MICRO-SHAFT-POKING

A Novel Instrument for Mechanically Characterizing Soft Biomimetic Membrance

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Abstract: Characterizing viscoelastic properties of soft biomimetic membranes has become increasingly important due to their biomedical applications such as tissue engineering/regenerative medicine and biosensors. This paper presents a new micro-shaft-poking (MSP) technique which is free from the complication of substrate backing, normally occurred as an intractable problem in the conventional indentation testing for soft membranes. A tailored indention apparatus with a spherical indenter was constructed to achieve the force resolution and displacement of 1μN and 1μm. The biomimetic membranes which were used for mechanical testing were made of two types of hydrogel, alginate and agarose. The results showed that the elastic modulus increased with gel concentration. A creep test has also been conducted to examine the time-dependent behaviors of various hydrogel and a viscoelastic model has been correspondingly developed and applied to describe the experimental results. Other potential applications of this new instrument to other membranes, both artificial and biological, have been addressed in the paper.

1 INTRODUCTION

Recent advancements in biomimetic materials have opened a new avenue for tissue regeneration/implant and the construction of next generation biomedical devices, such as developing engineered tissue and designing cell-based biosensor. In many cases, their viscoelastic/mechanical properties play an important role in the performance and durability of these membranes, and ultimately dictate whether the applications are successful or not. A great need therefore is required for the development of new techniques for mechanically characterizing these emerging biomimetic materials such as hydrogels (Ratner et al., 1996). However, mechanical characterization of these materials in a quantitative manner is highly challenging due to their unique mechanical characteristics, such as fragility and viscoelasticity

Despite the intractability in experimentally measuring the mechanical properties of soft biological materials, recent progress in the advanced instrument developments have made such microscale characterization more feasible (Lu et al., 2004). The common fundamental principle among these advanced methods is to measure the material deformation under an applied load. Among these,

bulge or blister testing, nanoindentation, and microtensile testing are prevailingly used for mechanical characterization of soft biomimetic materials (Espinosa, et al., 2003; Liu et al., 2004). Testing via bulging or nanoindentation alleviate many of the problems such as mounting specimen and providing sufficient resolution of measurements (Scott et al., 2004). Moreover, the data measured by using different testing techniques are often scattered. Such variations have been recognized to be attributed to the technological means employed by each technique and the calibration. Also the different requirements for conducting the experiments and interpretation of results will also contribute to the diecrepancy (Liu et al, 2004; Menciassi et al., 2001). Therefore the mechanical properties of the soft materials are difficult to be unequivocally determined when the various techniques are compared (Espinosa et al., 2003).

In this work, a new ultra-precise measuring instrument, the MSP, for characterizing mechanical properties of biomimetic materials are described. Based on simultaneous force-displacement measurements, the elastic modulus of soft membranes can be determined. This instrument provides a broad range of measurements to facilitate large deformation analysis as well as time dependant

force measurements, with microscale resolution. Firstly, the testing technique has been used to characterize circular biomimetic hydrogel membranes whose properties are of great interest for biomedical applications and have not been investigated well before.

2 EXPERIMENTAL SETUP

2.1 Instrumental Setup

The system, schematically shown as Figure 1, was newly developed for measuring force as a function of displacement. The instrument is based around an inverted optical microscope (Eclipse TE 2000S, Nikon, USA), incorporated with a XYZ motorized motion control interfaced with as external PC. The microscope's Z-axis motorized stage, which is capable of 1μm step and a travelling distance up to 8.5mm, is used as a displacement actuator (ESP300, Newport, Irvine, CA). Attached to Z-axis stage was a specially designed solid arm, on which edge a force transducer (404A, Aurora Scientific Inc., Canada), with 1μN force resolution and 100mN maximum force capability, was mounted firmly. The stability of the arm prevents from the "dead" weight effect of the transducer's head in the output signal and avoids bending of the interior housing of the transducer, which might offset the output. In addition, it ensures precise loading in a vertical position. In the final form the instrument had a force and displacement resolution of 1μN and 1μm respectively.
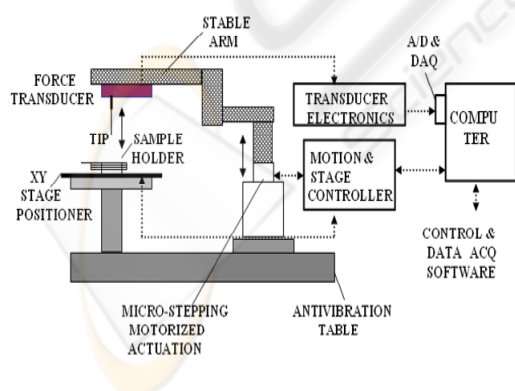

Figure 1: Schematic view of the instrumental set-up (not to scale).

