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Characterizing the viscoelastic properties of thin hydrogel-based constructs for tissue engineering applications

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We present a novel indentation method for characterizing the viscoelastic properties of alginate and agarose hydrogel based constructs, which are often used as a model system of soft biological tissues. A sensitive long working distance microscope was used for measuring the time-dependent deformation of the thin circular hydrogel membranes under a constant load. The deformation of the constructs was measured laterally. The elastic modulus as a function of time can be determined by a large deformation theory based on Mooney–Rivlin elasticity. A viscoelastic theory, Zener model, was applied to correlate the time-dependent deformation of the constructs with various gel concentrations, and the creep parameters can therefore be quantitatively estimated. The value of Young's modulus was shown to increase in proportion with gel concentration. This finding is consistent with other publications. Our results also showed the great capability of using the technique to measure gels with incorporated corneal stromal cells. This study demonstrates a novel and convenient technique to measure mechanical properties of hydrogel in a non-destructive, online and real-time fashion. Thus this novel technique can become a valuable tool for soft tissue engineering.

Keywords: Young's modulus; viscoelastic; indentation; hydrogel; creep deformation; tissue engineering

1. INTRODUCTION

The use of hydrogels in tissue engineering has become popular due to their viscoelastic characteristics, biocompatibility, amiability of fabrication into specific shapes and their ability to allow transfer of gases and nutrients. Among the most commonly used hydrogels for constructing engineered tissue are natural polymers such as agarose, alginate, chitosan, collagen, fibrin and hyaluronic acid as well as synthetic polymers such as poly(acrylic acid) (PAA) and poly(vinyl alcohol) (PVA). Hydrogels are particularly useful as engineering scaffolds for soft viscoelastic tissues and have been used to replicate cartilage (de Chalain *et al.* 1999), cornea (Minami *et al.* 1993), skin (Eaglstein & Falanga 1997) and vascular tissue (Weinberg & Bell 1986). Among the common features of all these tissues are their high degree of flexibility and their ability to withstand forces.

One of the major challenges facing the use of hydrogels for tissue engineering is the ability to replicate the tissues' mechanical and viscoelastic characteristics. In many cases, such hydrogel-based tissues have significantly weaker mechanical strength than their native counterpart (Orwin *et al.* 2003; Awad *et al.* 2004). The reason for these poor mechanical properties is that hydrogels do not form exactly the complex structure of the native tissue. The mechanical strength of the hydrogel can be improved by mechanically stimulating the seeded cells, leading to remodelling within the construct in some types of cells (Wakatsuki & Elson 2003). This eventually will lead to the formation of tissue equivalent. Thus, along with the creation of various mechanical stimulation conditions, the monitoring of mechanical properties of hydrogel constructs is of paramount importance to optimize stimulation conditions for facilitating tissue engineering applications.

At present there are few methods available for characterizing the mechanical properties of hydrogel constructs. The tensile test or extensometry is one of the most common methods of mechanical characterization (Drury *et al.* 2004). It involves deforming a material at a constant rate of elongation and recording the force required to maintain that rate of elongation. The force and material elongation are used to obtain a stress versus strain chart from which several mechanical parameters such as the Young's modulus, yield strength and ultimate tensile strength can be derived. Another method is the unconfined compression test

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(Quinn & Grodzinsky 1993; Stammen *et al.* 2001; Koob & Hernandez 2003), which involves compressing the hydrogel between two plates. The force required to compress the hydrogel and the amount of deformation are used to derive a stress versus strain graph from which the compressive modulus and compressive strength can be determined. A confined compression test may also be used to determine the mechanical properties of hydrogels (Behravesh *et al.* 2002; Gu *et al.* 2003). It differs from an unconfined compression tests in that the hydrogel is held within a chamber, preventing the hydrogel samples from lateral deformation as it is compressed. Another method is the bulge or blister test (Ranta-Eskola 1979), which involves the application of a uniform fluid pressure via a through window in the substrate to deform the membrane and measure its displacement as a function of the applied pressure, i.e. producing a compliance curve. In the bulge test, the stress of the deformed sample is bi-axial and axisymmetric and the gripping problems which often occur in the tensile test can be eliminated. These merits make the method most popular in soft polymeric tests (Briscoe & Panesar 1992). However, several shortcomings, including potential leakage and dissolved air or moisture in using fluid as a pressurized medium, may cause an error in the compliance measurement; such inaccuracy could be significant in the characterization of permeable or semi-permeable membrane systems (Wan & Mai 1995; Liu *et al.* 2004).

Recently, Liu & Ju (2001) and Ju & Liu (2002) have developed a novel experimental technique which enables the viscoelastic properties of polymer membranes to be characterized. It involves a central indention of a circumferentially clamped membrane using a ball of known weight and measurement of the corresponding displacement occurring at the centre. The advantages of this mechanical characterization method are compelling and can be briefly summarized: (i) the stress distribution in the deformed sample is bi-axial and axisymmetric, (ii) no need for force feedback control for creep test, (iii) applicable to permeable or semi-permeable membrane and (iv) the force and displacement resolution can be as accurate as 10 μN and 10 μm, respectively. More importantly, the measurement can be performed for specimens that are fully immersed in solution and at elevated temperatures with no risk of damaging the instrument. This is extremely valuable for biological systems which frequently require online, real-time and non-destructive measurement. In this study, the viscoelastic properties of soft biological tissues, using alginate and agarose hydrogel based constructs as a model system, have been investigated by this new indentation technique. The effects of seeded cells on the mechanical properties of the hydrogel have also been examined.

