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1 **Mechanical Characterization of Hydrogels and its Implications for Cellular Activities**

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16 **1. Introduction**

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

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

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

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

62

63 **2. Hydrogel Characterization Techniques**

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

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

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

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

110 A bulge or inflation test is a more novel technique where a hydrogel can be characterized by
111 inflating it. The hydrogel is held in a sample holder and fluid is pumped underneath it causing
112 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 suspend
152 around it 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

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

189

190 **3. Effect of Hydrogel Mechanical Properties on Cellular Activities**

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

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

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

223 The mechanical properties of hydrogels can have a profound influence on regulating the
224 phenotypic behaviour of cells. This is most noticeable with stem cells, where variations in
225 stiffness can promote differentiation towards different lineages. Engler et al.(30) showed that
226 the ability of stem cells to differentiate towards specific lineages was dependent on the
227 substrate stiff- ness of the materials on which the cells were cultured. They noted that
228 neurogenic differentiation was optimal at a stiffness of 0.1–1 kPa, myogenic differentiation at
229 8–17 kPa and osteogenic differentiation at 25–40 kPa. In hydrogels the effect of the
230 concentration, which is directly linked to mechanical properties, on the differentiation of
231 neuronal stem has been investigated.(31) Phenotypic neuronal markers were up regulated
232 when the hydrogel stiffness matched that of brain tissue. Bian et al.(32) found that the
233 chondrogenic capacity of mesenchymal stem cells could be optimized by varying the stiffness

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

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

250 4. Effect of Cellular Activity on Hydrogel Properties

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

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

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

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

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

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

300 Mechanical stimulation of the cells has been shown to affect the ability of cells to change
301 their surrounding matrix.(44) The application of force onto cells can initiate several cellular
302 processes including ion-channel activation, phosphorylation and cytoskeletal changes.
303 Another factor that plays an important role in dictating the cell–matrix mechanical
304 relationships is the initial conditions used to manufacture the hydrogels. Cell seeding density
305 can also influence the mechanical properties of hydrogels. Increasing the cell number often
306 leads to an increase in the rate of hydrogel remodelling and changes in mechanical properties.

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

312 **5. Mechanical Properties as a Marker of Cellular Activities**

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

323

324 *5.1. Indicator of Differentiation Status*

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

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

342

343 *5.2 Indicator of Cell Viability and Contractility*

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

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

357

358 *5.3. Indicator of Network Structure in the Hydrogel*

359 There have been a large number of reports dedicated the effect of ageing on protein
360 structures; in particular collagen type I, as it is a key lifelong structural protein in the body. A
361 prevalent ageing mechanism, concerned with the non-enzymatic glycation of collagen, is the
362 formation and accumulation of advanced glycation end-products (AGEs).48 Accumulation of
363 AGEs in relation to increasing chronological age has been linked to permanent alterations to
364 the intra- and intermolecular structure of collagen, which often manifests as compromised
365 mechanical properties to the tissue or construct being investigated. In recent work by Wilson
366 et al.,(49) type I collagen was extracted from the tendons of different aged rats, varying from
367 2–3 days (newborn) to 2 years (old adults). The mechanical properties of the resulting
368 reconstituted hydrogel constructs were then measured using an indentation technique.(50) It
369 was found that in acellular hydrogel scaffolds that there was a clear visible trend showing that
370 increasing age resulted in a reduced in the elastic modulus (**Figure 7**). The preliminary
371 examination of the elastic modulus of corneal stromal fibroblasts grown in these hydrogels
372 found that younger collagen induced higher contraction than older collagens manifesting as a
373 higher modulus. Hence, it has been postulated that at a given collagen concentration, the
374 younger collagen hydrogels (newborn and 2 months old) with a highly organized fibrous
375 structure, resulted in a higher construct modulus compared to the randomly and loosely
376 packaged older specimens (6 months and 2 years old). Thus, it is feasible to predict
377 microscopic differences in the collagen hydrogel through the measurement of mechanical
378 properties