A fine, spherical tip is attached at the end of the force transducer's output tube for deforming the sample. A handy sample holder facilitates mounting of a thin, circular membrane between a set of parallel plastic rings, without affecting the natural properties of the material. The position of the membrane for central alignment can be adjusted precisely by tuning a two dimensional (X-Y) translation stage (ASSY STAGE 25, Cell Robotics Inc., USA), with a resolution higher than 2μm. The force transducer signal is filtered and amplified by using differential amplification (S 400A, Aurora Scientific Inc., Canada). The amplified analogue signal is transmitted through a connector block (DAQ SCB-68, National Instruments, USA) into a data acquisition (DAQ) board (PCI DAQ-6036E, National Instruments, USA) for digitization and further processing. The acquired data were displayed and recorded by a tailored software design based on the Labview platform (National Instruments, USA). Figure 2 shows the interconnections among instrumentation, data acquisition and control.
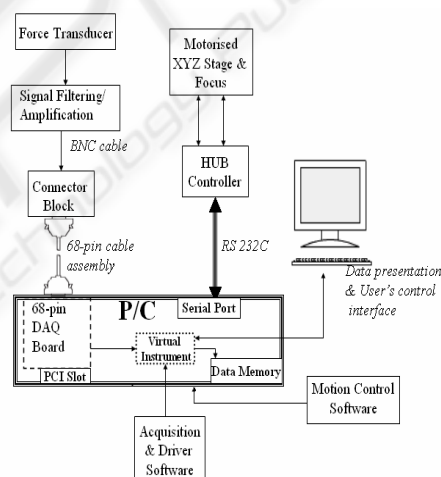

Figure 2: A block diagram of the system showing the interconnections between instrumentation, data acquisition and control interface.

2.2 Material Preparation

Two types of hydrogel, alginate and agarose, were prepared. Alginate is a co-polymer consisting 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 behaviours 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 deionised 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). Different concentrations of the

alginate solution were formed by adjusting the ratio of alginate powder to deionised water. When fully dissolved, the solution was autoclaved for sterilization. Autoclaving had the effect of decreasing the viscosity of the solution. To fabricate alginate hydrogels, rings made from filter paper (Millipore, USA) were placed on the bottom of small petri dish. These rings reduced the amount of shrinkage of the hydrogel after crosslinking and allowed the hydrogels to be lifted from the petri dishes. 200 µl alginate solution was poured inside a ring of inner diameter 11 mm. 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 a hydrogel. Once applied, the $CaCl_2$ solution had to cover the alginate quickly to prevent the hydrogel from forming unevenly. After 10 minutes the $CaCl_2$ solution was removed and the hydrogel was washed twice in phosphate buffered saline (PBS) (Sigma, UK).

Agarose hydrogels were made using agarose type 1 (Sigma, UK). A 2% (w/v) agarose solution was produced by dissolving 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 to over 60°C. When fully dissolved, the solution was filtered to remove any impurities. 200 µl of the solution was applied to a petri dishes with circular filter paper rings of inner diameters 11 mm. The hydrogels were formed by cooling at room temperature. Once the hydrogel had formed, water or PBS was added to the petri dish to prevent the agarose from dehydrating.

3 THEORETICAL ANALYSES

The Young's modulus was calculated from the indentation data using a previously described theoretical model (Scott et al., 2004). For a hydrogel material suspended around its outer edge (Figure 3), the total displacement that the indenter is lowered (δ) is equal to the sum of the depth of penetration into the hydrogel (δ_1) and the vertical deformation displacement of the hydrogel (δ_2). δ_1 can be calculated using the Hertz model (Johnson, 1985);

$$E^* = \frac{3F}{4R^{\frac{1}{2}}\delta_1^{\frac{3}{2}}} \quad (1)$$

where F is the force applied to the hydrogel by the indenter, R is the radius of the indenter tip and E^* is the elastic modulus and can be derived from the equation;

$$\frac{1}{E^*} = \frac{1-\nu_I^2}{E_I} + \frac{1-\nu_H^2}{E_H} \quad (2)$$

where E_I and E_H are the moduli of the indenter and hydrogel respectively and ν_I and ν_H are their Poisson's ratios. Since the modulus of the indenter is much larger than the hydrogel, the term $(1 - \nu_I)/E_I$ was neglected and equation (1) can be rewritten as;