2. MATERIAL AND METHODS

2.1. Sample preparation

Two types of hydrogel, alginate and agarose, were examined. Alginate is a copolymer of β-D-mannuronic (M block) and α-L-guluronic (G block) acid. The ratios and lengths of these blocks play an important role in the mechanical behaviour of the alginate. A 2% (w/v) solution of sodium alginate was formed by dissolving 2 g of Protanal LF200 S (FMC BioPolymer, Norway) in 100 ml of deionized water. The ratio of M block to G block in this type of alginate has been found to be 0.23 (Drury *et al.* 2004). Lower concentrations of the alginate solution were formed by diluting this solution with deionized water. When fully dissolved, the solution was autoclaved for sterilization. To fabricate alginate gel, a ring made from filter paper with inner diameter of 20 mm was placed on the bottom of a petri dish. This ring reduced the amount of shrinkage of the gel after crosslinking. Three hundred microlitre of alginate solution was poured inside the ring. Then 5 ml of 0.5 M filtered calcium chloride solution ($CaCl_2$) was added over the alginate. The application of $CaCl_2$ caused the sodium in the alginate to be replaced by calcium, which resulted in crosslinking and formation of gel. After 10 min the $CaCl_2$ solution was removed and the gel was washed twice in phosphate buffered saline (PBS; Sigma, UK).

Agarose gel was made using agarose type 1 (Sigma, UK). A 2% (w/v) agarose solution was produced by adding 0.2 g of agarose powder in 10 ml PBS. For lower concentrations, less agarose powder was required. The powder was dissolved by heating the solution. When fully dissolved, the solution was filtered for sterilization and 300 μl of the solution was then applied to a petri dish with a circular paper ring of inner diameter 20 mm on the bottom to avoid gel shrinking. The gels were set by cooling at room temperature.

In order to examine the effect of cells on the hydrogel constructs and verify the feasibility of online measurement, human keratocytes were used to form viable constructs. This study has received prior approval from the Birmingham NHS Health Authority Local Research Ethics Committee to use some remaining cornea tissue after corneal transplantation. Only tissues from donors that have been consented for research were used for this study. The cornea was left in 1.5 U ml^{-1} dispase (Roche, UK) for 1 h to help remove the outer layers. The remaining epithelial and endothelial layers were then removed physically using fine tipped tweezers. The remaining piece of cornea, the stroma, was sliced up and placed into cell culture flasks. Dulbecco's modified eagle medium (DMEM; Biowest, France) supplemented with 10% foetal calf serum (Biowest, France), 1% antibiotic and antimitotic solution (Sigma, UK) and 1% 200 mM L-glutamine (Sigma, UK) was added to each flask and the flasks were cultured at 37 °C, 5% CO_2 until the cells migrated out from the stromal pieces. The media was changed every 2–3 days. When the cells in the flask were fully confluent, they were passaged.

The keratocytes at passage three were seeded to hydrogels. In the cases of alginate, the required numbers of cells were suspended in the alginate before it was crosslinked by $CaCl_2$. In the cases of agarose, the cells were suspended in the warm agarose solution (approximately 35–40 °C) before it was set. The seeding density of 2 million cells ml^{-1} of solution was used for all the gels examined in this paper. After gelation, the hydrogel constructs were cultured overnight in supplemented DMEM media.

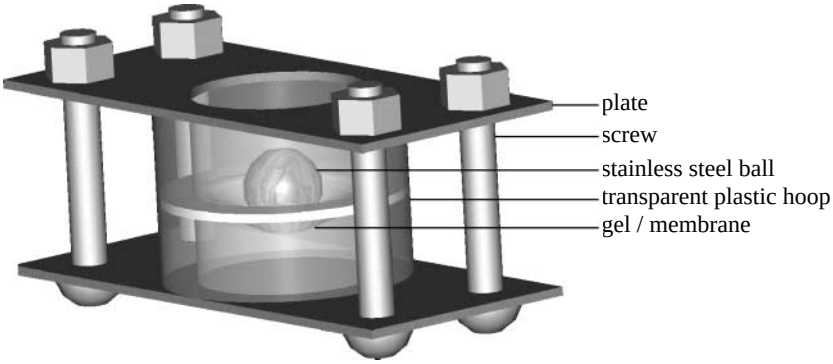

Figure 1. A schematic of the hydrogel sample holder and the loading ball.

