1   Title page

2   **Influence of cell and collagen concentration on the cell–matrix mechanical relationship**

3   **in a corneal stroma wound healing model**

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15   *Research highlights*

16   • Non-destructively measuring the modulus of corneal fibroblasts in hydrogel for 25 days.

17   • Initial hydrogel and cell concentration affect the contraction and modulus increase.

18   • Hydrogels with high initial cell seeding densities increased in elastic modulus faster.

19   • Hydrogels with low initial collagen concentrations increased in elastic modulus faster.

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1   **Abstract**

2   The effect of different collagen and cell concentrations on the mechanical and remodeling

3   behaviors of corneal stroma wound healing models consisting of collagen hydrogels seeded

4   with human corneal fibroblasts during a 25 day culture period were examined. Human

5   corneal fibroblasts were seeded at 1×10^5, 3×10^5 or 5×10^5 cells per hydrogel, and collagen

6   concentrations of 2.5 mg/ml, 3.5 mg/ml or 4.5 mg/ml were examined. Two non-destructive

7   techniques, spherical indentation and optical coherence tomography, were used to measure

8   the elastic modulus and dimensional changes respectively at several time-points over the

9   culture period. The elastic modulus of the hydrogels increased continuously over 25 days.

10   Hydrogels with higher initial cell seeding densities and lower initial collagen concentrations

11   were found to increase in elastic modulus faster and possessed a higher elastic modulus by

12   the end of the culture period when compared to the other hydrogels. A mathematical equation

13   was applied to accurately fit the change in elastic modulus over time. This study

14   demonstrates a robust *in vitro* technique able to monitor the effect of different parameters on

15   the cell–matrix mechanical relationship in a corneal stroma model during prolonged culture

16   periods and enhances our understanding on corneal wound healing processes.

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1. Introduction

Examination of corneal fibroblasts *in vitro* in three-dimensional environments can provide a useful model for understanding the cell behavior *in vivo* after injury. In laser eye surgery, there is an interest in how the procedure results in corneal thinning and how the creation of a corneal flap may risk corneal ectasia. Scar tissue formation results from phenotypic changes in keratocyte behavior after injury which leads to extracellular matrix contraction. Some corneal diseases are influenced by the collagen structure in the stroma, such as keratoconus in which structural changes within the cornea results in stromal thinning and a more conical shape than its normal aspherical curve. Hydrogels have been used to replicate extracellular matrix by providing a three-dimensional structure through which cells can survive, move and communicate (Bell et al., 1979). Collagen based hydrogels have become particularly popular for replicating corneal extracellular matrix *in vitro* because of their viscoelastic characteristics, similar composition to native cornea, their biocompatibility and their ability to be remodeled by cells (Kim et al., 2010; Duan et al., 2007). Fibroblasts seeded in collagen hydrogels have been shown to influence the mechanical and structural characteristics of such hydrogels (Bell et al., 1979, Ahearne et al., 2010, Philips et al., 2003). Fibroblasts can manipulate the mechanical properties of their surrounding hydrogel matrix through a number of mechanisms including the release of extracellular matrix products and matrix digestion enzymes (Webb et al., 2006, Saddiq et al., 2009), attachment and integration of fibroblasts throughout the hydrogels (Takakuda and Miyairi, 1996) and contraction of the hydrogels (Bell et al., 1979). Changes to the mechanical properties of the matrix can in turn induce a change in the cell's behavior (Pelham and Wang, 1997, Engler et al., 2006, Breuls et al., 2009). This creates a reciprocal cell–matrix mechanical relationship where both cell behavior and matrix properties are dependent upon each other (Wakatsuki and Elson, 2003, Brown, 2006).

There are many factors that can influence the cells' abilities to manipulate the mechanical properties of their surroundings. It has been shown that the addition of matrix metalloproteinases inhibitors (Daniels et al., 2003) and other reagents can influence the cell–matrix mechanical relationship. Chemical and photochemical crosslinking of matrix components such as collagen can also be used to influence mechanical characteristics (Cheung et al., 1985, Ahearne et al., 2008a). Mechanical stimulation of cells has been shown to affect the ability of cells to change their surrounding matrix (Nirmalanandhan et al., 2008). In addition to these factors, the initial conditions used to manufacture the hydrogels also play an important role in dictating the cell–matrix mechanical relationship. For cell-seeded collagen hydrogels this may include the number of cells seeded and the collagen concentration used to produce them.