6. Strategies for Improving the Mechanical Properties of Hydrogels

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

6.1. Concentration

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

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

411

412 *6.2. Crosslinking*

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

422

423 *6.3. Composition*

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

435

436 *6.4. Orientation of Fibrous Components*

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

445

446 *6.5. Micro- and Nanopatterning*

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

453 *6.6. Magnetically Aligned Collagen*

454 Magnetic fields have been utilized in an attempt to create orientated collagen type I
455 fibrils.(57) The use of magnetic fields to induce collagen orientation is advantageous in that it
456 is non-destructive.(57) It has been reported that molecules of collagen can be assembled into
457 orientated fibrils *via* the application of a magnetic force.(57) In brief, this can be achieved by
458 loading an aliquot of collagen into a shallow sample holder and positioning it horizontally in
459 the central region of a split coil superconducting magnet and increasing the temperature from
460 20 to 30 °C for approximately 30 min. The collagen molecules assemble into orientated
461 fibrils perpendicular to the applied field and transform into a viscous gel that is stable and
462 orientated after the magnetic field is removed. A limitation of this technique is that fibril
463 diameter cannot be regulated using this technique. Furthermore, there is conflicting evidence
464 suggesting that the application of strong magnetic forces can in fact impair cell function and
465 viability.(58)

466

467 *6.7. Electrospinning of Nanofibres*

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

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

480

481 **7. Conclusion**

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

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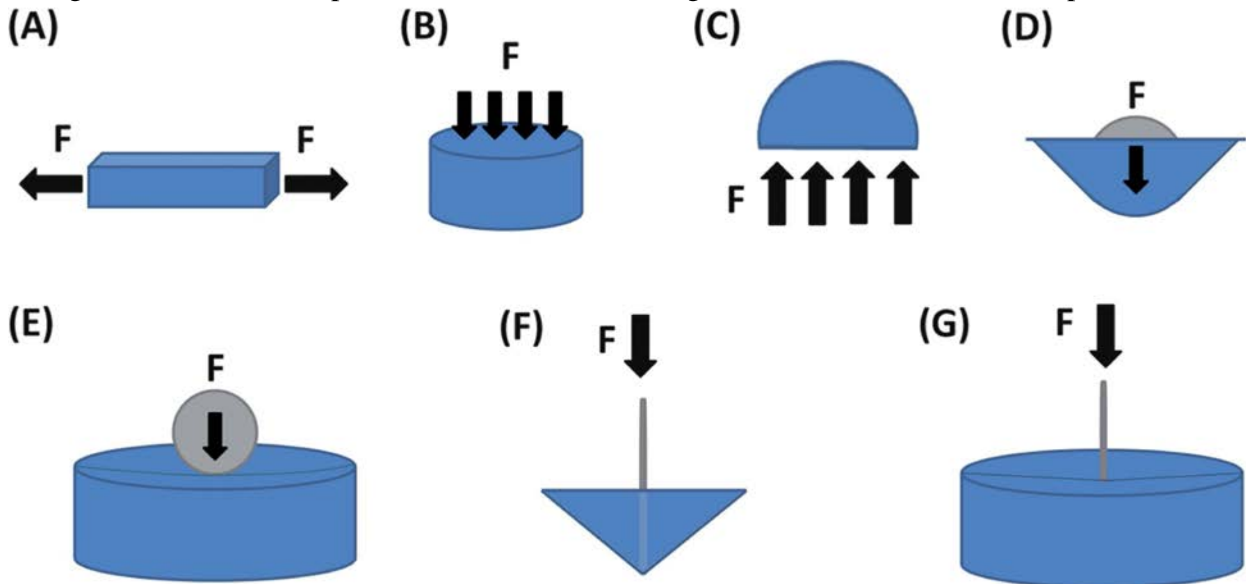
590

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).