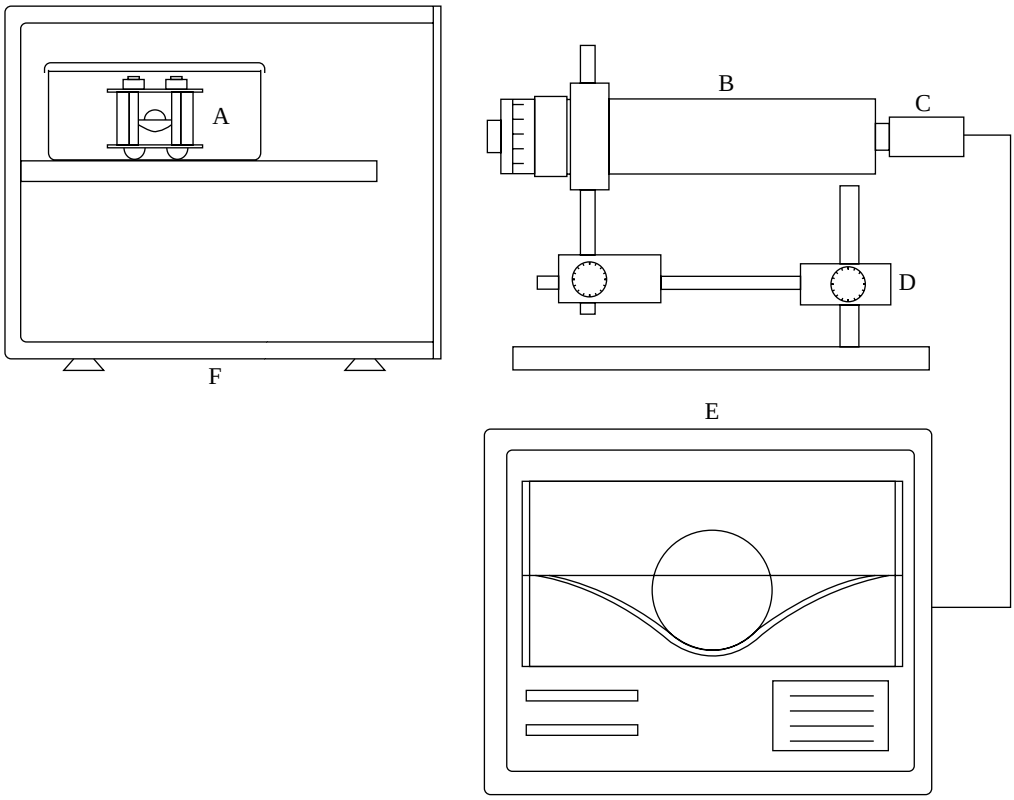

Figure 2. A schematic of the instrument system used to measure the deformation and creep behaviour of membranes. (*a*) Sample holder and ball; (*b*) long working distance microscope; (*c*) CCD camera; (*d*) precision X–Y translation stage; (*e*) image analysis system; (*f*) incubator at 37 °C, 5% CO_2.

Cell viability tests were performed on the alginate and agarose hydrogel constructs 24 h after they were prepared. A 50 mM EDTA (Sigma, UK) solution in PBS were used to dissolve the alginate hydrogels, allowing the release of their cells (Yoon *et al.* 2002). The pH of the EDTA solution was adjusted to 7.4 by adding a controlled volume of sodium hydroxide (Sigma, UK). Two millilitre of EDTA solution were applied to each gel for 5 min to dissolve the gel. The dissolved gel was centrifuged to form a cell pellet, the supernatant was removed and then the cells suspended in a 1 ml solution of 50% Trypan blue (Sigma, UK) and 50% PBS. After 5 min, a live–dead cell count was performed using a haemocytometer. Cell viability was then calculated from the total number of live cells divided by the total number of cells used in the construct.

For the agarose constructs, a different approach was utilised to examine the cell viability as the trypan blue method would have involved heating the construct to over 45 °C to liquefy the gel and this elevated temperature would damage the cells. A live/dead fluorescent dye kit, L-3224 (Molecular Probes, The Netherlands), was used to test the cell viability in the agarose hydrogels. After 24 h culture, a section was sliced from the agarose construct and placed onto a glass microscope slide. One hundred microlitre of the dye solution which consists of 8 µM EthD-1 and 4 µM calcein were added to the piece of agarose. After 30 min, cell viability was assessed using confocal microscopy (Olympus, Japan). The mixture fluorescent dye stains live cells green and dead cells red.

PKH26 red fluorescent cell linker kit (Sigma, UK) was used to examine the morphology of cells within the constructs 24 h after seeding. For this procedure, the fluorescent dye was added to the cells before the cells were seeded to the gels. After the cells were spun down

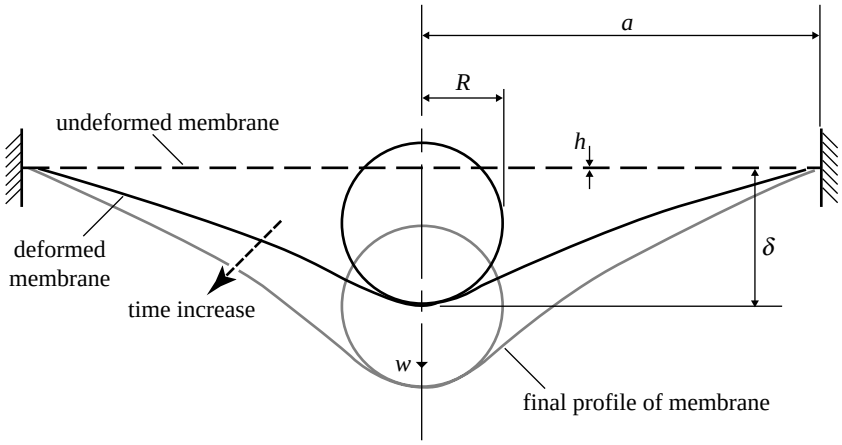

Figure 3. Indentation of a viscoelastic membrane by a sphere.

