CHAPTER 12

Mechanical Characterisation of Hydrogels for Tissue Engineering Applications

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Summary

Hydrogels have been extensively investigated for use as constructs to engineer tissues in-vitro. Among the principal limitations with using hydrogels for engineering tissues are their poor mechanical characteristics. Many techniques exist to measure the mechanical properties of hydrogels but few allow non-destructive monitoring of these properties under cells culture conditions. Two recently developed techniques shall be discussed in detail in the current chapter. Thin hydrogels have been clamped around their outer edge and deformed using a spherical load. The time-dependent deformation has been measured in-situ using a long focal distance microscope connected to a CCD camera and the deformation displacement has been used with a theoretical model to quantify the mechanical and viscoelastic properties of the hydrogels. For thicker hydrogels, optical coherence tomography has been used to measure the time-dependent depth of indentation caused by a spherical load on top of the hydrogel. Hertz contact theory has been applied to calculate the hydrogels mechanical properties. The mechanical and viscoelastic properties of several different hydrogel materials were examined. The principal advantages of these techniques have over conventional mechanical characterisation techniques are that the measurement can be performed on cell-seeded hydrogels and under sterile conditions while allowing non-destructive, in-situ and real-time examination of the changes in mechanical properties.

KEYWORDS: Hydrogel, Mechanical characterisation, Indentation, Viscoelastic, Optical coherence tomography.

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Topics in Tissue Engineering, Vol. 4. Eds. N Ashammakhi, R Reis, & F Chiellini © 2008.

INTRODUCTION

Hydrogels are biomaterials that consist of a water-swollen network of crosslinked polymer chains [1]. They can be made from chains of natural polymers such as collagen or alginate or from synthetic polymers such as poly(vinyl alcohol) (PVA) or poly(acrylic acid) (PAA). Their biocompatibility, ease of fabrication and viscoelastic properties makes them highly suitable for use as constructs to engineer tissues. Attempts at engineering several different tissue types using hydrogels have previously been examined including cartilage [2,3], cornea [4,5], skin [6], tendon [7] and vascular tissue [8].

One of the main limitations with using hydrogels to engineer tissues *in vitro* is their poor mechanical properties. Previous attempts at engineering tissues using hydrogels have produced tissues with significantly poorer mechanical strength than the real tissues [9,10]. There are several reasons for this including the random alignment of fibres and high water content within the hydrogel. When cells are applied to a hydrogel they can improve the mechanical strength of the hydrogel construct through the reorganisation of fibres, production of extracellular matrix products and the application of intrinsic strains [11-14]. The ability to record changes in the mechanical properties of hydrogels over time is important in the quest to engineer functional tissues *in vitro*.

Several methods have been used to examine the mechanical properties of hydrogels but few can be used to measure the mechanical properties of cell-seeded hydrogels. This chapter will discuss the different methods of mechanically characterising hydrogels and will review two recently developed methods in detail, which are capable of examining the mechanical characteristics of cell-seeded hydrogels non-destructively under cell culture conditions.

MECHANICAL CHARACTERISATION OF HYDROGELS

Extensiometry

At present the most commonly used method to determine the mechanical properties of hydrogels are by tensile testing or strip extensiometry. These methods have been extensively used to study the mechanical behaviour of various hydrogels [13, 15]. The technique involves applying a tensile force to strips of material held between two grips (Figure 1a). Alternatively, the force can be applied to a ring instead of a single strip (Figure 1b). Applied force and the elongation of the

material are used to obtain a stress-strain chart. This chart can be used to derive several mechanical properties of the hydrogel including Young's modulus, yield strength and ultimate tensile strength. Extensiometry can also be used to examine the viscoelastic characteristics of a hydrogel material by elongating the material strip to a particular length and examining the stress relaxation response over time at a constant strain. There are several shortcomings with using extensiometry, which include only hydrogel strips or rings can be measured, measurements can only be performed once on each test piece, the potential misalignment of grips and the strain is limited to being uniaxial. The destructive nature of this type of test makes it difficult to monitor the change in mechanical properties of the hydrogels over time, an important parameter in engineering tissues.