$$\delta_1 = \left(\frac{9F^2(1-\nu^2)^2}{16RE^2}\right)^{\frac{1}{3}} \quad (3)$$

where E and ν are the Young's modulus and Poisson's ratio of the hydrogel materials. The vertical deformation displacement of the hydrogel was calculated using plate-bending theory (Timoshenko & Woinowsky-Krieger, 1959) from the equation;

$$\delta_2 = \frac{3Fa^2(1-\nu^2)}{4\pi Eh^3} \quad (4)$$

where h is the thickness and a is the radius of the hydrogel inside the sample holder. By adding equations (3) and (4), the total displacement of the indenter on the bending-governing deformation was determined from the equation;

$$\delta = \left(\frac{9F^2(1-\nu^2)^2}{16RE^2}\right)^{\frac{1}{3}} + \frac{3Fa^2(1-\nu^2)}{4\pi Eh^3} \quad (5)$$

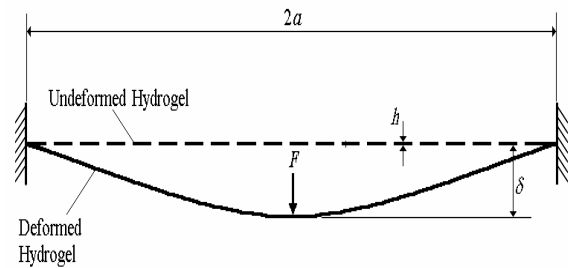The Young's modulus was calculated from equation (5) using Matlab software (MathWorks, USA).

Figure 3: Schematic representations of a membrane deformed by micro-shaft poking (MSP) indentation.

Two theoretical models were used to examine the relaxation behaviour of the hydrogels during indentation. The 3-parameter standard linear model and 5-parameter Maxwell-Weichert model were both used to describe the viscoelastic relaxation response under a constant strain (Figure 4).
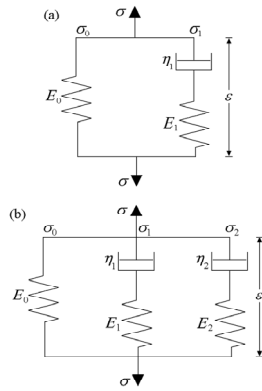
Figure 4: Schematic representation of the (a) 3-parameter standard linear model and (b) 5-parameter Maxwell-Weichert model.

For a 5-parameter Maxwell-Weichert model, which consists of a single spring and two Maxwell elements in parallel, the total stress, $\sigma(t)$, equals to the sum of the stresses applied to the spring and the Maxwell elements;

$$\sigma(t) = \sigma_0 + \sigma_1 + \sigma_2 \qquad (6)$$

where σ_0 is the stress applied to the spring and σ_1 and σ_2 are the stresses applied to each Maxwell element and whose values can be described as;

$$\sigma_0 = \varepsilon E_0 \qquad (7)$$

$$\sigma_1 = \varepsilon E_1 e^{\frac{-E_1 t}{\eta_1}} \qquad (8)$$

$$\sigma_2 = \varepsilon E_2 e^{\frac{-E_2 t}{\eta_2}} \qquad (9)$$

where η refers to the dashpot viscosity. By substituting equations (7), (8) and (9) into equation (6), the stress relaxation function $g(t)$, which equates to $\sigma(t)/\sigma(0)$, can be described as;

$$g(t) = A_0 + A_1 e^{\frac{-t}{\tau_1}} + A_2 e^{\frac{-t}{\tau_2}} \qquad (10)$$

where A_0, A_1 and A_2 represents the strain dependent amplitudes, and τ_1 ($=\eta_1/E_1$) & τ_2 ($=\eta_2/E_2$) represent strain dependent time constants.

For the standard linear model, the stress relaxation function is written as;

$$g(t) = A_0 + A_1 e^{\frac{-t}{\tau_1}} \qquad (11)$$

The values for A and τ were determined using non-linear regression analysis for both relaxation models.