to form a pellet, they were washed in DMEM, centrifuged and the supernatant was removed. For a pellet of 4 million cells, 200 µl of Dilutent C and 200 µl of 4 µM PKH26 dye were added. After 10 min incubation, the labelled cells were washed three times in supplemented media before being placed into the hydrogel constructs. The cells were applied to the hydrogels as previously described. Confocal microscopy was used to examine the morphology of the cells within the constructs.

2.2. Measurement instrumentation

The instrument that has been developed to measure the deformation consisted of two separated parts; a sample holder with a spherical indenter and an image acquisition system. The hydrogel was clamped around its outer circumference by a specifically designed sample holder in which the hydrogel was held between two transparent plastic circular hoops of inner diameter 20 mm. Two hard, thin plastic sheets were placed above and below the hoops. The plastic sheets were then held together by a number of stainless steel screws as shown schematically in figure 1. The whole assembly could be submerged in liquid within a large rectangular petri dish while still allowing the hydrogel to be viewed laterally. The hydrogel was deformed (indented) by using a stainless steel ball (Spheric-Trafalgar, UK) of weight 0.27 g and diameter 4 mm. The ball which served as a spherical indenter was placed at the centre of the hydrogel and the weight of the ball caused the deformation to occur. All the deformation and measurements of the hydrogels were carried out in deionized water except for the tests carried out on hydrogels that contained cells and their non-cell controls, which were carried out in PBS. The weight of the ball was recalculated to compensate for its submersion. For cell-hydrogel constructs, the whole assembly was maintained in a large square petri dish which was placed in an incubator at 37 °C, 5% CO_2. In this study, the diameter ratio of the hydrogel to the ball was kept constant at 5.0, which is identical with the value used in the previous analyses (Yang & Hsu 1971; Liu & Ju 2001). With this sample holder, large displacements, as high as 5 mm, can be visualized.

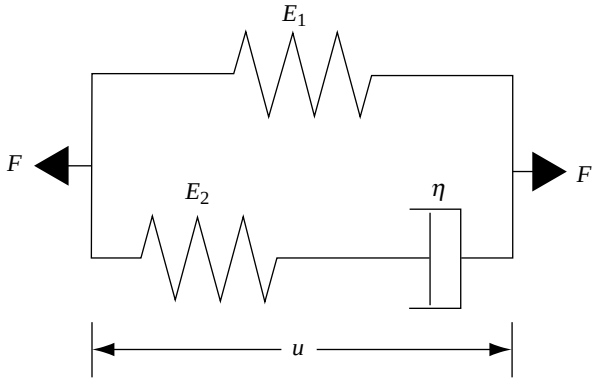

Figure 4. A schematic of the three-parameter Zener model. The parameter F is the applied force, u is the displacement, η is the viscosity of the dashpot and E_1 and E_2 are the elastic moduli of the two springs.

The image acquisition system consisted of a long focal distance objective microscope (Edmund Industrial Optics, USA) with a computer-linked CCD camera (XC-ST50CE, Sony, Japan) as schematically shown in figure 2. The system allowed a high magnification up to 120 times for acquiring the side-view images of the deformation profile from outside the incubator through a glass window. LabView (National Instruments, UK) was used to write a programme for recording the images of deformation profiles. This programme also allowed images to be recorded automatically at different time intervals. The viscoelastic characteristics of the hydrogels were examined by measuring the central displacement of the deformed hydrogels over time, with a time interval of 1–5 min between recorded images. A high intensity light source with fibre optic cable (Stokeryale, USA) and a multi-LED ultra bright torch were used to increase the brightness of the sample area and allow the images to be captured more clearly. The magnification of the system was calibrated with the computer-acquired images of a micro-ruler placed alongside the investigated samples. As well as the deformation, the thickness of the samples was measured using the same approach. The samples were placed onto a flat surface and the profile of their thickness was recorded laterally using the image acquisition system.