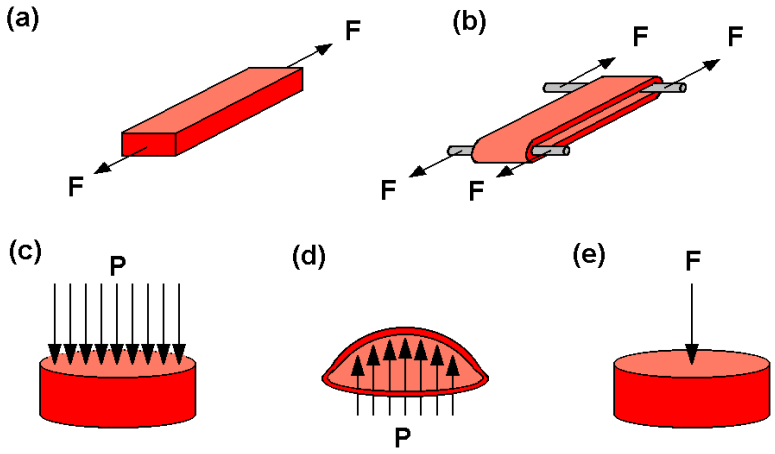

Fig. 1. Conventional techniques to mechanically characterize hydrogels: (a) strip extensiometry; (b) ring extensiometry; (c) compression test; (d) bulge test; (e) indentation (F = force, P = pressure).

Compression test

Compression test is another technique that has previously been used to examine the mechanical properties of several different types of hydrogels [16,17]. This technique involves placing the material between two plates and compressing it (Figure 1c). The pressure applied to the surface of the hydrogel and distance the hydrogel is compressed, can be used to calculate the mechanical properties of the hydrogels using a theoretical model. One of the advantages of the compression test over extensiometry is that it does not limit the hydrogel geometry to strips or rings although it does require a flat surface. This approach has several limitations including bulging of the hydrogel under compression and difficulty in applying pressure evenly. Bulging can be

overcome by confining the hydrogel around its outer edge although this changes the nature of the measurements. A number of studies have used the compression test to examine the mechanical properties of cell-seeded hydrogels [16,17]. However, non-destructive online characterisation of the mechanical properties of hydrogels is not achievable with this technique.

Bulge test

Another commonly used method to examine the mechanical characteristics of hydrogels is the bulge test also referred to as the inflation test [18,19]. The test involves inflating the hydrogel through a window in the substrate and measuring the resulting displacement as a function of the applied pressure (Figure 1d). The displacement can be measured using a CCD camera or a laser. A finite element model is then used to analyse the data and derive values for the mechanical properties of the hydrogel. The bulge test has a number of shortcomings including potential leakage, difficulty to control applied pressure and dissolved air becoming trapped in the solution [20]. These shortcomings make this technique unsuitable for examining cell-seeded hydrogels online in a cell culture environment.

Indentation test

Indentation has become an increasingly popular method of characterising the mechanical properties of hydrogels. This technique works by indenting a hydrogel at a single point to a predetermined displacement depth and measuring the reaction force required to cause the indentation (Figure 1e). The most common approach utilizes an indenter connected to a force transducer to record the force required for indentation. A force-displacement curve is used to calculate the elastic modulus of the material. For this type of indentation the tip geometry plays an important role in determining the materials mechanical strength [21]. More recently, advances in sensor and computer technology have made indentation an increasingly attractive option in examination of viscoelastic as well as elastic materials. By applying the indenter to a sample material for a fixed period of time at a constant indentation depth, stress relaxation data can be obtained. Recent advances in technology have also allowed indentation to be performed on a nanometric scale level [22]. This is particularly useful in examining the mechanical characteristics of thin materials or coatings on a substrate. Indentation has several advantages over other conventional mechanical characterisation techniques [23]. Indentation allows quick,

online and real time measurements of materials. It can be used to measure localised mechanical strength at several different points on a material surface. One drawback with using this type of indentation is that it cannot be using in a sterile environment under cell culture conditions, as this would damage the force transducer.