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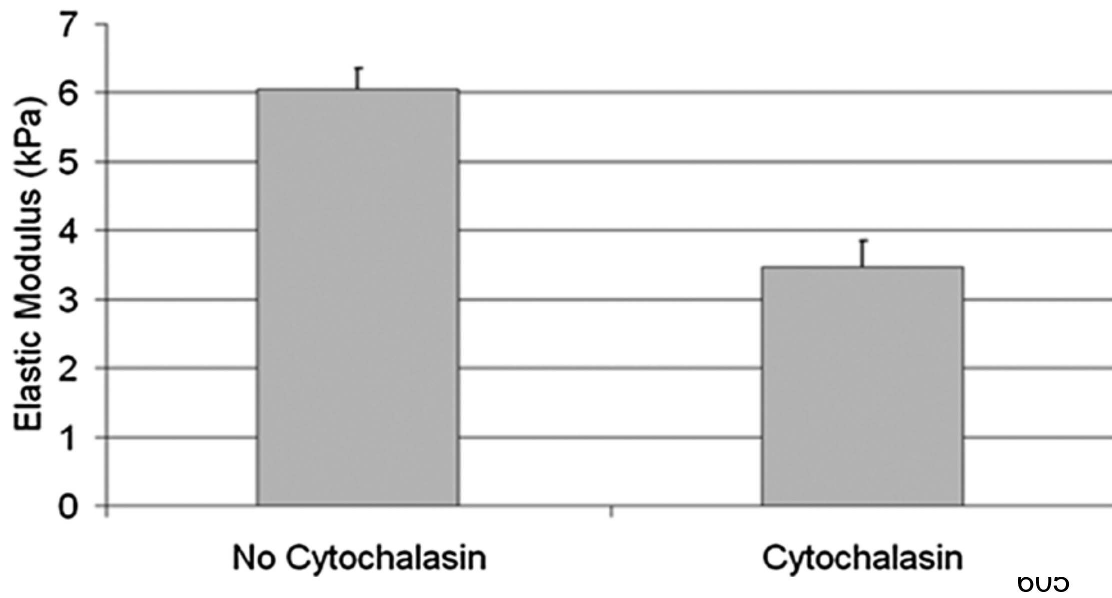
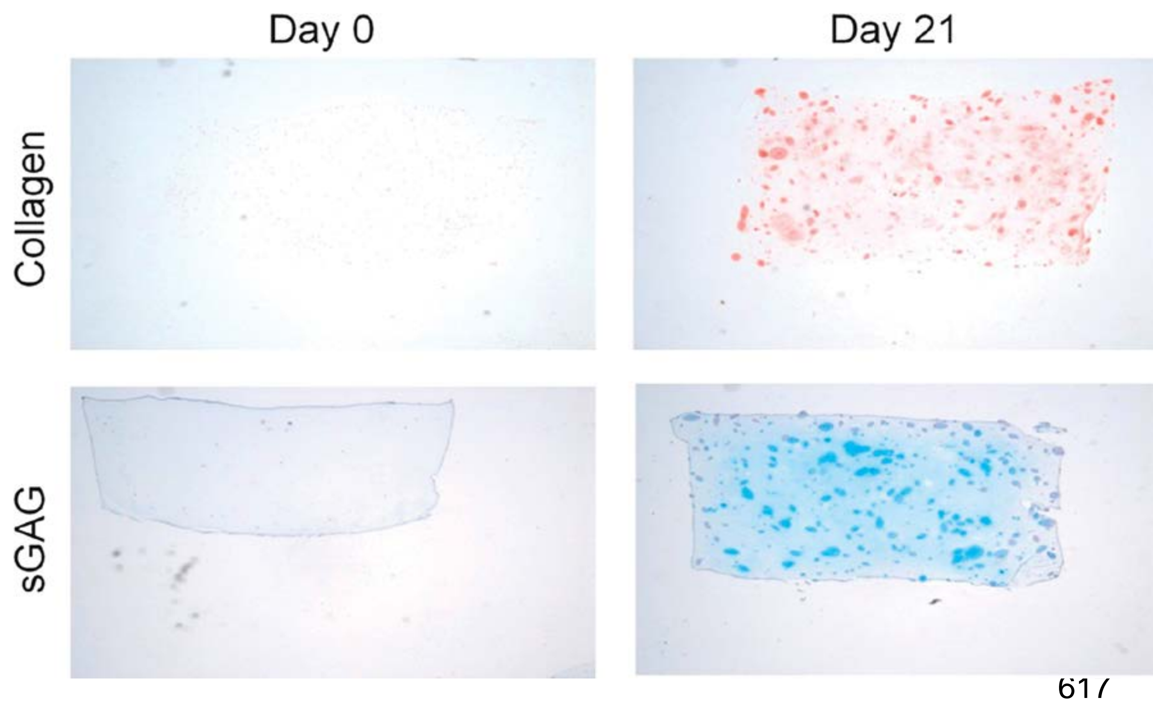