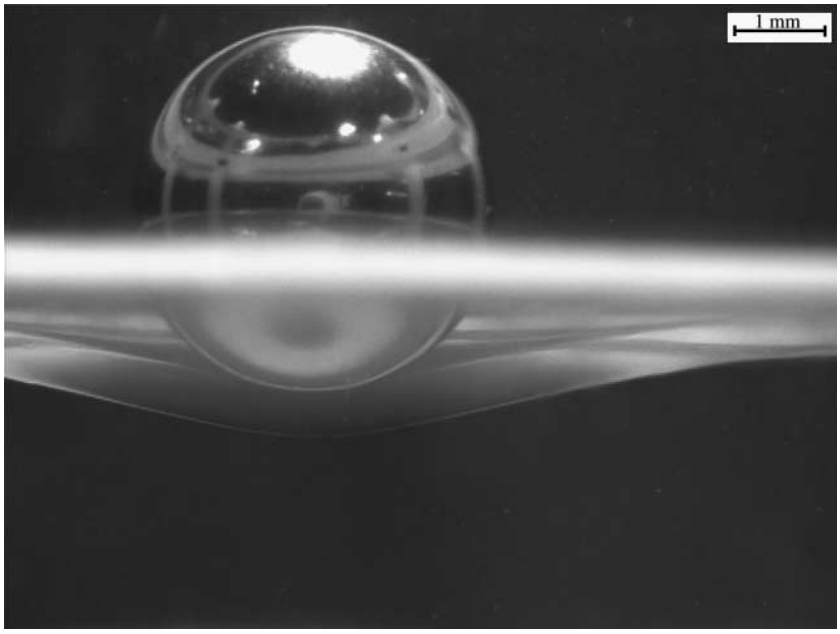

Figure 5. An image of the alginate membrane under ball indentation using the long working distance microscope.

3. THEORETICAL ANALYSES

Hydrogels such as alginate and agarose can be modelled as viscoelastic materials that exhibit rubber like characteristics (Anseth *et al.* 1996). Their mechanical behaviours can be characterized using Mooney–Rivlin equations (Rivlin 1948), which are widely accepted to characterize isotropic and incompressible materials. These types of materials are often referred to as Mooney materials.

The weight of the ball caused a deformation to occur in the clamped hydrogel. Subsequently, the images were recorded by the long working distance microscope system and then analysed to determine the mechanical and viscoelastic properties of the hydrogel. In order to calculate these properties, a mathematical model was required. Yang & Hsu (1971) developed equations for Mooney materials, which describe the deformation of a membrane due to the weight of a ball. These equations have been applied to find the Young's modulus of membrane materials (Liu & Ju 2001).

When the ball caused the deformation to occur in the membrane, as shown in figure 3, the central displacement (δ) was measured by the microscope system and then used to determine the Young's modulus (E) from the following equation:

$$\frac{6w}{EhR} = 0.075\left(\frac{\delta}{R}\right)^2 + 0.78\left(\frac{\delta}{R}\right), \tag{3.1}$$

where w is the weight of the ball, h is the membrane thickness and R is the radius of the ball (Liu & Ju 2001). This equation has been developed for a ball and sample with the dimensional characteristics of $a/R=5$ and $\delta/R \leq 1.7$, where a is the radius of the membrane. This model also assumes that the ratio of thickness to the radius is low and the deformation is large, hence stretching of the membrane dominates over bending. Overall, this assumption can be validated by using the dimensionless parameter, defined as the following equation:

$$\lambda = [12(1-\nu^2)]^{3/2}\left(\frac{wa^2}{Eh^4}\right), \tag{3.2}$$

where ν is the Poisson ratio. When hydrogel material is fully swollen, its mechanical behaviours can be considered as similar to those of rubber-like materials (Anseth *et al.* 1996), which have a Poisson ratio of about 0.5. In our experiments, the hydrogels were always immersed in physiochemical solution and adequately swollen. Hence, the Poisson ratio of the hydrogels was assumed to be 0.5 in this paper. When λ is large (greater than 2000 for a clamped material), stretching dominates and the effect of bending can be neglected (Begley & Mackin 2004). The values obtained for λ in our experiments were found to range from 2000 to 5000; hence stretching dominated and equation (3.1) was valid for our calculations.

The viscoelastic properties of the hydrogel can be found by measuring the creep behaviour of the sample and applying the standard linear model or Zener model as shown in figure 4, where F represents the force and u represents the displacement. This viscoelastic model consists of two springs and one dashpot to describe the material's elasticity and viscosity, respectively. By measuring the central displacement against time, the time-dependent modulus can be determined and the effective viscosity can then be calculated by applying the following set of equations (Ju & Liu 2002):

$$\frac{\varepsilon(t)-\varepsilon(0)}{\varepsilon(\infty)-\varepsilon(0)} = 1-\mathrm{e}^{-t/\mu_\sigma}, \tag{3.3}$$

$$E_2 = \left(\frac{\varepsilon(\infty)}{\varepsilon(0)}-1\right)E_1, \tag{3.4}$$

$$\mu_\sigma = \frac{\eta}{E_1}\left(1+\frac{E_1}{E_2}\right), \tag{3.5}$$

where $\varepsilon(t)$ is the deformation strain at time t, $\varepsilon(0)$ is the initial deformation strain, $\varepsilon(\infty)$ is the final deformation strain, E_1 and E_2 are the elastic moduli, η is the effective viscosity, and μ_σ is called the relaxation time for constant stress (Fung 1993).

4. RESULTS

Figure 5 shows a portion of the deformation profile of a 1% (w/v) agarose hydrogel recorded by the microscope system. It can be seen that this system can obtain clear images of the deformation profile with a high resolution. The depth of indentation, i.e. the central displacement of the deformation profile, can easily be measured from these images. The microscope system can also be used to measure the thickness of the hydrogels. In general, agarose hydrogels were slightly thicker than alginate hydrogels despite the same volume of gel solution being used to make each type. Crosslinking of the alginate solution by $CaCl_2$ caused the hydrogel to shrink.