Alternative tests

Several alternative techniques have been developed to characterise the mechanical properties of hydrogels. Spherical ball inclusion involves embedding a metallic sphere within a hydrogel [24]. A magnetic force is applied to the sphere causing it to move inside the hydrogel. This displacement can then be measured and used to calculate the mechanical properties of the hydrogel. This technique has a number of limitations including the sphere cannot be removed after measurements and it can only be used on transparent materials. Micropipette aspiration is another technique that can be used to measure the mechanical properties of hydrogels and biological materials [25]. This technique involves aspirating the hydrogel into a tube and comparing the amount of material in the tube with the force being applied to aspirate the material. This technique is useful for measuring the mechanical properties of hydrogels when micrometer or nanometer deformations are required but it also has several limitations most notably damage to materials being aspirated.

All these techniques can be used to determine the mechanical properties of hydrogels but they cannot be used to monitor the mechanical properties of hydrogels online over long culture periods. To overcome this problem two techniques involving spherical indentation have been developed to monitor the mechanical properties of hydrogels over time under cell culture conditions.

LONG-FOCAL-MICROSCOPY-BASED SPHERICAL MICROINDENTATION

A long-focal-microscopy-based spherical microindentation system has been developed to measure the mechanical properties of hydrogels on-line under cell culture conditions. This technique involves the central indention of a circumferentially suspended hydrogel using a sphere of a known weight and measurement of the resulting central deformation displacement.

Liu and Ju originally developed this technique to measure the mechanical properties of membrane materials including polymers and egg membranes [26,27]. Ahearne *et al*., modified this technique to allow characterisation of cell-seeded hydrogels under cell culture conditions [20]. Hydrogels of thickness up to 1 mm could be examined using this technique.

Instrumentation

The instrument consisted of two separated parts; a sample holder with a spherical indenter and an image acquisition system. The hydrogel sample was suspended around its outer circumference using a specifically designed sample holder (Figure 2) in which the hydrogel was held between two transparent circular rings of inner diameter 20 mm. Two hard thin stainless steel plates were placed above and below the hoops. The plates were then held together by a number of stainless steel screws. The sample holders could be submerged in liquid within a large rectangular petri dish while still allowing the hydrogel to be viewed from the side. The hydrogel was deformed (indented) by using a sphere of diameter 4 mm. PTFE, 316L stainless steel and 440 stainless steel spheres (Spheric-Trafalgar, UK) were used to perform experiments. The expected stiffness of the sample was important in deciding which materials were most suitable for use. The sphere was placed at the centre of the sample and the weight of the sphere caused the deformation to occur. The weight of the sphere was recalculated to compensate for its submersion in fluid. In this study, the diameter ratio of the hydrogel to the sphere was kept constant at 5.0, which is consistent with the values used in the previous analyses [26,28]. With this sample holder, large displacements, as high as 5 mm, can be visualized.

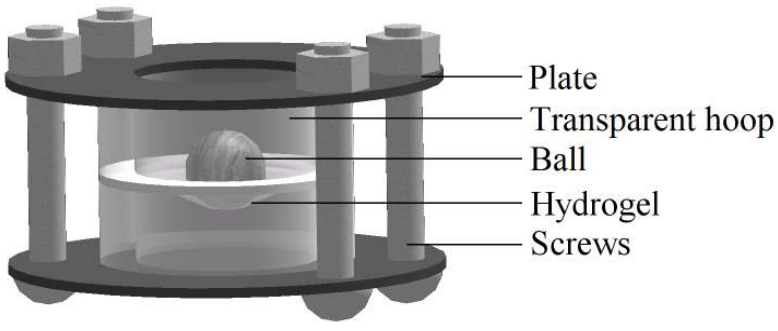

Fig. 2. Hydrogel sample holder and sphere for long focal microscope system.

For cell-hydrogel constructs, the whole assembly was submerged in a sterile buffer solution (PBS, Sigma, UK) and maintained in a large square petri dish which was placed in an incubator at 37°C, 5% CO_2. The environmental conditions have been shown to play a significant role when measuring the mechanical properties of hydrogels [29,30]. A window in the incubator allowed measurements to be taken of the hydrogels deformation profile from outside the incubator. All the components of the sample holder were autoclaved before use. The holder was assembled in a flow hood using autoclaved tweezers. Care had to be taken not to contaminate the holder as this could lead to infection in the hydrogel construct.