One of the difficulties in examining the cell–matrix mechanical relationship is that there are few methods for monitoring the changes in their mechanical behavior over long cell culture periods. Recently a novel online spherical indentation technique has been developed to non-destructively monitor the changes in elastic modulus of hydrogels over several weeks in culture (Ahearne et al., 2010). A non-destructive imaging technique, optical coherence tomography (OCT), has also been developed to reveal and monitor the morphology of three-dimensional cell culture samples during prolonged culture periods (Ahearne et al., 2008b). In this study, the role of initial cell seeding density and collagen concentration on the mechanical and contractile behavior of collagen hydrogels has been examined using these two techniques over a 25 day culture period.

2. Materials and methods

2.1. Sample preparation

All chemicals used were from Sigma–Aldrich unless otherwise stated. Human corneal tissue was used to cultivate corneal fibroblasts as previously described (Ahearne et al., 2005). This research has received approval from Black Country Research Ethics Committee (06/Q2702/44) and informed consent has been obtained. Hydrogels with a total volume of 500 μl were manufactured with rat-tail collagen type 1 (BD Bioscience, Erembodegem, Belgium) using a previously described protocol (Ahearne et al., 2008a). Human corneal fibroblasts were suspended inside the collagen hydrogel solution prior to gelation. Collagen concentrations of 2.5 mg/ml, 3.5 mg/ml and 4.5 mg/ml and cell concentrations of 5×10^5, 3×10^5 and 1×10^5 cells per hydrogel were examined. Hydrogels without cells were also manufactured as a control. The hydrogels were cultured in six-well plates with 5 ml culture media per well consisting of low glucose Dulbecco's Modified Eagle's Medium supplemented by 10% fetal calf serum, 1% l-glutamine and 1% antibiotic–antimycotic solution. The media was changed every 3–4 days.

2.2. Characterization of elastic modulus and dimension changes

A sample holder was built to hold the hydrogels during mechanical measurements (**Fig. 1A**). The hydrogels were suspended between two transparent plastic circular rings of inner diameter 20 mm. The rings were held in place between two thin stainless steel plates. The lower base plate had a number of screws attached which were used to keep the sample holder together. Three hydrogels could be held in a single sample holder at any one time. Each sample holder was assembled in phosphate buffered saline (PBS) within a large rectangular Petri dish in a Class II laminar flow hood followed by incubation at 37 °C. A multi-LED lamp was placed in the incubator to illuminate the sample. The hydrogels were deformed

1   using a PTFE sphere (Insley, Berks, UK) of diameter 4 mm. The sphere was placed in the

2   centre of each hydrogel. The weight (0.072 g) of the sphere caused the hydrogel to deform.

3   After measurements had taken place, the sphere was removed. The hydrogel was taken out

4   from the sample holder and then placed back in culture medium until the next measurement

5   time-point. An image acquisition system was used to record images of the spherically

6   deformed hydrogels (Ahearne et al., 2005). It consisted of a long-working-distance objective

7   microscope (Edmund Industrial Optics, York, UK) with a computer-linked CCD camera

8   (XC-ST50CE, Sony, Japan). The camera was linked to an image acquisition card (National

9   Instruments, USA) which was used to acquire and process the images. An example of a

10   hydrogel deformation image acquired by this system is shown (**Fig. 1B**). This system allowed

11   images to be recorded of the deformation profile of the hydrogel from outside the incubator

12   through a glass window. The magnification of the system was calibrated with the computer-

13   acquired images of a stage micrometer. Images of the spherically deformed hydrogels were

14   recorded 10 min after the sphere was initially placed onto the hydrogel. This was to allow

15   time-dependent deformation to reach equilibrium. The elastic modulus was measured one day

16   after hydrogel formation and every 3–4 days subsequently for up to 25 days.

17   A home-built optical coherence tomography (OCT) system was used to examine the cross-

18   sectional thickness of each specimen at the same examining frequency as the modulus

19   measurement. A filter paper ring around the outer edge of each hydrogel prevented

20   contraction in all directions except the thickness. OCT is a low coherence interferometric

21   technique which measures backscattered light after the light source has passed through a

22   material (Bagnaninchi et al., 2007). This technique allowed non-destructive imaging of the

23   hydrogels cross-sectional thickness to be measured under sterile conditions. A

24   superluminescent diode with a central wavelength of 1310 nm and bandwidth of 52 nm was

1 used as the source of the OCT. The system has an axial resolution of 14 µm in free space and

2 penetration depth of up to 2 mm.

