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Mechanical Characterization of Hydrogels and its Implications for Cellular Activities

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1. Introduction

One of the challenges faced by scientists and clinicians is to fabricate physiologically relevant three-dimensional (3D) culture models with controllable biochemical and biophysical properties that can provide an *in vitro* platform to develop and test new clinical therapies.(1) The use of hydrogels is among some of the more promising approaches for the development of culture models for use in tissue engineering and regenerative medicine. These biomaterials consist of a water-swollen network of crosslinked hydrophilic polymer chains. The limited availability of native tissues for transplantation and *in vitro* testing has propelled the need to develop new hydrogels that replicate native tissue extracellular matrix (ECM). Hydrogel materials can be fabricated from natural protein polymers such as collagen, fibrin, agarose, gelatine or alginate, or from synthetic polymers such as poly(ethylene glycol) (PEG), poly(vinyl acetate) (PVA) or poly(acrylic acid) (PAA). The choice of polymer is vital when determining the suitability of a particular hydrogel material for a given application.(2) Natural protein hydrogels are advantageous in that they provide native biochemical cues and are able to simulate many aspects of the natural ECM. Synthetic hydrogels are valuable in that more well-defined, easily tuneable structures and mechanical properties can be achieved in comparison to protein-based hydrogels. Frequently a combination of natural and synthetic hydrogels is utilized in order to more closely mimic the dynamic native culture environments that change in response to cellular behaviour. Hydrogels provide a popular method of culturing cells in a 3D environment as they provide a structure in which a tissue can develop. Hydrogels act as a temporary matrix that allows cells to grow, move and communicate. Their viscoelastic characteristics, biocompatibility, availability and their ability to be remodelled by cells make them a suitable material for tissue regeneration. Cell-seeded hydrogel constructs can also replicate the close contact/adhesion that occurs between cells and ECM. Hence the mechanical properties of the resulting hydrogel construct become a unique property that

mutually affects the constructed hydrogel and the cells. Characterization of the mechanical properties of hydrogel constructs may ultimately have implications on cellular activities.

In vivo and *in vitro* the extracellular environment is vital in controlling cell health and provides both chemical and mechanical stimuli that influence cellular behaviour.(1,3) *In vivo* cells are organized into tissues and organs with complex mechanical and structural architectures.(3) Both endogenous and exogenous forces act upon the cells and their surrounding environment. Endogenous factors include cell–integrin binding to the ECM and cellular responses to soluble factors such as growth factors and cytokines. Exogenous forces include gravity, substrate stiffness, polarity and surface to volume ratios (1) and tissue-specific interactions including traction forces generated by cells.(4)

Many cell types can be described as ‘anchorage dependent’ in that, to remain viable, they require a substrate to attach to.(5) Most soft tissues including vascular, cardiac, dermal, muscle, brain, tendon and cornea consist of ECM combined with adherent cells that possess elastic or visco- elastic characteristics(5) *in vivo*. Tissue culture plastic and glass coverslips provide a relatively rigid microenvironment (lacking many mechanical and biophysical cues) for cells to be cultured *in vitro* when compared to cells in native tissues. *In vivo* it is the combination of the cellular microenvironment and chemical and physical cues that mediate cellular behaviour. These niche environments are often very difficult to replicate *in vitro*. Cellular behaviour can vary markedly based upon the mechanical properties of the culture substrate.(6) Cellular migration, adhesion, proliferation, migration and differentiation can all affect and be affected by the mechanical properties of a tissue.(3)

2. Hydrogel Characterization Techniques

The mechanical and viscoelastic properties of hydrogel materials are important parameters when considering their suitability for use with cells and tissues. These same properties are highly reliant on environmental factors such as temperature and pH, in addition to the cell activity. Thus, it is imperative to be able to determine the material properties of hydrogel constructs under conditions similar to the in-situ environment in which they will be utilized.⁽²⁾ Many techniques exist to measure the mechanical properties of hydrogels and are centred on theories of rubber elasticity and viscoelasticity.⁽²⁾ In general, hydrogels can be considered to behave in a viscoelastic manner, meaning they exhibit both viscous and elastic characteristics. The relationship between stress and strain in viscoelastic materials is dependent on time. Frequently used methods for measuring the mechanical properties of hydrogels include tensile, compression or indentation techniques.