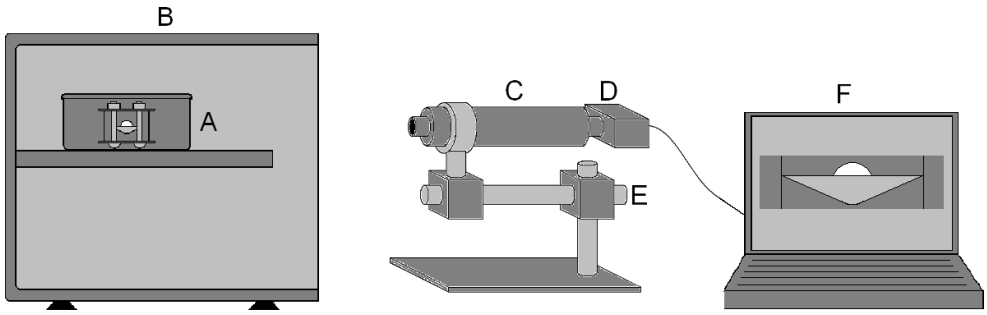

Fig. 3. Schematic representation of the long focal microscope spherical microindentation system: (A) sample holder and sphere; (B) incubator at 37°C, 5% CO_2; (C) long focal distance microscope; (D) CCD camera; (E) precision X-Y translation stage; (F) image analysis system.

The image acquisition system (Figure 3) consisted of a long focal distance objective microscope (Edmund Industrial Optics, USA) with a computer-linked CCD camera (XC-ST50CE, Sony, Japan). This 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. The magnification of the system was calibrated with the computer-acquired images of a stage micrometer. LabVIEW (National Instruments, USA) 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 change in central displacement of the deformed hydrogels over time, with a time interval of 1 to 5 minutes 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. As well as the deformation, the thickness of the hydrogels was measured using a similar

approach. The hydrogels were placed onto a flat surface and the profile of their thickness was recorded laterally using the image acquisition system.

Materials and Methods

In order to examine the effect of cells on the hydrogel constructs and verify the feasibility of on-line measurement for cell-seeded hydrogels, MG-63 bone cells and human corneal fibroblasts were used to form viable constructs. The use of human corneal fibroblasts for this research has received approval from Birmingham NHS Health Authority Local Research Ethics Committee. Alginate, agarose and collagen hydrogels were examined for use in these experiments. Alginate and agarose hydrogels were prepared as previously described [20]. Alginate hydrogels were made using sodium alginate (Protoanal LF200, FMC Biopolymer, Norway) dissolved in sterile water. The alginate solution was poured inside a filter paper ring of inner diameter 20 mm and crosslinked using 0.5 M sterile calcium chloride for 10 minutes. The crosslinked alginate forms a hydrogel. Cells were suspended in the sodium alginate prior to crosslinking. Agarose hydrogels were formed by heating agarose powder (Sigma, UK) in buffer solution and then cooling to room temperature where the solution forms a hydrogel. Cells were suspended in the warm agarose solution before it was set. Collagen hydrogels were formed using high concentration rat-tail collagen type 1 (BD Biosciences, USA) according to the manufacturers specifications. NaOH was used to neutralise the pH of the collagen solutions, which was necessary for hydrogel formation and maintenance of cell viability. Hydrogels were formed by setting the collagen solution as 37°C for 1 hour.

Mechanical characterisation of the hydrogels was performed using the long focal microscope spherical microindentation technique. The hydrogels were clamped and submerged in a square petri dish containing PBS and positioned in an incubator at 37°C. A sphere was placed on the construct resulting in a deformation to the construct. The central deformation of the construct was measured and the central deformation used to calculate the Young's modulus. The central displacement was measured 10 minutes after the sphere was left on the hydrogel to allow for creeping. The thickness of the constructs was also measured.

Results and Discussion

The long-focal-microscopy-based spherical microindentation technique has been used to examine the mechanical and viscoelastic characteristics of several different hydrogels including agarose and alginate [20]. The deformation profiles of an agarose, an alginate and a collagen hydrogel, as recorded by the microscope system, are shown (Figure 4). These hydrogels were seeded with human corneal fibroblasts. 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 long focal microscope system can also be used to measure the thickness of the hydrogels.