606 Figure 2 .The elastic modulus change in response to the addition of cytochalasin in collagen
607 hydrogel seeded with fibroblasts, which disrupted actin filament in fibroblasts.(13)

608



617

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

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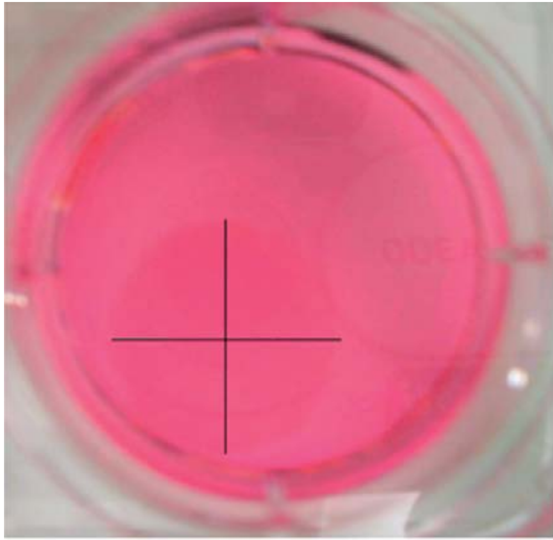

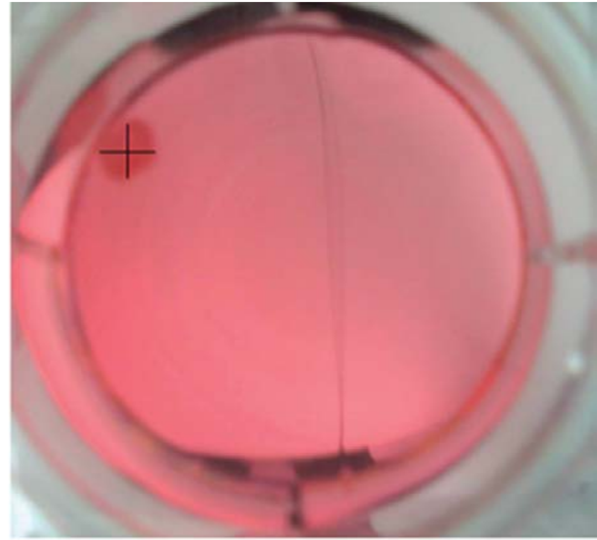

1 hour 5 days

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