4 RESULTS AND DISCUSSIONS

4.1 Young's Moduli of Hydrogels

A typical force-displacement curve for a 2% w/v alginate and 2% w/v agarose hydrogel indented to 1000μm are shown (Figure 5 (a) & (b)). It can be seen that the loading and unloading curves did not match. This type of behaviour, referred as hysteresis, is common in viscoelastic materials and is the result of energy dissipation during loading.
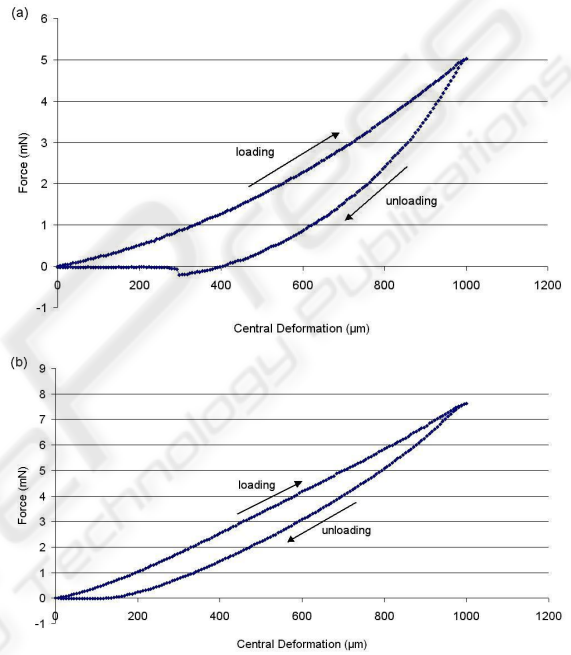

Figure 5: The MSP-measured loading-unloading curves of a hydrogel membrane with (a) 2% alginate & (b) 2% agarose.

The loading-unloading curves appear less linear for alginate hydrogel than agarose hydrogels and there was a more significant difference between the loading and unloading curves for alginate. This suggests that alginate has non-linear viscoelastic characteristics while agarose has more linear elastic characteristics. This type of mechanical behaviour agrees with the findings from previous publications (Bonn et al., 1998; Zhang et al., 2005). It was also found for some of the alginate hydrogels that the indentation force became negative for a short period during unloading. This can be explained by adhesion forces between the indenter and the hydrogel causing the indenter to adhere onto the hydrogel while unloading (Gupta et al., 2007).

Several loading and unloading cycles were recorded for individual hydrogels. It was noticeable

that different indentation cycles on the same hydrogel did not match although later indentation cycles appeared to match much more closely than earlier cycles. It can be seen (Figure 6) that for a 2% alginate hydrogel, after the first indentation cycle an increase in force was only detected after the indenter has been lowered by over 200 μm. This would suggest that plastic deformation of the alginate hydrogels had occurred in addition to elastic and viscoelastic deformation. The plastic deformation, in addition to the viscous properties of the hydrogel, prevented the hydrogel from fully returning to its original pre-indentation shape. There was also a small decrease in the amount of force required to indent the hydrogel with each cycle. This decrease in force was reduced with each cycle until reaching there was no functional force decrease was detectable. This phenomenon is common in biological materials (Fung, 1993) and is the combination of fibre reorganization and fluid movement within the tissue. The loading-unloading cycles for agarose appeared to match more closely than for alginate.
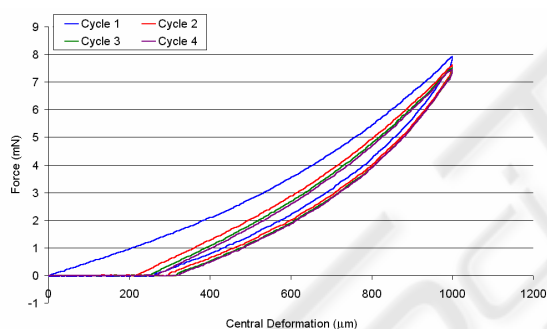

Figure 6: The MSP-measured loading-unloading cycles for a 2% alginate hydrogel.

The Young's moduli of agarose hydrogels of different concentrations indented up to 1000 μm are displayed (Figure 7). Agarose was preferred to alginate for calculating the Young's modulus since it had a more linear elastic response to indentation. The Young's modulus of alginate hydrogels varies in literature with a range between 1 kPa and 100 kPa (Awad et al., 2004; Drury et al., 2004). A non-linear loading curve would result in the values for Young's modulus becoming dependent on the indentation depth. It can be seen that there was an almost linear increase in Young's modulus with agarose concentration. Simple regression analysis was used to confirm the linearity of Young's modulus with agarose concentration between 0.4–1.2% with a coefficient of determination (R^2) equal to 0.9935.
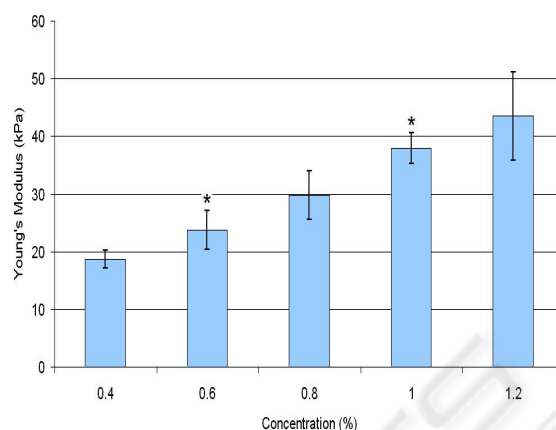