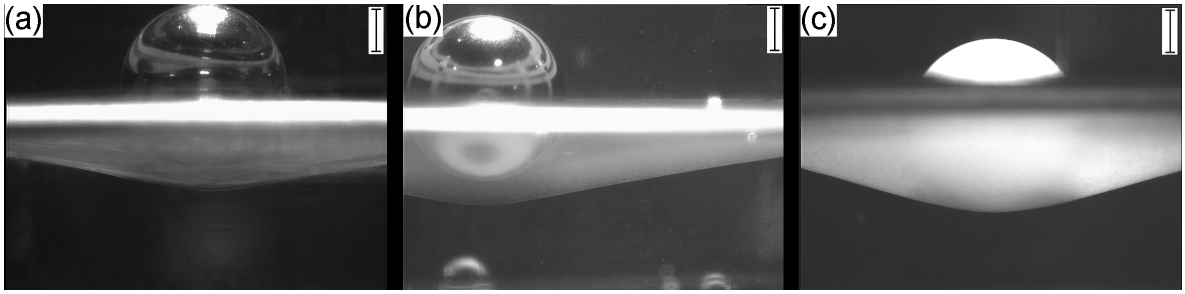

Fig. 4. Images of cell seeded (a) alginate, (b) agarose and (c) collagen hydrogels under spherical indentation recorded by the long focal imaging system (scalebar = 1 mm).

The Young's moduli of 2% alginate and 1% agarose hydrogels seeded with keratocytes and MG-63 cells are shown (Figure 5). It can be seen that the presence of the cells did not have any significant effect on the Young's modulus after just 1 day's incubation. This was confirmed using one-way ANOVA with a Tukey test with a 95% confidence interval. There did appear to be a reduction in the Young's modulus of the 2% alginate when compared to a sample with the same alginate concentration but not incubated in media overnight. This was due to sodium ions in the media replacing calcium in the alginate [31] that resulted in a reversal of the crosslinking caused by the $CaCl_2$.

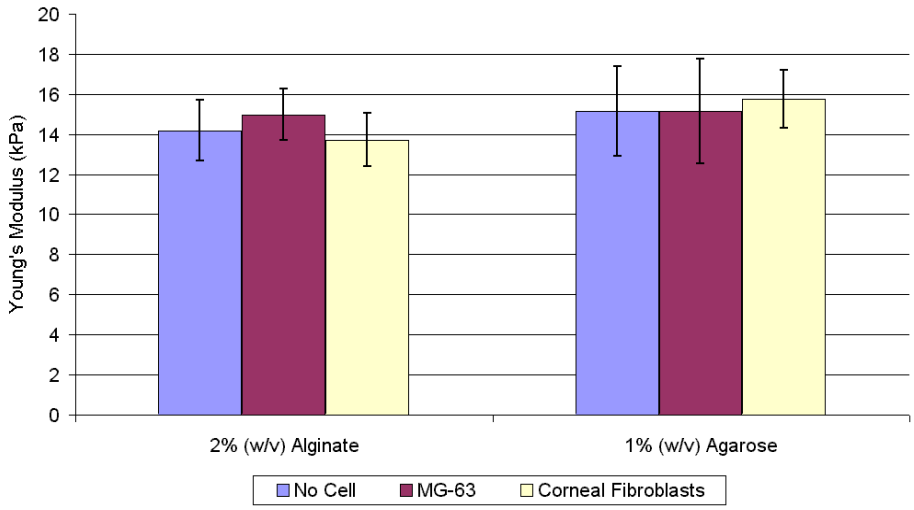

Fig 5. Young's modulus (± standard deviations) of the alginate and agarose hydrogels with and without cells after 1 day (2 million cells per ml, n = 3).

The advantages of this mechanical characterisation technique are compelling and can be briefly summarised: *i)* the stress distribution in the deformed sample is bi-axial and axisymmetric, *ii)* there is no need for force feedback control for creep test, *iii)* it is applicable to permeable or semi-permeable thin hydrogels, and *iv)* the force and displacement resolution can be as accurate as 10 μN and 10 μm respectively. More importantly, real-time measurements can be performed online on cell-seeded hydrogels while fully immersed in solution and at elevated temperatures without risk of contaminating the hydrogels or damaging the instrument. In addition the non-destructive nature of this test allows repeated measurements of the same hydrogel at several different time points.