Tensile testing or strip extensimetry testing are the most frequently used methods for the mechanical characterization of hydrogels.⁽⁷⁾ This test involves clamping the hydrogel between two grips and then stretching it (**Figure 1A**). The amount of force required to stretch the hydrogel is measured and plotted against the distance the hydrogel has been stretched. The force and distance can be used to determine the stress and strain applied to the hydrogels and from this the Young's modulus can be calculated. Other parameters such as the ultimate tensile strength and yield strength of the hydrogels may also be determined using this test although these would require testing the sample to failure. This test can also be used to determine the viscoelastic characteristics of the hydrogel by stretching the hydrogel a predetermined distance and measuring the change in force required to maintain that elongation over time. If the hydrogel is viscoelastic, it should undergo stress relaxation resulting in a reduction in force over time until reaching equilibrium. The dynamic modulus of the hydrogels may also be determined by repetitively loading and unloading of the

hydrogel. A variation on the standard tensile test involves using a hydrogel ring that is stretched between two posts. The advantage of this approach is that no grips are required. For both approaches hydrogels may be immersed in solution to ensure that they are maintained in a swollen state. The principle advantage of using these tests are their relative simplicity compared to other techniques. One limitation is that in general only uniform strips or rings can be tested; more complicated geometries would require more complex mechanical models to calculate values. In addition, the fragility of hydrogels can make them difficult to handle and grip in this system.

Compression testing has also been used to examine the mechanical properties of hydrogels. For this technique the hydrogel is placed under a uniform load that results in the hydrogel being compressed (**Figure 1B**). Depending on how the system is set up, either the load or the distance can be controlled while the other is measured. The resulting stress–strain relationship can be used to calculate the compressive modulus of the hydrogel. Due to the viscoelastic nature of hydrogels, typically dynamic moduli at specified frequencies or equilibrium modulus are determined. The equilibrium modulus is calculated from the stress–strain data after the hydrogel has undergone stress relaxation. This technique has previously been used to measure the mechanical properties of several types of cell-seeded hydrogels including fibrin, agarose and gellan gum hydrogels.(8) The samples can be fully submerged in solution during testing to prevent dehydration. Unlike extensometry, the geometry of the hydrogel is not limited to strips or rings, although a flat surface is required. Usually cylindrical hydrogel constructs are used. Limitations include bulging of the material and difficulties in applying even pressure to the sample.

A bulge or inflation test is a more novel technique where a hydrogel can be characterized by inflating it. The hydrogel is held in a sample holder and fluid is pumped underneath it causing it to bulge (**Figure 1C**). The bulge displacement as a function of the applied fluid pressure is

113 measured using a charge-coupled device (CCD) camera or a laser.(7) The relationship
114 between the applied pressure and the resultant strain on the hydrogel can be incorporated into
115 a mathematical model to calculate the elastic or viscoelastic properties of the hydrogel.
116 Leakage, difficulties controlling and measuring the applied pressure and dissolved air
117 becoming trapped in the solution are all problems associated with bulge and indentation
118 testing. The test is also only suitable for flat uniform hydrogels.

119 Indentation techniques have been widely used to characterize soft biomaterials including
120 hydrogels. Hayes et al. (9) were one of the first groups to use indentation to examine the
121 mechanical properties of a tissue. They used indentation to examine the mechanical
122 properties of human cartilage. Indentation has also been used to examine the adhesive
123 characteristics of tissues (10) by measuring the adhesion force between the indenter and the
124 tissue. There are several variations of the indentation techniques used to characterize
125 hydrogels including spherical indentation, micro-indentation and nano-indentation.

126 Spherical indentation involves suspending a thin circular hydrogel around its outer
127 circumferences in a specifically designed sample holder and placing a ball of known weight
128 and size onto the hydrogel (**Figure 1D**). The weight of the ball causes the hydrogel to
129 deform. The deformation is recorded *via* a CCD camera and the depth of indentation is used
130 to calculate the elastic modulus of the hydrogel.(11) The viscoelastic properties of the
131 hydrogel can also be monitored by measuring the change in central deformation over
132 time.(12) This approach is particularly suitable for cell-seeded hydrogels as the whole
133 assembly can be fully submerged in solution and be kept in an incubator at 37 °C while
134 testing. This technique has been used to examine the effect of fibroblasts on the mechanical
135 properties of collagen hydrogels (13) and the influence of nano fibres on hydrogel properties,
136 (14) and for optimizing crosslinking conditions for hydrogels.(15) This technique allows for

137 online, real-time and non-destructive measurements to be taken over prolonged culture
138 periods.