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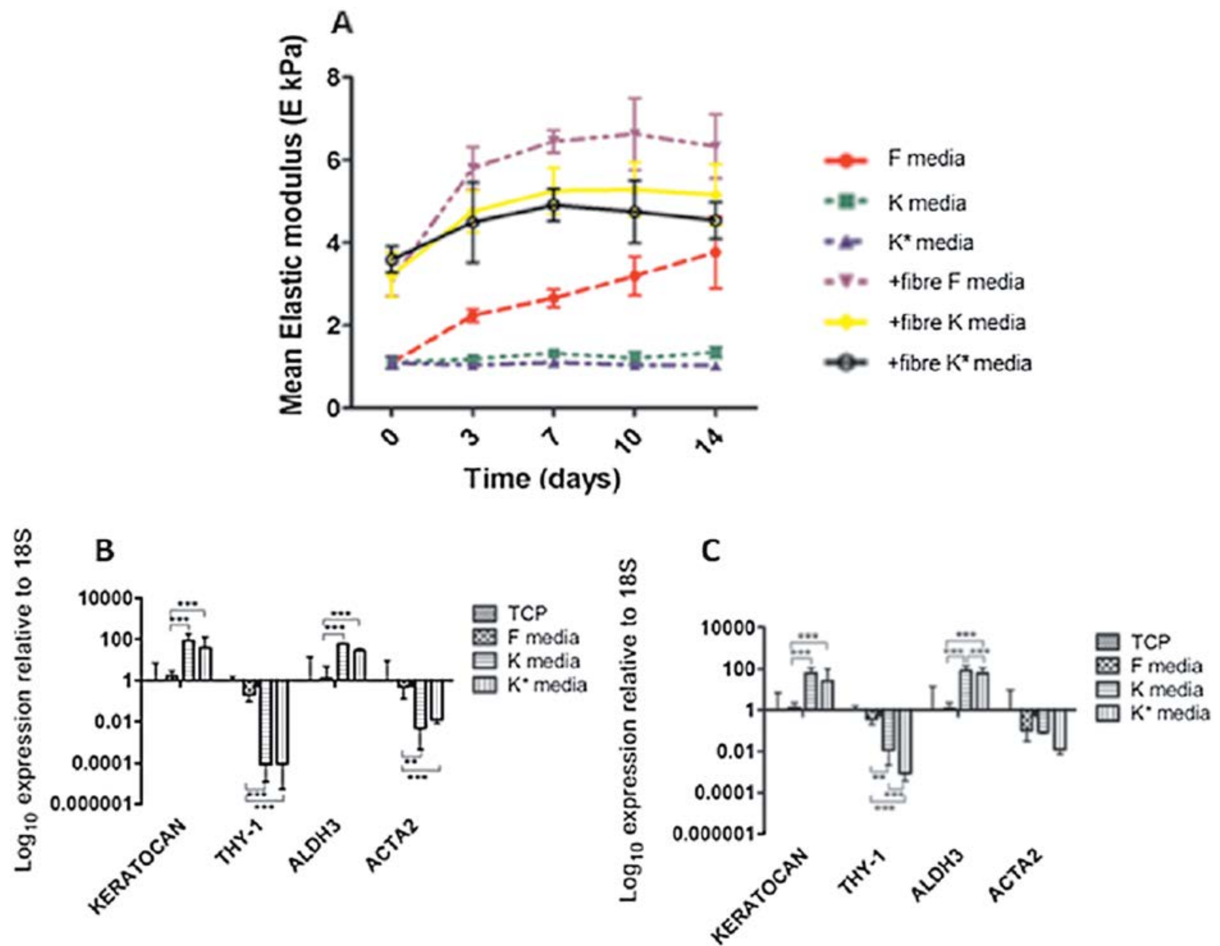

626 Figure 5. (A) The elastic modulus and gene expression of corneal stromal cells grown in
627 collagen hydrogels for 14 days in response to chemical and topographic regulation. F denotes
628 serum-containing medium, K denotes serum-free, insulin supplemented medium, and K0
629 denotes serum-free b-FGF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