OPTICAL-COHERENCE-TOMOGRAPHY-BASED SPHERICAL MICROINDENTATION

An alternative to the previous technique is the optical-coherence-tomography (OCT) based spherical microindentation technique [32]. This technique is based on Hertz contact theory where the depth of indentation of a sphere into a hydrogel resting on a substrate can be used to calculate the mechanical properties of the hydrogel. OCT is a non-invasive imaging technique capable of three-dimensional imaging with micrometer resolution [33]. OCT operates on the principle of interferometric backscattering of a beam of light passing though a sample material. A beam of

light is split in two; one of the beams of light passes through the sample of interest while the other acts as a reference (Figure 6). The intensity of backscattered light is measured using photo detectors and used to reconstruct an image of the cross-sectional microstructure of the sample. This technique may be used to measure the depth of indentation of the sphere into a hydrogel in addition to the thickness and geometry of a hydrogel. Images of the samples can be obtained non-destructively and online making it an ideal method of monitoring the structure of engineered tissues in culture.

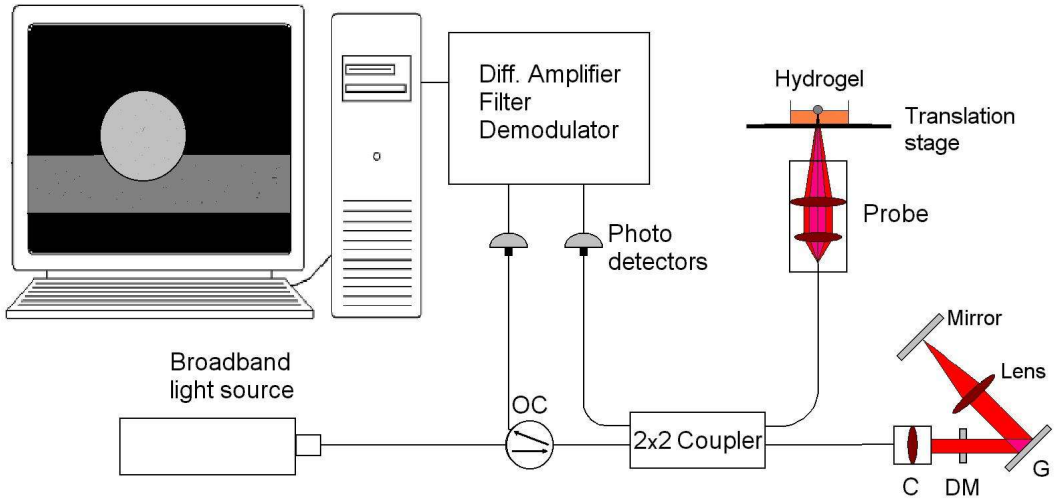

Fig. 6. Schematic representation of the optical coherence tomography system (C = collimator, DM = double pass mirror, G = grating, OC = optical circulator).

Materials and methods

Agarose hydrogels were used to test the feasibility of using this technique to measure the mechanical properties. Unlike the long focal indentation technique where the hydrogel thickness was limited up to 1 mm, thicker hydrogels could be measured using the OCT system. 3 ml of the heated agarose was poured into a small petri dish (diameter 35 mm) and allowed to set. The hydrogels that were formed were between 2 mm and 3 mm in thickness. Several different sized stainless steel spheres of diameter 1.5 to 3 mm were used in these experiments.

A previously described bench-top fibre based time-domain OCT system was used. A 1300 nm superluminescent diode light source was used with a bandwidth of 52 nm [32,34]. The light source yielded a 14 μm axial resolution in free space, or 10 μm in the hydrogel sample when the mean refractive index of the material was assumed to be 1.4. The OCT image was

formed by scanning the sample at 100 Hz with the frame consisting of 400 by 350 pixels, corresponding to a physical size of 5.8 mm by 2.9 mm for the image. The signal-to-noise ratio of the system was found to be 90 dB for the current investigation. An automated precision x-y stage was used to obtain images throughout the sample width thus enabling three-dimensional scanning of the sample.