139 A variation of the spherical indentation technique involves placing a hydrogel onto a flat
140 substrate rather than suspending it (**Figure 1E**). This approach is more suitable to thicker
141 hydrogels while the suspension approach is more suited to thinner hydrogels. The weight of
142 the ball causes the hydrogel to deform and the deformation depth and weight of the ball can
143 be applied to mathematical model called the Hertz model, to calculate the elastic modulus of
144 the hydrogel. The main difficulty with this technique is accurately measuring the depth of
145 indentation. One method around this problem is to use optical coherence tomography (OCT).
146 The combination of spherical indentation and OCT has previously been used to measure the
147 mechanical properties of agarose hydrogels.(16)

148 Micro-indentation involves deforming a hydrogel using a rigid indenter connected to a force
149 transducer. The indenter is lowered onto the hydrogel and deforms it to a particular depth.
150 The depth of indentation and the amount of force applied are both applied to theoretical
151 model to calculate the mechanical properties of the hydrogel. The hydrogel may be suspended
152 around its outer edge or placed flat on a substrate (**Figure 1F and G**). For suspended
153 hydrogels a number of different theoretical models can be used to calculate the mechanical
154 properties of the hydrogel.(17) For hydrogels on a flat substrate, the Hertz model is used to
155 calculate the modulus of the hydrogel.(18) Micro-indentation can be used to examine
156 regional variation across different areas of a hydrogel.

157 Nano-indentation works on the same principle as micro-indentation, but the tip size and
158 indentation depth are on a nanometric scale. This apparatus consists of a sharp-tipped
159 indenter attached to a cantilever beam. Mechanical characterization at this scale is limited to
160 producing data on the surface properties of the hydrogel. The difficulties associated with

161 using nano-indentation with hydrogels include accurate calibration of the instrument,
162 applying a suitable mechanical model and the elimination of other sources of error.(19)

163 Eastwood et al.(20) developed a tensioning-culture force monitor system which can apply a
164 predetermined loading to the hydrogel, in particular collagen hydrogel, and monitor the
165 contraction strain generated by resident fibroblasts. The beauty of this system is that it can
166 monitor the strain development for days continuously and visualize the associated global
167 morphology change.

168 Ultrasound elastography is a technique that works by transmitting ultra- sonic waves through
169 the hydrogel and then reading backscattered waves, which can be used to form 2D images.
170 When a force is applied to the hydrogel, the resulting displacement can be detected
171 throughout the hydrogel. This information can then be processed to characterize the
172 mechanical properties of the hydrogel. Fromageau et al.(21) used several variations of this
173 technique to measure the Young's modulus of PVA hydrogels. They found that elastography
174 produced similar mechanical values to standard mechanical testing techniques. The main
175 limitation with this technique is the costs involved in the purchasing and running of the
176 ultrasound equipment.

177 Recently, Li et al.(22) explored a novel approach that utilizes a low-coherence interferometer
178 to detect the laser-induced surface acoustic waves (SAW) from agar hydrogels to mimic soft
179 tissues. This technique allows for rapid characterization of the elastic properties of soft
180 biological tissues and has the advantage of being a non-destructive technique.

181 There is a widespread demand for the development of non-destructive techniques that permit
182 the continuous measurement of hydrogel constructs for prolonged culture periods. The use of
183 non-destructive mechanical characterization techniques is extremely valuable in that they
184 allow for changes in mechanical properties over time to be characterized. Such changes can

then be more accurately linked to cell activity and remodelling of the hydrogel matrix. Among other techniques, micro-indentation, ultrasound elastography and the combination of OCT and surface acoustic wave or with indentation techniques are extremely powerful tools for the characterization of the mechanical properties of hydrogels or soft tissues.