633

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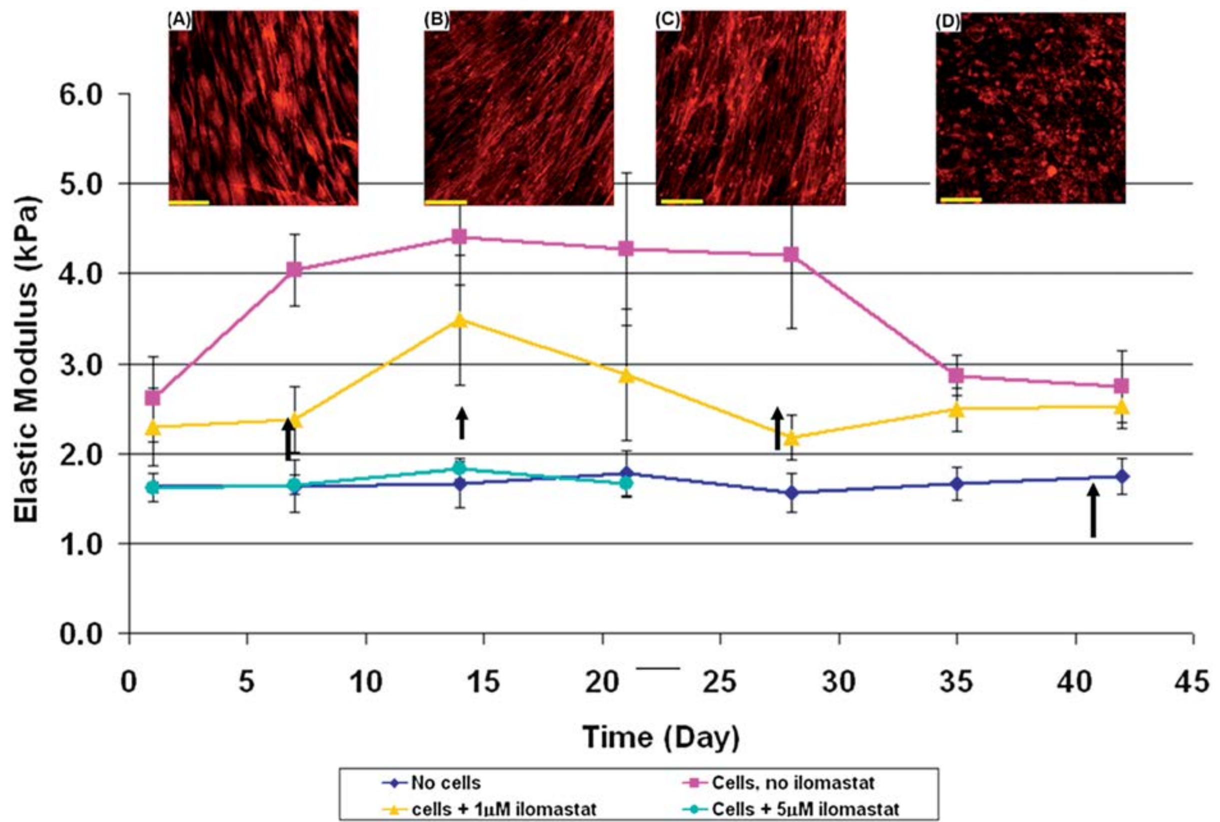