3 *2.3. Mathematical modeling*

4 The elastic modulus of the hydrogels was calculated from the images using a large

5 deformation model (Ju and Liu, 2002). From the acquired images, the central deformation (δ)

6 of the hydrogel was calculated and substituted into **Equation 1** to find the elastic modulus

7 *6 wr = Eh (0.075 δ^2 + 0.78rδ)*

8   *(Equation 1)*

9 where *h* is the hydrogel thickness, r is the radius of the sphere and w is the weight of the

10 sphere. This equation is applicable when the ratio of *a/r* = 5 and *δ/r* < 1.7, where a is the

11 radius of the clamped portion of the hydrogel. This model also assumes that the ratio of

12 thickness to the radius is low and the deformation is large, hence stretching of the membrane

13 dominates over bending.

14 A simple equation was used to simulate the change in elastic modulus with respect to culture

15 time as shown in **Equation 2**

16 $E_c = E_0 e^{\alpha t}$

17   *(Equation 2)*

18 where E_c is the elastic modulus of the cell-seeded hydrogels, E_0 is the equivalent elastic

19 modulus of the hydrogel with the same initial collagen concentrations but without cells, α is a

20 constant and *t* is the culture time in days. The constant α can be calculated using regression

21 analysis and is dependent on the initial cell and collagen concentration used. When no cells

22 are present in the hydrogel α = 0, therefore *Ec = E0*.

2.4. MTT assay

Methylthiazolyldiphenyl-tetrazolium bromide (MTT) was used to monitor cell proliferation within the hydrogels. MTT was dissolved in PBS at a concentration of 5 mg/ml and sterile filtered. Each hydrogel examined was placed into a cell culture well containing 10% MTT solution in culture media. The wells were placed into an incubator at 37 °C, 5% CO_2 for 3 h. The MTT/culture media was removed and each hydrogel was washed twice in PBS. DMSO solution was then added to each well for 1 h. The DMSO solution was transferred into a 96 well plate and the absorbance of light at wavelengths of 570 nm and 690 nm were measured using a microplate reader (Synergy 2, BioTek, USA). Readings were obtained at the two wavelengths of 570 nm and 690 nm and were subtracted from each other to give single values for each well. A standard curve was created by suspending a known numbers of cells in a hydrogel. From the standard curve, the number of viable cells was calculated.

2.5. Fluorescence staining and cell viability

Cell viability was assessed using a live–dead cell double staining kit. The kit contained Calcein–AM and Propidium Iodide which fluorescently stain live cells green and dead cells red respectively. 2 μl of Calcein–AM and 1 μl Propidium Iodide in 500 μl PBS were added to each hydrogel and incubated for 20 min at 37 °C. The cells were examined using an FV300 confocal microscope (Olympus, Japan). To fluorescently stain f-actin, a phalloidin–TRITC staining solution was applied to hydrogel specimens following the manufacturers protocol. The stained cells were viewed by confocal microscopy.

2.6. Statistical analysis

ANOVA (Minitab, USA) was used to find which of the factors under investigation were statistically significant with 95% confidence interval.

3. Results

Cell proliferation was examined using MTT (**Fig. 2**). Cells were shown to have proliferated by 3–4 times their original cell number in all the hydrogels after 7 days in culture. This proliferation along with hydrogel contraction led to a dramatic increase in the cell density, i.e. the number of cells per unit volume of hydrogel, especially for the hydrogel group with 3.5 mg/ml collagen concentration (**Fig. 2A**). The cell density from hydrogels seeded with 1×10^5, 3×10^5, 5×10^5 cells per hydrogel increased to approximately 4×10^5, 9×10^5, 18×10^5 cells per hydrogel respectively. Hydrogels with a lower initial collagen concentration appeared to show a greater increase in cell proliferation when compared to hydrogels with higher initial collagen concentration but the same cell seeding concentration. The group of the hydrogels that were seeded with 5×10^5 cells (**Fig. 2B**) showed less change in cell population between 7 and 25 days. Hydrogels with 1×10^5 and 3×10^5 cells continued to proliferate between 7 and 25 days but at a slower rate. Interestingly, hydrogels with lower initial collagen concentration (2.5 mg/ml and 3.5 mg/ml) but the highest cell seeding density, 5×10^5, showed a decrease in cell population between 7 and 25 days. These hydrogels had more dead cells than the 4.5 mg/ml hydrogels (**Fig. 2C and D**).