3. Effect of Hydrogel Mechanical Properties on Cellular Activities

In most native tissues, anchoring cells attach to the surrounding ECM. This ECM provides an inner physical support and its composition, topography and stiffness provides biochemical and biophysical cues that are necessary to the development and maintenance of these tissues. Until recently, chemical regulators within the extracellular environment have primarily been investigated, with little emphasis regarding the influence of mechanical regulation.(3) Similar to surface chemistry, the mechanical properties affect the local behaviours of tissues and cells. Normally cells embedded in tissues are able to ‘probe’, ‘feel’ or ‘sense’ the elasticity or stiffness of their surrounding matrix (5,6) or substrate as they anchor and pull themselves along during cell migration. ‘Stiffness’ refers to the measure of a material’s ability to resist deformation and this can change during physiological processes including embryonic development, wound healing and pathological conditions.(4) In the body, the magnitude of stiffness is vast, ranging from a few kPa in adipose tissue²³ to GPa in bone.(4,24) A wide variation in matrix stiffness along with biochemical signals influence focal-adhesion structures and the cytoskeleton.(5) Previous studies using cells committed to a particular lineage, especially fibroblasts, on coating collagen gels and wrinkling-silicone sheets also suggest some responsiveness to the physical state of the matrix.(25)

In addition to applying force to its surroundings, the cells themselves respond to the resistance of the surrounding environment.(26) As the physical conditions of tissues can

be altered during pathological conditions, this can affect cellular behaviour and differentiation. Cells adapt their adhesions, cytoskeletal configuration and general morphology in response to changes in substrate resistance or stiffness.(5) For example, cells attached to stiff, rigid constructs will form stable focal adhesions, whereas cells attached to less stiff materials will have diffuse and dynamic adhesion complexes.(5,26)This can have a direct impact on cellular migration and proliferation, such as increased proliferation in cells seeded onto stiffer substrates.(6) This can be linked to cellular wound healing responses as, often, the granulation and change in mechanical properties of scar tissue (6) is related to cellular infiltration and remodelling. In general, cells appear to adhere, spread and survive better on stiffer materials, although there are exceptions to this including neutrophils, which do not appear to be affected by substrate stiffness,(27) and neurons, which actually show improved survival on stiffer materials.(28) Studies on fibroblasts cultured on hydrogels have demonstrated that substrate stiffness significantly alters ECM assembly, cell spreading and motility.(6,29)

The mechanical properties of hydrogels can have a profound influence on regulating the phenotypic behaviour of cells. This is most noticeable with stem cells, where variations in stiffness can promote differentiation towards different lineages. Engler et al.(30) showed that the ability of stem cells to differentiate towards specific lineages was dependent on the substrate stiffness of the materials on which the cells were cultured. They noted that neurogenic differentiation was optimal at a stiffness of 0.1–1 kPa, myogenic differentiation at 8–17 kPa and osteogenic differentiation at 25–40 kPa. In hydrogels the effect of the concentration, which is directly linked to mechanical properties, on the differentiation of neuronal stem has been investigated.(31) Phenotypic neuronal markers were up regulated when the hydrogel stiffness matched that of brain tissue. Bian et al.(32) found that the chondrogenic capacity of mesenchymal stem cells could be optimized by varying the stiffness

of hyaluronic acid hydrogels. Likewise, Steward et al.(33)found that differentiation of mesenchymal stem cells could be partially regulated by hydrogel stiffness towards osteogenic or chondrogenic lineages.

Cell adhesion can also play a significant role on the mechanical properties of hydrogels and is vital for many other processes such as matrix contraction and cell migration. Hydrogel stiffness is dependent on the polymer concentration and the crosslinking density. A consequence of increasing the polymer concentration often results in a subsequent increase in adhesion sites. Cell adhesion can have a profound effect on cell behaviour and cell phenotype. Trappmann et al.(34) showed that differing protein anchorage densities can be used to regulate stem cell fate. This finding is important as it demonstrates the interdependent relationship between matrix stiffness, binding site availability and cell phenotype. Of course, not all hydrogels facilitate binding by cells. Steward et al.(35) compared the phenotypic and cytoskeletal behaviour of cells in a hydrogel that facilitates binding, i.e. fibrin, and cells in a hydrogel that lack binding sites, i.e. agarose. Cells in hydrogels that allowed binding had a spread morphology while those in hydrogels that lacked binding sites maintained a spherical morphology.

4. Effect of Cellular Activity on Hydrogel Properties

In addition to hydrogels having an effect on the behaviour of cells, reciprocally the cell activity within the hydrogels can affect their bulk mechanical properties. Similar phenomena are found in diseases such as those that affect connective tissues and alter the mechanical properties of the tissue. The result is often the formation of hard tumours or the generation of ulcers. In these cases, it is the cellular activities that regulate the ECM. Hence, cells seeded in hydrogels can affect their mechanical properties through several metabolic activities

including digestion *via* the production of enzymes; proliferation; matrix synthesis; contraction; ECM deposition and crosslinking.

