical structure [17,20,21]. Furthermore, the application of a pressure gradient to a porous construct and measurement of the resulting fluid flow velocity through the construct has been employed to measure k through Darcy's law [22–24]. Yet, pressure-induced deformation and significant microstructural changes within highly hydrated constructs can cause substantial variations in the measured k [9,25]. This phenomenon is analogous to concentration polarization and cake layer (gel) formation during ultrafiltration of macromolecular solutions using permeable filters. In these experiments, fluid flow through the membrane can be described using Darcy's law [26,27]. By applying the boundary conditions existing within the cake layer during ultrafiltration, the

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hydraulic resistance of the cake layer can be measured [9,26]. It is noteworthy that the formation of a dense collagen region during confined compression of collagen gels has been shown to locally increase the compressive stiffness and hydraulic resistance of hydrated gels, as well as diminish the fluid flow through the construct [9,28].

Previously, we reported a micromechanical model to describe fluid flow and k of hydrated collagen gels during radially confined self-compression (SC) [29]. Confined SC resulted in a unidirectional fluid flow through the gel, which transformed the highly hydrated gel without structural competence into a considerably dense lamella at the fluid expulsion boundary (FEB) (~ 44 -fold increase in solid volume fraction, σ) with a significantly reduced k (100- to 1000-fold). Thereupon, as revealed by confocal and electron microscopy, a two-layer model, consisting of an upper highly hydrated collagen layer with a dense lamella at the bottom, was developed. The initial boundary conditions, along with the mass loss fraction data obtained from confined SC of collagen gels, were applied to Darcy's law, to measure the k of the lamella.

Since the two-layer model is best applied to measure the k of hydrated gels with uniform fibrillar structure, further development of this model is necessary in order to analyse more complex boundary conditions, such as the presence of a heterogeneous structure or an external stress through the application of plastic compression (PC). PC is a rapid method for generating extracellular matrix (ECM)-like dense collagen scaffolds for tissue engineering through fluid expulsion via the application of compressive stress on collagen gels. Therefore, a modified form of the two-layer model was employed to define the permeability of different collagen gel microstructures, produced by applying increasing levels of PC, followed by SC. Using this model, the microstructure and permeability of multi-layered scaffolds generated by PC can be defined. Such analysis would enable the development of a novel approach to measure the k of biomimetic collagen gels, developed as scaffolds and tissue equivalents. The modelling also allows for the precise control of the physical and mechanical properties in hydrated biomimetic scaffolds, and in the creation of bulkier (e.g. layered or rolled) heterogeneous structures.

2. Materials and methods

2.1. Collagen gel preparation

Collagen gel scaffolds were prepared by adding 0.8 ml of $10\times$ Dulbecco's modified Eagle's medium to 3.2 ml of rat-tail type I collagen dissolved in acetic acid (2.10 mg ml^{-1} , First Link Ltd., UK). This solution was neutralized by initially introducing $70 \mu\text{l}$ of 5 M NaOH followed by the sequential addition of $0.5 \mu\text{l}$ of 1 M NaOH until physiological pH (7.4) was attained. Thereafter, 0.9 ml of the solution was pipetted into impermeable circular moulds (16 mm in diameter) and left for 30 min in an incubator to set at 37°C .

2.2. Determination of mass loss following radially confined plastic and self-compression of collagen gels

Cast collagen gels underwent radially confined PC by applying different static stresses of 0, 340, 690 and 1022 N m^{-2} (referred to as PC0, PC1, PC2 and PC3 respectively) for 2 min (Fig. 1A). Highly hydrated collagen gels were transferred to a saturated porous support consisting of (bottom to top) absorbent paper blot layers, stainless steel mesh and a polymer mesh. The wet substrate was maintained at the level of a water bath to allow for fluid flow. As shown in Fig. 1A, an impermeable polystyrene tube was used to laterally support the gel, inhibiting radial fluid flow. Post-PC, colla-

gen gels were allowed to undergo SC at room temperature in a closed chamber maintained at 100% relative humidity (Fig. 1B). Confined SC allowed unidirectional fluid flow out of the gel, parallel to the direction of the driving force for flow (gravity, x direction in Fig. 1).

The initial weight of the hydrated collagen gel was recorded (Mettler Toledo AL204, Canada) before and after PC and also monitored over time during SC up to 50 min, until equilibrium was reached. Thereupon, the mass loss fraction $\lambda(t)$ (the fraction of gel mass lost divided by the initial mass at casting) was measured for each gel specimen. The collagen volume fractions of highly hydrated collagen gel (σ_b) and lamella (σ_c) were determined as described previously [29], and found to be 0.0016 ± 0.0001 and 0.0708 ± 0.0043 , respectively. A minimum of four replicate specimens were tested for all experimental conditions. Statistical analysis was performed using Student's t -test to determine p -values at a significance level of 0.05.

2.3. Microscopic characterization of collagen gel microstructure undergoing plastic and self-compression

Confocal laser scanning microscopy (CLSM; Carl Zeiss, LSM5 Exciter, Canada) was used to further investigate gel microstructure following either PC or SC. Gel specimen preparation for collagen fibril immunostaining was performed as described previously [29]. Briefly, after either 0 or 20 min of SC, collagen gels were fixed using a 4% formalin fixative and incubated overnight in phosphate-buffered saline (PBS) containing rabbit anti-rat collagen type I antibody (ab24133 , $1 \mu\text{g ml}^{-1}$, Abcam, USA). Gel specimens were then transferred to PBS containing $1 \mu\text{g ml}^{-1}$ goat anti-rabbit polyclonal antibodies conjugated to Alexa488 (Invitrogen Inc, USA) for 2 h. Using a $\times 20$ objective, Z-stacks of fluorescent immunoreactivity towards collagen type I throughout the thickness of each specimen were acquired at 1 airy unit using a slice interval thickness of $15 \mu\text{m}$. Maximum intensity projections and orthogonal images of collagen scaffolds were generated using NIH ImageJ v. 1.43.

2.4. Modelling of gel mechanics during plastic and self-compression

In concert with our previous study [29], a number of assumptions were made here in order to describe collagen gel PC using the two-layer model. A highly hydrated collagen gel without structural competence (zero stiffness) was assumed. Furthermore, the stiffness of the lamella was assumed to be high enough to prevent flow-induced deformation [15,26,30], i.e. the k of lamella was uniform [15,26]. Therefore, by applying a radially confined compressive stress, the gel undergoes compression (Fig. 1A), while the highly hydrated gel loses water and consolidates against FEB. The static compressive load and gravitational force are driving forces for downstream fluid flow. This fluid loss results in a fluid velocity of \bar{U} through the lamella. The cast gel was assumed to have uniform fibrillar density. Hence, the osmotic pressure gradient within each layer was zero.

Accordingly, a two-layer model was developed to describe the PC of collagen gel, by modifying Darcy's law to define the fluid flow through both the highly hydrated gel and the lamella,

$$\bar{U} = -\frac{k}{\mu} \bar{\nabla}(p + \rho gx) \quad (1)$$

where μ is the fluid viscosity, p is the fluid pressure, ρ is the fluid density, g is the acceleration due to gravity and x defines the upwards vertical direction. By applying the existing boundary conditions under confined PC (Fig. 1A), we found:

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