The mechanical and viscoelastic behaviour of hydrogels was examined by placing a sphere of known weight and size on top of a hydrogel. The weight of the sphere caused a deformation to occur in the hydrogel. The OCT instrument scanned the hydrogel from underneath and recorded images of the deformed hydrogel, which were analysed using ImageJ software (NIH, USA). The weight and size of the ball and the central indentation depth were used to determine the Young's modulus of the hydrogel by applying a simple theoretical model. The change in deformation over time can be used to characterise the viscoelastic creep response of the hydrogel. The current version of our OCT-indentation instrument has a force resolution and displacement resolution as accurate as 10 μN and 15 μm, respectively and the penetration depth of sample scanning is approximately 2 mm. The penetration depth is dependent on the material properties of the sample. Unlike depth sensing microindentation, the OCT based spherical indentation technique does not require any force feedback monitoring system, it enables online measurement and it can be used on samples submerged in fluid. Hertz contact model [35] was used to calculate the Young's modulus of the hydrogels. For this model the hydrogel thickness needed to be at least 10 times the indentation displacement.

Results and Discussion

An image of an agarose hydrogel recorded by OCT is displayed below (Figure 7). It can be clearly seen that the sphere caused a deformation to occur in the hydrogel. This deformation can be used to calculate the Young's modulus of the hydrogel using a simple theoretical model [35]. When we compare the results for 1% (w/v) agarose hydrogels measured by both the long focal

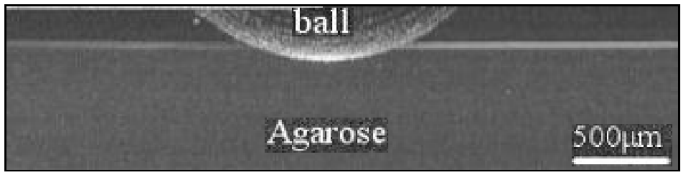

Fig. 7. OCT image of a 1% agarose hydrogel indented using a 2.5 mm diameter stainless steel sphere.

and OCT techniques, we find that both produce similar modulus values (Figure 8). There was no significant difference was found when using a student t-test with a 95% confidence interval. The small difference between results can be explained by the different deformation behaviours of the hydrogels. In the long-focal-microscopy-based spherical microindentation technique the hydrogel undergoes bending and stretching deformations while in the OCT technique the hydrogels undergo simple indentation.

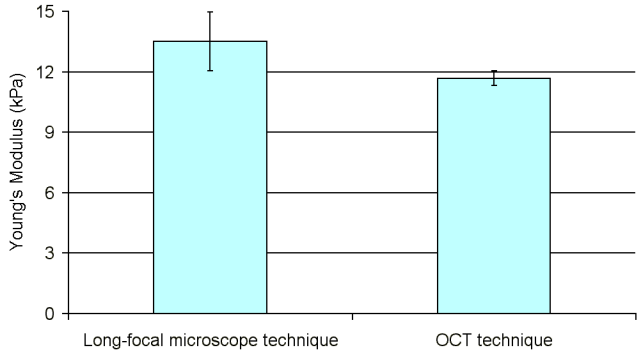

Fig. 8. Young's modulus of 1% (w/v) agarose hydrogels (± standard deviation) found using long-focal microscope spherical microindentation (n = 3) and OCT spherical microindentation (n = 2).

The OCT spherical indentation technique can be used to measure the mechanical behaviour of hydrogels including cell-seeded hydrogels online. The hydrogels can be set up in a sterile, temperature-controlled chamber. Since there is no feed for force feedback control the risk of contamination is substantially reduced. This system enables quick, reproducible measurements of the mechanical properties of hydrogels non-destructively and suitable monitoring of the mechanical properties of engineered tissues.

CONCLUSION

Online monitoring of the mechanical properties of cell-seeded hydrogels is important when examining the suitability of these hydrogels for use in engineering tissues *in vitro*. Two non-destructive approaches for examining the mechanical properties of hydrogels online have been discussed. The long-focal-microscopy-based spherical microindentation approach can be used to measure the mechanical properties of thin hydrogels on-line and non-destructively at different time points. For thicker hydrogels the optical coherence tomography approach can be used. Both these techniques will be of benefit to researchers using hydrogels to try to engineer soft tissues *in vitro*.

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