Enzymes are produced by cells to initiate matrix remodelling and cell migration. Among the most prominent enzymes produced by cells are a family of enzymes called matrix metalloproteinases (MMPs). Mauch et al.(36) showed that fibroblasts seeded in collagen hydrogels release MMPs, resulting in the degradation of the hydrogel matrix. The enzymatic degradation of hydrogels by cells can be easily controlled by the addition of MMP inhibitors and other reagents that can influence the cell–matrix mechanical relationship. In addition to preventing matrix degradation, the inhibition of MMPs may also prevent the contraction and remodelling of hydrogels. Ahearne et al.(13) found that exogenous addition e.g. cytochalasin, to fibroblast-seeded collagen hydrogels reduced the fibroblasts' contractibility and capacity of adhesion to hydrogel, which significantly decreased the elastic modulus of the hydrogel construct (**Figure 2**).

One of the most prominent mechanisms by which cells affect the mechanical properties of hydrogels is through the production of ECM proteins. For example, hydrogels seeded with chondrocytes, mesenchymal stem cells or infrapatellar fat-pad derived progenitor cells have been shown to increase the stiffness of these hydrogels when they were cultured in a chemically defined prochondrogenic medium.(8,37-39) This increase in stiffness has been attributed to the release of collagen and sulfated glycosaminoglycans (sGAG) by cells that accumulate in the hydrogel and can be determined using biochemical analysis and histological staining, as shown in **Figure 3**. Hydrogels may also undergo calcification when cells are cultured in a pro-osteogenic medium. The increase in calcium deposition can increase the bulk stiffness of the hydrogel. Calcium phosphate may also be incorporated into the hydrogel prior to fabrication to increase the hydrogel stiffness or induce it to be formed. Douglas et al.(40) incorporated alkaline phosphatase into collagen and PEG-based hydrogels

to induce their mineralization into calcium phosphate. They found that calcium phosphate was formed, and mineralization increased the Young's modulus of the hydrogel. One factor that needs to be considered when relating hydrogel stiffness to ECM production is the ability of the hydrogel to retain the newly formed matrix components. Hydrogels with a high porosity or high-water content may not retain matrix proteins as easily as other hydrogels. The loss of matrix proteins in this manner would affect the change in hydrogel stiffness.

Many hydrogels undergo contraction when seeded with cells (**Figure 4**). Hydrogels such as collagen and fibrin contain ligands that enable cell adhesion. Due to their limited mechanical strength, the cells are able to contract these hydrogels. Contraction can be a problem when attempting to design hydrogels that incorporate specific geometries that replicate the native tissues. These geometries can be destroyed by cells contracting their surrounding matrix.(41) Bell et al.(42) have suggested that contraction can be advantageous and may be used to enable the cells to engineer hydrogels into tissue-like structures. It has previously been shown that increasing the stiffness of these hydrogels reduces the rate of contraction. Ahearne et al.(43) showed that following 25 days in culture, collagen hydrogels seeded with fibroblasts at a concentration of 2.5 mg mL^{-1} reduced in thickness by 85% as a result of contraction, while stiffer hydrogels fabricated at a concentration of 4.5 mg mL^{-1} showed a reduced in thickness by approximately 60%.

Mechanical stimulation of the cells has been shown to affect the ability of cells to change their surrounding matrix.(44) The application of force onto cells can initiate several cellular processes including ion-channel activation, phosphorylation and cytoskeletal changes.

Another factor that plays an important role in dictating the cell–matrix mechanical relationships is the initial conditions used to manufacture the hydrogels. Cell seeding density can also influence the mechanical properties of hydrogels. Increasing the cell number often leads to an increase in the rate of hydrogel remodelling and changes in mechanical properties.

For fibroblast-seeded collagen hydrogels, the initial cell and collagen concentration used was found to affect the ability of cells to change the mechanical properties of the hydrogels.(43) Varying the initial cell density in turn varies the amount of force that can be generated to remodel the hydrogels. This phenomenon demonstrates a clear mechano feedback response between fibroblasts and their surrounding hydrogel matrix.

5. Mechanical Properties as a Marker of Cellular Activities

The mechanical properties of soft tissues are often closely related to their physiological function. For example, *in vivo* tissue contraction, remodelling and fibrosis (or scarring) following injury or disease often results in an alteration to the mechanical properties of the affected tissue (45) due to an ‘activation’ of the native cell phenotypes into their injury subtypes.(46) The dynamic reciprocity between hydrogel mechanical properties and cell activity has driven researchers to investigate how the mechanical properties of hydrogel constructs affects cell behaviour and whether these properties can act as a marker to predict cellular activity. This relationship has to be considered when designing hydrogels that need to be suitable for implantation. Here we present several examples to demonstrate how the mechanical properties of hydrogel constructs can reflect cellular activities.