The effect of cell seeding concentration on the elastic modulus of the hydrogels was examined (**Fig. 3**). Measurements were taken of hydrogel samples run in parallel ($n = 6$ for each group). Cell seeding densities of 5×10^5, 3×10^5 and 1×10^5 cells per hydrogel were examined as well as hydrogels without cells. Higher cell seeding concentrations led to a faster and more substantial increase in elastic modulus. Regardless of the initial collagen concentration used, the hydrogels with the highest number of cells (5×10^5) had the highest modulus after 25 days. Two-way ANOVA showed that cell seeding concentration had a significant effect on the change in elastic modulus with culture time. Hydrogels with higher cell concentrations also decreased in thickness more quickly than those with lower cell

1   concentrations. The higher cell number resulted in more extensive contraction of the

2   hydrogels which caused a reduction in water content. The increased contraction and reduction

3   in volume of the hydrogels resulted in higher cell and collagen densities.

4   The effect of initial collagen concentration (2.5 mg/ml, 3.5 mg/ml and 4.5 mg/ml) on the

5   elastic modulus of the hydrogels was also examined. Measurements were again taken of

6   hydrogel samples run in parallel ($n = 6$ for each group). Hydrogels with the lowest initial

7   collagen concentration had a faster rate of increase in elastic modulus (**Fig. 3A–C**). These

8   hydrogels were more contractile than hydrogels with a higher collagen concentration (**Fig.**

9   **3D–F**). The increased rate of contraction led to an increase in cell density and collagen

10   density. Two-way ANOVA showed that the initial collagen concentration of cell-seeded

11   hydrogels had a significant effect on the change in elastic modulus over the culture period.

12   Representative actin staining images are demonstrated in **Fig. 4**. At week 4 when collagen

13   concentration was fixed (3.5 mg/ml), higher cell seeding density resulted in less organized

14   and less strong actin filament expression (**Fig. 4 A–C**); whilst when cell density was fixed

15   (500,000 cells), lower collagen concentration in the specimens results in less organized actin

16   expression (**Fig. 4 D–F**). Culture time also affect actin expression. Longer culture time

17   resulted in less actin organization in the specimen with lower collagen concentration (2.5

18   mg/ml) and higher cell density (500,000 cells) (**Fig. 4 G–I**), whilst actin organization

19   improved in higher collagen concentration (4.5 mg/ml) and lower cell density (100,000 cells)

20   (**Fig. 4 J–L**) hydrogels.

21   **Equation 2** was used to plot the change in elastic modulus over the culture period from the

22   data for different cell and collagen concentrations. The values for the constant α and the

23   correlation coefficient R (in brackets) are displayed in **Table 1**. The value of α was dependent

24   on the cell and collagen concentrations used to manufacture the hydrogels. As can be seen in

Fig. 3 the rate of increase in elastic modulus over time was influenced by the initial cell and collagen concentrations. When there was no or little change in the elastic modulus the value of α approached zero.

4. Discussion

We have demonstrated an online approach to monitoring the cell–matrix mechanical relationship in cell-seeded hydrogels which may be used as a corneal stroma wound healing model. The non-destructive nature of the spherical indentation technique described here allowed repeated measurement of the hydrogels at different time-points. OCT allowed repeated online measurement of the hydrogels thickness. The combination of these two techniques provides an excellent approach to examine the remodeling behavior and the cell–matrix mechanical relationship in a corneal stroma model over several weeks in culture. This approach is also useful in the development of a tissue engineered corneal substitute since it can be used to monitor the mechanical changes in the samples over a prolonged culture period.

Hydrogels seeded with cells provide a useful model for understanding cell behavior and tissue remodeling. Collagen provides the most suitable hydrogel material for examining these behaviors since it can be easily remodeled and collagen type 1 is the most abundant protein in the cornea. Previous work using agarose and alginate hydrogels showed limited remodeling by corneal cells (Ahearne et al., 2005). Hydrogels seeded with corneal fibroblasts have been used to model the corneal stroma and in particular stromal wound healing and remodeling behaviors (Kim et al., 2010, Kurosaka et al., 1998). *In vitro* corneal fibroblasts behave similarly to activated keratocytes after corneal injury (Fini, 1999). These cells are more contractile than inactivated keratocytes and exhibit fibroblastic characteristics. It has previously been reported that corneal fibroblasts affect the elastic modulus of collagen

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1   hydrogels through a number of mechanisms including by integrating themselves throughout

2   the hydrogels, attaching and applying intrinsic strain in the hydrogels and releasing

3   extracellular matrix products (Ahearne et al., 2010). The attachment and application of force

4   by corneal fibroblasts results in contraction of the surrounding matrix. This contraction

5   directly affects the hydrogels properties by removing water and increasing the overall

6   collagen density which in turn affect the elastic modulus and thickness of the hydrogels.