The central displacement of deformation profile increased over time due to creep. Figure 6 shows a typical curve of creep test for a 1.25% (w/v) alginate and a 1.25% (w/v) agarose hydrogel, i.e. the deformation rate at the centre slowed down over time until the displacement reached a plateau. The time-dependent measurement of central displacement showed that the alginate samples exhibited viscoelastic deformation, while agarose demonstrated more elastic behaviours since it showed almost little change in central displacement over time.

The values for the Young's modulus were found for a number of alginate and agarose hydrogels at different concentrations. The measurements were carried out in triplicate. The values obtained for the Young's modulus of the alginate and agarose at different concentrations are shown in figures 7 and 8. For both the agarose and alginate materials, the creep deformation reached saturation after the load was applied for approximately 60 min, as shown in figure 6. At such saturated deformation, the elastic modulus E_1 used in the three-parameter viscoelastic model corresponded to the E obtained from equation (3.1), which was derived based on elastic theory (Ju & Liu 2002). In general, the values of E_2 calculated from equation (3.4) are 1 to 2 order magnitudes smaller than those of E_1. For simplicity, only the values of E_1, which is equivalent to the Young's modulus in elastic theory, are shown in figures 7 and 8. It can be seen that the standard deviation bars for these charts were reasonably small which demonstrates good reproducibility of the measurements using this technique. There was an increase in Young's modulus with increased gel concentration for both alginate and agarose, and this phenomenon coincides both with our general physical intuition and with other people's findings (Gu *et al.* 2003; Ng *et al.* 2005). In general, the Young's modulus of agarose increased almost in a linear manner with gel concentration. Such linearity is ensured by a simple regression analysis, achieving a value of 0.9846 for the coefficient of determination.

In order to demonstrate and evaluate the viscoelastic characteristics of the hydrogels, creep tests were performed. The viscous properties of the alginate were

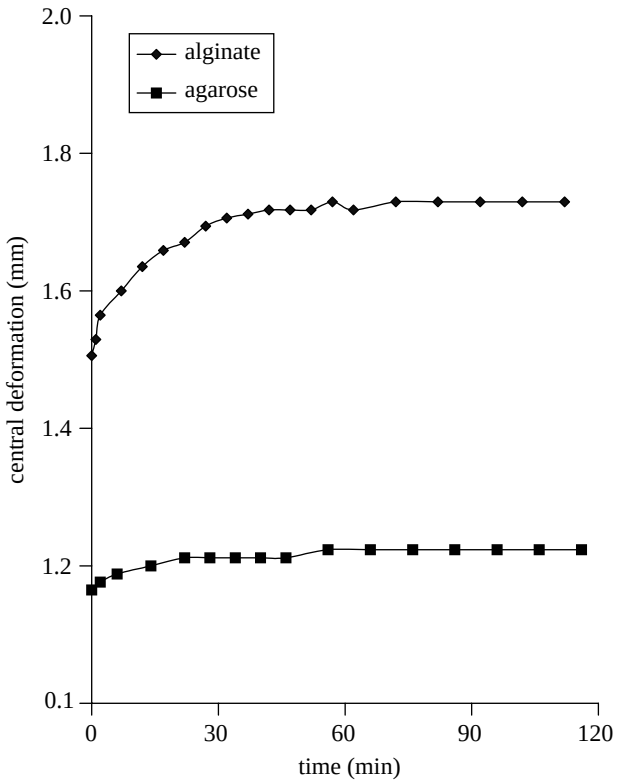

Figure 6. Creep deformation, i.e. central displacement versus time, of 1.25% alginate and agarose hydrogels.

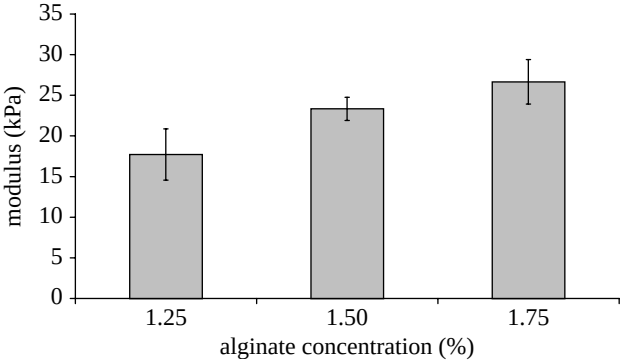

Figure 7. Young's modulus of the alginate at different concentrations (n=3).

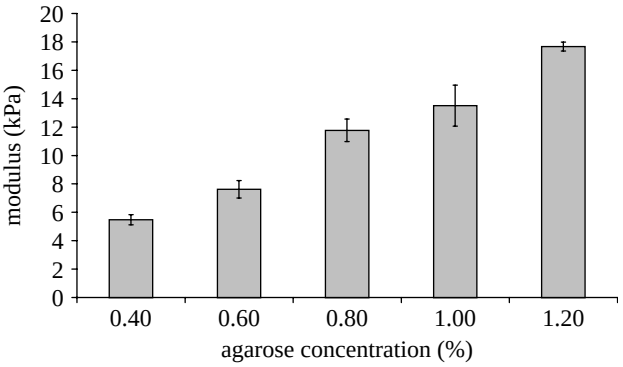

Figure 8. Young's modulus of the agarose at different concentrations (n=3).