5.1. Indicator of Differentiation Status

A recent study by Wilson et al.(14) demonstrated how the assessment of mechanical properties in terms of elastic modulus measurement could be used to determine the effect of biochemical ingredients and topographic features on corneal stromal cell differentiation in collagen hydrogels. The aim was to determine the most suitable culture condition whereby cultured corneal stromal cells that were initially ‘activated or fibroblastic in phenotype could

be restored to the native ‘inactivated’ keratocyte phenotype *in vitro*. The basis of using matrix contraction capacity as an additional marker of corneal stromal cell phenotype differentiation in response to different culture conditions is that corneal keratocytes are quiescent and noncontractile and corneal fibroblasts are contractile and motile.(47) Thus, the construct contraction and elastic modulus measurements combined provided a descriptive insight into what was happening at a cellular level within the constructs when culture conditions change. This was used to indicate that the synergistic effect of nanofibre incorporation, serum removal, plus insulin medium supplementation provided the most suitable environment for the restoration of the native corneal keratocyte cell phenotype. These results were then corroborated with microscopic and genotypic characterization data to further validate that mechanical characterization can act as a sensitive marker of cellular activities such as cell phenotype and differentiation (**Figure 5**).

5.2 Indicator of Cell Viability and Contractility

Ahearne et al.(13) used a spherical indentation technique to investigate the relationship between cell viability, hydrogel contraction and hydrogel elastic modulus in response to long-term culture (**Figure 6**). It was found that an initial increase in elastic modulus coincided with contraction of the hydrogel while a reduction in cell viability over several weeks in culture resulted in a reduced modulus. Inhibition of contraction using an MMP inhibitor found that when contraction was prevented, there was no subsequent increase in modulus. It was also found that the inhibition of actin stress fibres resulted in a reduction in elastic modulus, suggesting that the intrinsic strain applied by these cells was instrumental in controlling the bulk mechanical properties of the hydrogel. The actin staining images at corresponding time points exhibited clear morphology difference in responding to the associated modulus the

specimens exhibited higher modulus expressed highly stretched actin filaments (**Figure 6B** and **C**), while the destroyed actin morphology (**Figure 6D**) appeared at the specimens which had low modulus with long culture duration, implying the low cell viability

5.3. Indicator of Network Structure in the Hydrogel

There have been a large number of reports dedicated the effect of ageing on protein structures; in particular collagen type I, as it is a key lifelong structural protein in the body. A prevalent ageing mechanism, concerned with the non-enzymatic glycation of collagen, is the formation and accumulation of advanced glycation end-products (AGEs).⁴⁸ Accumulation of AGEs in relation to increasing chronological age has been linked to permanent alterations to the intra- and intermolecular structure of collagen, which often manifests as compromised mechanical properties to the tissue or construct being investigated. In recent work by Wilson et al.,⁽⁴⁹⁾ type I collagen was extracted from the tendons of different aged rats, varying from 2–3 days (newborn) to 2 years (old adults). The mechanical properties of the resulting reconstituted hydrogel constructs were then measured using an indentation technique.⁽⁵⁰⁾ It was found that in acellular hydrogel scaffolds that there was a clear visible trend showing that increasing age resulted in a reduced in the elastic modulus (**Figure 7**). The preliminary examination of the elastic modulus of corneal stromal fibroblasts grown in these hydrogels found that younger collagen induced higher contraction than older collagens manifesting as a higher modulus. Hence, it has been postulated that at a given collagen concentration, the younger collagen hydrogels (newborn and 2 months old) with a highly organized fibrous structure, resulted in a higher construct modulus compared to the randomly and loosely packaged older specimens (6 months and 2 years old). Thus, it is feasible to predict microscopic differences in the collagen hydrogel through the measurement of mechanical properties