635

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

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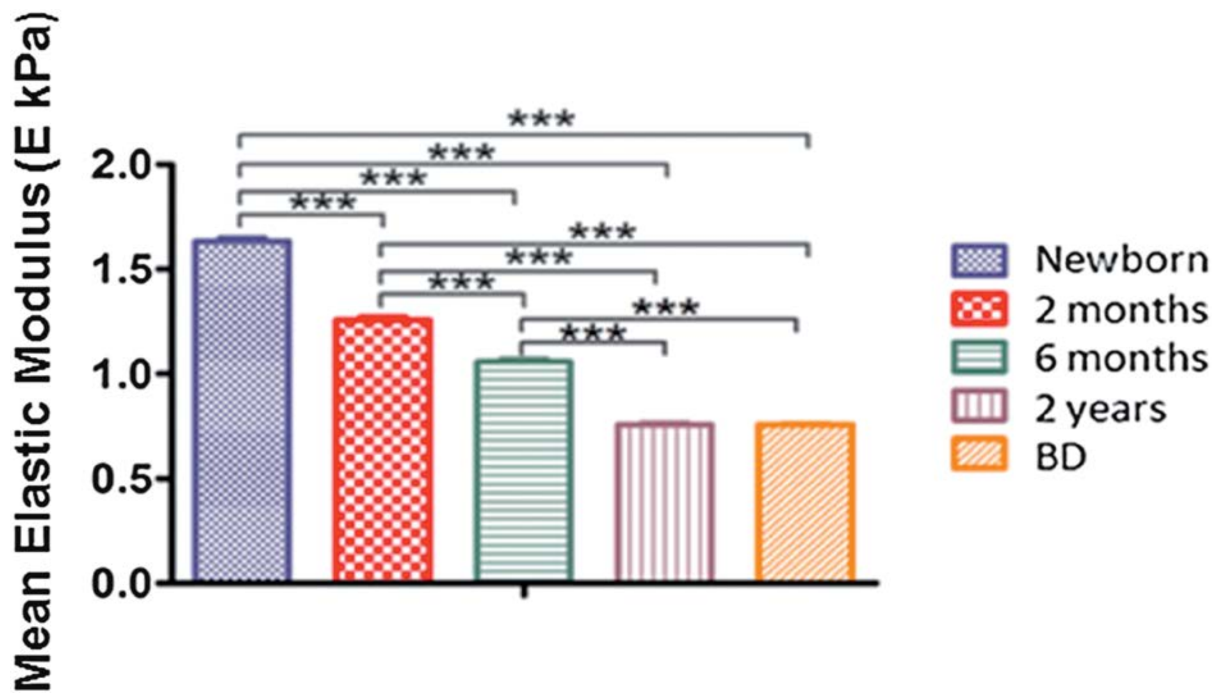

641

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

644

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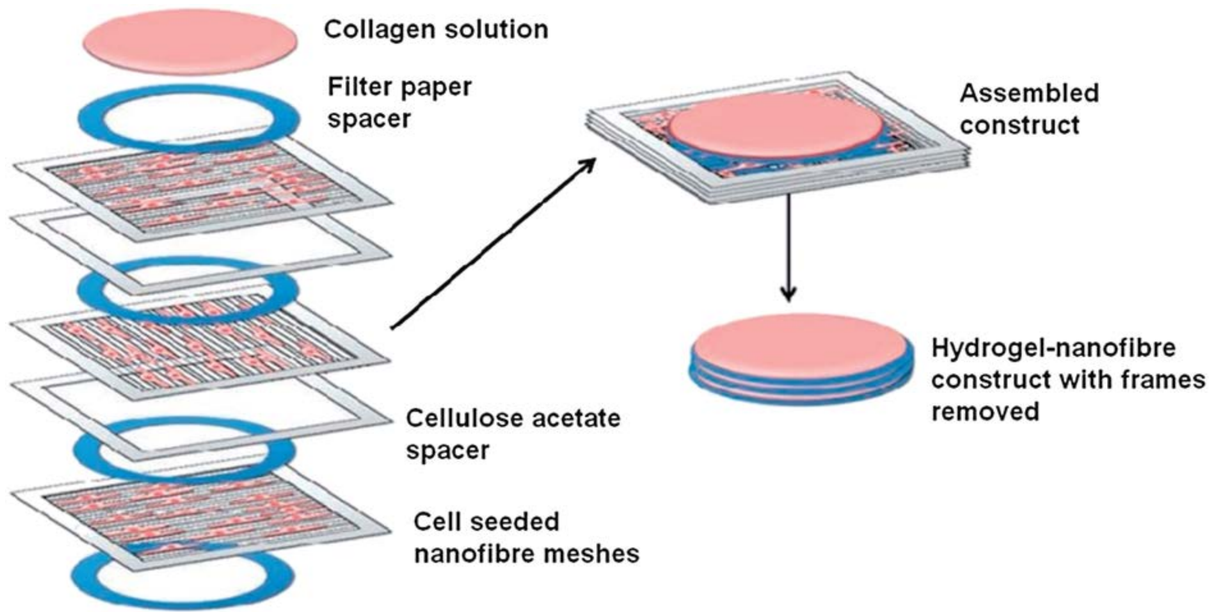

646 Figure 8. Schematic representation of the assembly process used to fabricate a nanofibre –
647 hydrogel construct