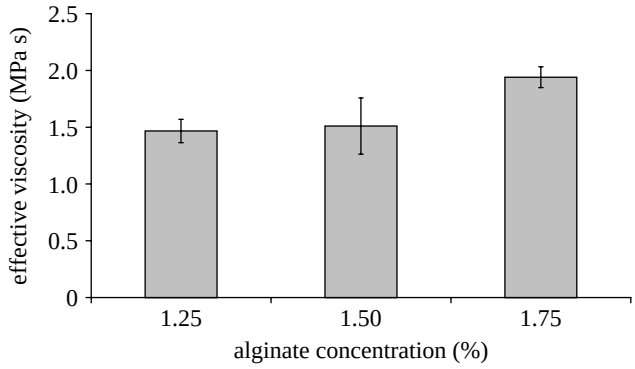

Figure 9. Effective viscosity of the alginate at different concentrations ($n=3$).

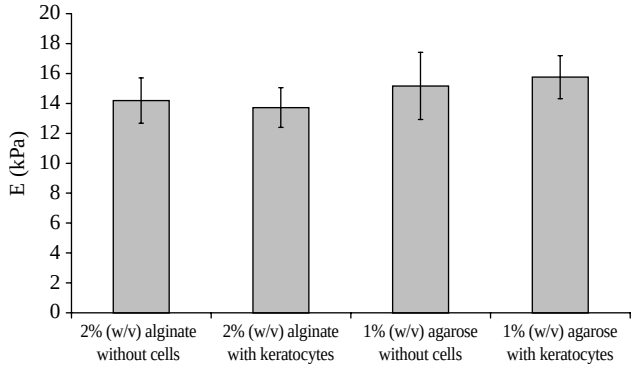

Figure 10. Young's modulus of the alginate and agarose hydrogel with and without keratocytes (2 million cells ml^{-1}, $n=3$).

calculated based on the equations (3.3)–(3.5) and the values are shown in figure 9. The trend of the results, i.e. the effective viscosity increased with the gel concentration increase, coincides with our physical intuition and other publications (Kong *et al.* 2003). Small variations in the thickness of the alginate may be a responsible for the variation in effective viscosity values with gels of the same concentration.

The Young's modulus of 2% alginate and 1% agarose hydrogels seeded with keratocytes is shown in figure 10. It can been seen that the presence of the cells did not appear to have any significant effect on the Young's modulus after just 1 day's incubation. There did appear to be a reduction in the Young's modulus of the 2% alginate when compared to the sample with the same alginate concentration but not incubated in media overnight. This was possibly due to sodium ions in the media replacing calcium in the alginate (LeRoux *et al.* 1999), which resulted in a reversal of the crosslinking caused by the $CaCl_2$. Confocal microscopy images of the cell-gel constructs, shown in figure 11, exhibited a round cell morphology in both gel types after 24 h culture. Cell viability appeared positive within the hydrogels with a value of 69% for alginate and 86% for agarose after 1 day's incubation.

5. DISCUSSION

A novel indentation system has been used to characterize the mechanical and viscoelastic properties of alginate and agarose hydrogels constructs. This system has a number of advantages over standard mechanical characterization techniques. One of the main advantages is that this system is capable of working within a biological environment. The entire sample holding equipment can be autoclaved and measurements can be taken under sterile conditions. It has also been shown that the system can be operated under physiological conditions (37 °C, 5% CO_2) and testing of the hydrogel constructs can be done while submerged in culture media. These environmental properties are of extreme importance when measuring mechanical properties of hydrogel as changes in environment can significantly affect the mechanical characteristics of the hydrogel constructs (Zvanut & Rodriguez 1977; Moe *et al.* 1992).

The Zener model appeared to be a suitable viscoelastic model for the two types of hydrogel in this study. There are other viscoelastic models, such as Maxwell and Voigt, available for describing the creeping deformation (Fung 1993). The Maxwell model predicts that the deformation increases linearly against the time but never reaches a plateau, while the Voigt model depicts the deformation initially as zero and then gradually increasing with time. However, the deformations of our materials were initially finite and then increase with time and finally reach a plateau. Such deformation phenomena cannot be well described either by Maxwell or by Voigt, but the Zener model generally provides a good description.

As well as being able to work in a biological environment, obtaining reliable and reproducible data is important in any mechanical characterization system. When a number of hydrogels were made with similar concentrations and under similar conditions, reproducible data of their mechanical properties have been obtained when tested by this new system. Since there are numerous factors that could affect the mechanical properties of the hydrogels, the values of Young's modulus of alginate hydrogels reported in literature vary with a wide range from 1 to over 100 kPa (Kuo & Ma 2001; Awad *et al.* 2004; Drury *et al.* 2004). Our results, which give Young's modulus values for alginate ranging from 14 to 29 kPa, appear reasonable by comparison. However, it is worthwhile to point out that in the case of alginate, the particle size, gel size, ratio of G block to M block, method of crosslinking, sterilization method, incubation time and measurement temperature are some of the many factors that may affect the mechanical characteristics of the gel. More importantly, different methods used for mechanical characterization, e.g. tensile test or ball indentation, are expected to generate a variation in the determined values of the Young's modulus, due to the dissimilar testing mechanisms and analyses.