379

380 **6. Strategies for Improving the Mechanical Properties of Hydrogels**

381 When using hydrogels to study cell–ECM interactions, it becomes critical to tailor the
382 hydrogels’ mechanical properties. Various strategies have therefore been proposed to
383 improve their mechanical characteristics. A fundamental limitation of hydrogels for tissue
384 engineering is their inferior mechanical strength and stiffness in comparison to the native
385 tissue that they are being used to replicate. These mechanical properties result from the high
386 water content and random fibre orientations found in hydrogels.(7) Once the mechanical
387 properties of a hydrogel material have been determined, it is often desirable to improve the
388 mechanical strength of the construct so that it is more suitable for a given application.(2) The
389 mechanical properties of hydrogels can be improved using numerous strategies including the
390 alteration of the co-monomer composition, increasing/decreasing the crosslinking density,
391 alterations to the conditions in which the polymer is formed,(2) the addition of cells onto or
392 into the matrix *via* matrix remodelling, ECM secretions and the application of intrinsic strain.

393

394 *6.1. Concentration*

395 One approach to improving the mechanical properties of hydrogels is to increase the polymer
396 concentration. Several studies have examined the relationship between mechanical properties
397 and polymer concentration in hydrogels. Ahearne et al.(50) found that there was an almost
398 linear increase in elastic modulus with hydrogel concentration when examining agarose and
399 alginate hydrogels. Buckley et al.(37) found a similar trend when measuring the equilibrium
400 and dynamic moduli of agarose hydrogels of increasing concentration. The elastic modulus of

collagen hydrogels has also been shown to increase with concentration.⁽⁴³⁾ Interestingly, the initial collagen concentration also affected the subsequent rate of hydrogel contraction and matrix remodelling, with a lower initial collagen concentration having a faster rate of contraction. This faster rate of contraction led to these hydrogels having a higher cell density and a higher overall collagen density compared to the other hydrogels after 25 days in culture.⁽⁴³⁾ Methods of increasing the concentration of hydrogels such as plastic compression has also demonstrated the relationship between hydrogel stiffness and hydrogel concentration.^{51,52} By pushing fluid out of the hydrogels, this led to an increase in concentration thus an increase in stiffness. It has been reported that the polymer concentration in these hydrogels can increase by a factor of over 100.

6.2. Crosslinking

Chemical and photochemical crosslinking of matrix components such as collagen can also be used to influence the mechanical characteristics of hydrogels. Glutaraldehyde crosslinking of hydrogels has been shown to enhance the mechanical strength of several types of hydrogel.⁽⁵³⁾ The main problem with using glutaraldehyde is its toxicity. Alternative crosslinking agents such as genipin have been suggested as these are less toxic than glutaraldehyde.⁵³ UVA-crosslinking in the presence of riboflavin has been shown to increase the stiffness of collagen hydrogels without damage to the cells in those hydrogels.¹⁵ UV light has also been used to develop hydrogels with a stiffness matrix gradient to allow for the study of hydrogel stiffness and cell behaviour.⁽⁵⁴⁾

6.3. Composition

Altering the ratio of different monomers used to prepare a hydrogel is one of the simplest methods to increase the mechanical properties of the construct.(2) Provided that the hydrogel is not fabricated using identical monomer units, then by increasing the concentration of the physically stronger component, this should give a favourable outcome. Alteration of the polymerization conditions can dramatically alter the final formed product.(2) Time, temperature and the amount and type of solvent used can all be altered accordingly. The volume of solvent used is of particular importance since it can alter crosslinking density, the type or nature of the solvent can alter the copolymer structure.(2) Post-polymerization techniques can also alter the network structure of a hydrogel, causing alterations to mechanical strength. In addition, thermal cycling of the polymer, which involves successive freezing and thawing cycles can also increase the mechanical properties of hydrogels.(2,55)

6.4. Orientation of Fibrous Components

Often, the native tissue architecture is pivotal to the *in vivo* mechanical strength and function of a tissue. Much research has focused upon the mimicking of native tissue architecture in both 2D and 3D cultures. Contact guidance techniques have been extensively researched as they affect several cell characteristics including orientation, morphology, differentiation and secretion of ECM proteins. It is the material composition and more specically the 3D nano- and microscale structure (the mesostructure) of bioartificial constructs that are pivotal to their success.(51) Micro- and nanopatterned surfaces, magnetic alignment and electrospinning techniques are among a variety of techniques utilized in order to achieve this.