Figure 7: Young's modulus of agarose hydrogels measured by the MSP indentation (± standard deviation, n=4). *represents a significant difference with a 95% confidence over the previous concentration determined using ANOVA-tukey test.

The standard deviation bars show a high degree of repeatability in measuring different agarose hydrogel with the same concentration. Interestingly when the same hydrogel was measured several times, the standard deviation was further reduced i.e. for 0.5 % agarose hydrogel indented to 1000 μm four times, $E = 20.9 ± 0.7$ kPa. The values obtained for 1% agarose by the MSP method appear resemble those found by Bonn et al. (1998) using 3-point bending and Nyland & Maughan (2000) using atomic force microscopy.

4.2 Stress Relaxation of Hydrogels

Normalized force relaxation data was collected for both agarose and alginate hydrogels. The hydrogels were indented to a central displacement depth of 1000μm, which was maintained for 45 minutes. For times longer than 45 minutes, dehydration of the hydrogel would affect the measurement readings. Both agarose and alginate appeared to exhibit relaxation behaviour consistent with viscoelastic materials (Figure 8 (a) & (b)). The amount of force required to maintain the indentation displacement was reduced over time. The normalized force initially decreased quickly but then slowed until reaching a plateau. Alginate appeared to demonstrate a greater relaxation response than agarose, which suggests it is more viscoelastic than agarose, which has more elastic characteristics.

Nonlinear regression analysis, performed using XLStat (Addinsoft, USA), was used to determine the ability of the 3-parameter standard

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linear model and 5-parameter Maxwell-Weichert model to describe the hydrogels' relaxation responses to the deformation applied by MSP. Both models appear to show a high degree of correlation between the actual data and the theoretical model data (Figure 8). The values for coefficients of determination (R^2) for agarose and alginate hydrogels were found to be greater than 0.9 using the standard linear model and greater than 0.95 for the Maxwell-Weichert model. This suggests that the 5-parameter Maxwell-Weichert model is capable of providing a more accurate representation of the relaxation response than the standard linear model.
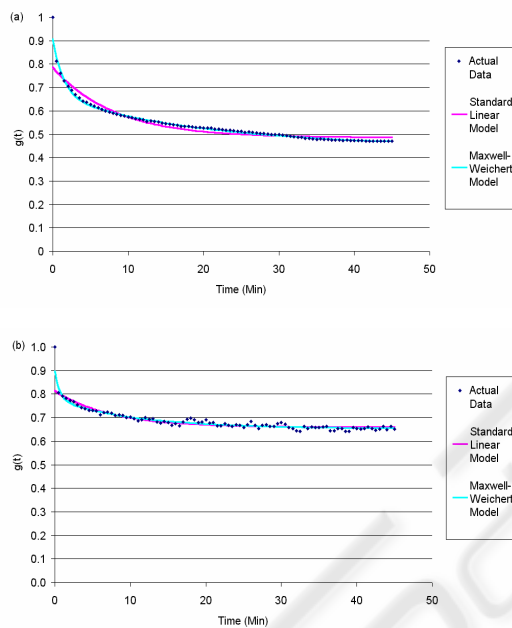

Figure 8: Actual and theoretical model normalised force data for (a) a 2% alginate and (b) a 1% agarose hydrogel at a constant indentation of 1000μm.

5 CONCLUSIONS

The MSP method has been applied to examine the mechanical and viscoelastic characteristics of various biomimetic materials, i.e., agarose and alginate hydrogel membranes and their results have been demonstrated to be satisfactory. Incorporated with simple analyses, the new instrument has been shown to be capable of determining quantitatively viscoelastic and mechanical properties based on experimental data of loading/unloading and stress relaxation curves. The instrument has potentials for testing other soft biological materials, such as human and animal skins.

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