A number of differences between the mechanical characteristics of agarose and alginate can be seen from the data produced using our system. The main difference is that agarose appeared to exhibit more elastic behaviour than alginate which appeared to exhibit more viscoelastic behaviour, as has been demonstrated in figure 6. These properties are important as they demonstrate how the hydrogel will deform and recover when subjected to forces over a long period of time. Since

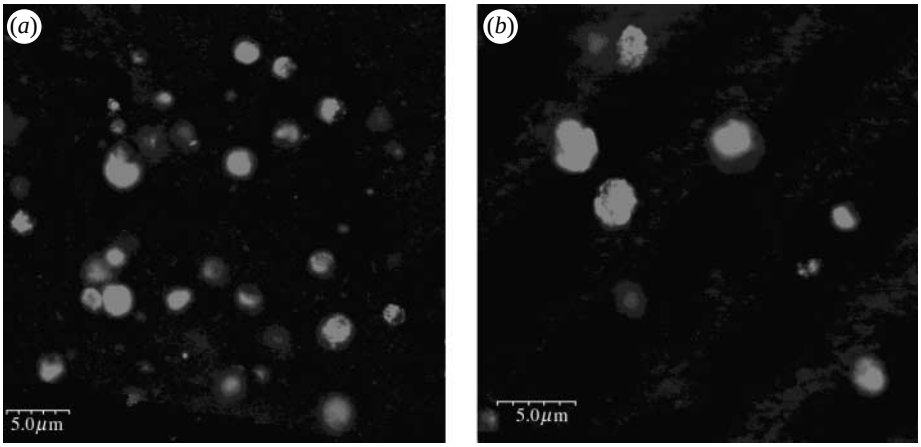

Figure 11. Confocal microscope images of PKH26 fluorescent labelled keratocytes in (*a*) 2% alginate and (*b*) 1% agarose hydrogel constructs after 24 h culture.

most tissues show signs of viscoelasticity, alginate would appear to have more applications than agarose for mimicking deformation behaviours of natural systems. However, another important difference between these two materials is that alginate loses its mechanical strength quickly under physiological conditions where sodium ions are present, while agarose appears to be comparatively stable. The required rate of degradation of the hydrogel is another factor to consider. When a hydrogel starts to degrade, it will lose mechanical integrity and strength. This loss of strength can be compensated by the activity of cells and production of extracellular matrix which can increase the strength of the hydrogel construct. Other factors that have to be considered are cell mobility within the gel, cell viability over time and the structure of the tissue that is being engineered. It is also worth noting that the values obtained for the Young's modulus of both the alginate and agarose hydrogels are significantly lower than the modulus of real viscoelastic tissues such as cartilage (Kempson *et al.* 1971) or cornea (Hoeltzel *et al.* 1992). This demonstrates the need for greater cell activity and the higher production rate of extracellular matrix to strengthen the hydrogel construct.

The reproducible data of cell-hydrogel constructs demonstrate the ability of our system to examine hydrogel constructs while still maintaining standard tissue cultivation conditions (37 °C, 5% CO_2). This cell culture experiment was to test whether the cells have any significant influence on the mechanical properties of the constructs in the short term. Keratocytes, which are cells found in the cornea, exhibit fibroblastic phenotypes when cultured under certain conditions (Beales *et al.* 1999). In general, the presence of cells within both alginate and agarose constructs appeared to have little effect in the short time periods examined. Confocal microscopy images of the constructs suggest that the cells, after staining with the fluorescent dye PKH26, maintained a spherical morphology. This type of cell behaviour has previously been found in both agarose and alginate hydrogels (Gruber *et al.* 1997; Awad *et al.* 2004). It indicates that after a short culture time, the cells did not adhere completely to the gel. This might explain the insignificance of the change in the mechanical properties of the cell embedded constructs, compared to the hydrogels without cells. In addition, the stiffness of the hydrogel may have overwhelmed the cellular contribution, which would also explain why there was no measurable effect on the constructs mechanical properties in the presence of cells. Examination of longer incubation periods may produce more significant data, as it has previously been shown that agarose containing chondrocytes showed a three-fold increase in modulus when incubated for eight weeks (Hu & Athanasiou 2005).

6. CONCLUSIONS

Characterizing the mechanical and viscoelastic properties of agarose and alginate hydrogel-based constructs was performed by using a novel indentation system. It has been demonstrated that this characterization system is able to produce reliable and reproducible data for both the pure hydrogels and cell embedded hydrogel constructs in a non-destructive, online and real-time manner. These unique features offer a powerful tool for numerous applications in the areas of tissue engineering and regenerative medicine, which often require monitoring and characterizing constantly the mechanical properties of target materials. In particular, this technique has a great potential for soft tissue engineering constructs including cornea, skin and vascular tissue during *in vitro* conditioning.

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