6.5. Micro- and Nanopatterning

Micro- and nanopatterned surfaces are often manufactured by the use of templates with well-defined groove widths and depths into which cells with and without matrix materials are added.(56) The patterned surfaces effectively restrict random cell growth *via* the incorporation of either physical or biochemical barriers. Orientated deposition of ECM components is capable of reinforcing the substrate in a given direction, which enhances the global mechanical properties of the original construct.(56)

6.6. *Magnetically Aligned Collagen*

Magnetic fields have been utilized in an attempt to create orientated collagen type I fibrils.(57) The use of magnetic fields to induce collagen orientation is advantageous in that it is non-destructive.(57) It has been reported that molecules of collagen can be assembled into orientated fibrils *via* the application of a magnetic force.(57) In brief, this can be achieved by loading an aliquot of collagen into a shallow sample holder and positioning it horizontally in the central region of a split coil superconducting magnet and increasing the temperature from 20 to 30 °C for approximately 30 min. The collagen molecules assemble into orientated fibrils perpendicular to the applied field and transform into a viscous gel that is stable and orientated after the magnetic field is removed. A limitation of this technique is that fibril diameter cannot be regulated using this technique. Furthermore, there is conflicting evidence suggesting that the application of strong magnetic forces can in fact impair cell function and viability.(58)

6.7. *Electrospinning of Nanofibres*

Electrospinning is a process that is able to produce continuous fibres from the submicron down to the nanometre–diameter range.(59) These fibres can then be arranged to recreate the

in vivo tissue microstructures and arrangements. Several studies have incorporated electrospun aligned nanofibres into hydrogels to improve mechanical properties and regulate cell behaviour. A schematic showing how aligned nanofibres meshes can be incorporated into a collagen hydrogel is shown in **Figure 8**. Wilson et al.(60) found that there was an increase in elastic modulus of collagen hydrogels seeded with corneal stromal cells after PLDLA nanofibres were added. The nanofibers also influenced the cell phenotype and cell orientation and reduced the rate of hydrogel contraction. Tonsomboon and Oyne (61) found a 10-fold increase in modulus after incorporating crosslinked gelatine nanofibres into alginate hydrogels. The combination of electrospun nanofibres and hydrogels represents an exciting new approach to engineering tissues with improved mechanical properties.

7. Conclusion

It has been demonstrated that the mechanical properties of hydrogels play a key role in the regulation of cellular activities and those cells are capable of remodelling the structural and mechanical properties of their surrounding hydrogel matrix. Understanding this reciprocal relationship is vital in the development of new tissue engineering and regenerative medicine strategies. It is envisioned that, by tailoring the mechanical characteristics of hydrogels to particular applications, more anatomically accurate tissues could be engineered.

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591 **Figures captions and captions**

592 Figure 1. Schematic representation of the following mechanical tests where F represents the

593 applied force: (A) tensile; (B) compression; (C) inflation; (D) spherical indentation
 594 (suspended); (E) spherical indentation (on substrate); (F) micro-indentation (suspended); (G)
 595 micro-indentation (on substrate).

596

Figure 2 .The elastic modulus change in response to the addition of cytochalasin in collagen hydrogel seeded with fibroblasts, which disrupted actin filament in fibroblasts.(13)

618 Figure 3. Increase in collagen (stained with picosirius red) and sGAG (stained with Alcian
619 blue) in cell-seeded agarose hydrogels after 21 days in culture in a chondrogenic medium

620

1 hour

5 days

Figure 4: Images of a collagen hydrogel seeded with corneal fibroblasts that have undergone contraction.(13)

Figure 5. (A) The elastic modulus and gene expression of corneal stromal cells grown in collagen hydrogels for 14 days in response to chemical and topographic regulation. F denotes serum-containing medium, K denotes serum-free, insulin supplemented medium, and K0 denotes serum-free b-FGF supplemented medium; +fibre indicates the incorporation of nanofibres in the hydrogel. (B) The gene expression without nanofibre incorporation; (C) the gene expression with nanofibre incorporation; keratocan and ALDH3 are keratocyte-specific genes, while Thy-1 and AQ2 ACTA2 are corneal fibroblast-specific genes

Figure 6: Change in elastic modulus of collagen hydrogels seeded with corneal stromal fibroblasts in response to culture time and the MMP inhibitor ilomastat. (13) Corresponding actin stained specimens at (A) 7 days; (B) 14 days; (C) 21 days and (D) 42 days

Figure 7. The elastic modulus of acellular hydrogel scaffolds using collagen extracted from rats of different ages.

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Figure 8. Schematic representation of the assembly process used to fabricate a nanofibre –

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hydrogel construct