7   Hence any factors which influence hydrogel contraction by fibroblasts in our corneal stroma

8   models, such as the cell seeding concentration or collagen concentration, will speed up or

9   slow down the changes of these properties.

10 Awad et al. (2000) and Nirmalanandhan et al. (2006) found that increasing the number of

11 cells led to an increased rate of collagen contraction although both also found that there was a

12 maximum threshold where increasing the number of cells no longer had any affect. Our

13 experiments showed that increasing the number of cells resulted in an increased rate of

14 contraction, a faster increase in elastic modulus and a higher modulus value was obtained at

15 any given culture time-point. This phenomenon can be explained by the increased attachment

16 points to collagen matrix due to higher cell numbers. As the hydrogels contract, water is

17 pushed out of the hydrogel leading to an increase in collagen density and an increase in

18 overall stiffness. In addition, cells attach and spread themselves throughout the hydrogel

19 forming an integral part of the hydrogels structure and mechanical properties. Since cells

20 respond to strain when attached to a matrix (Dartsch et al., 1986, Petroll et al., 2004) and

21 stress fibers found in cells are substantially stiffer than the hydrogels (Deguchi et al., 2006), it

22 is not surprising that increasing the number of cells led to an increase in elastic modulus of

23 the hydrogels once these cells had attached themselves.

24 The initial collagen concentration used was found to affect the ability of cells to change the

25 mechanical properties of the hydrogels. A higher initial collagen concentration resulted in a

1   higher initial stiffness for the hydrogels. However, the initial collagen concentration also

2   affected the rate of hydrogel contraction with a lower collagen concentration allowing a faster

3   rate of contraction. This faster rate of contraction led to these hydrogels having a higher cell

4   density and a higher overall collagen density by the end of the culture period. This

5   phenomenon demonstrates a clear mechano-feedback response between fibroblasts and the

6   matrix. In wound healing, fibroblast-generated tension compacts newly deposited collagen

7   into a dense aligned matrix in order to carry muscle and loads (Brown, 2006). Hydrogels with

8   a low initial collagen concentration had less mechanical resistance when fibroblasts

9   contracted the matrix hence the contraction became easier and faster compared to hydrogels

10   with higher initial collagen concentrations. Increasing the cell density increases the amount of

11   force which can be generated to contract the hydrogels.

12   A relationship was to found to exist between cell proliferation and elastic modulus of the

13   hydrogel. Our data shows that cells proliferated over the first week in culture in all the

14   samples tested. This increase in cell number led to an increase in cell density which in turn

15   increased the amount of contraction and led to a higher overall elastic modulus. Between day

16   7 and day 25, the total number of viable cells was reduced in hydrogels seeded with 5×10^5

17   cells except those of initial concentration 4.5 mg/ml in which the contraction of the hydrogel

18   was low. This suggests that the more contractile hydrogels were less able to maintain cell

19   proliferation and viability compared to the less contractile hydrogels. The live/dead staining

20   experiments confirmed that the hydrogels seeded with 5×10^5 cells and an initial collagen

21   concentration of 2.5 mg/ml had more dead cells than those with an initial collagen

22   concentration of 4.5 mg/ml. The increased rate of contraction would have resulted in

23   additional strain being applied to the cells and also increased the overall cell density, both of

24   which could have resulted in reduced cell viability. These results were consistent with our

25   previous study in which the addition of a matrix metalloproteinases (MMP) inhibitor,

ilomastat, to the hydrogels restricted contraction and led to higher cell viability in comparison

to the controls (Duan et al., 2007). This proposes a hypothesis that increased strain and

overpopulation of cells that results from contraction in hydrogels can reduce cell viability. A

more comprehensive study could be carried out in the future to directly relate cell

proliferation with changes in elastic modulus and thickness of the hydrogels, although the

large number of cells required for MTT makes this study complicated.

The expression pattern of actin filaments mirrored well the mechanical properties of the

hydrogels and the cell proliferation and viability. Hydrogels with low cell densities due to a

low initial cell seeding number and a high initial collagen concentration experienced the most

contraction and displayed higher modulus. At the early culture time, these specimens showed

dense and aligned actin filaments. By the 4th week, cells in the hydrogels exhibited thin actin

filaments and no particular cell alignment, manifesting as distorted actin expression. This

could be due to the reduction in cell viability in these samples. The least contracted

specimens in which lower modulus has been measured displayed highly organized and dense

actin filament by week 4.

The change in elastic modulus over the culture period was accurately represented using our

mathematical equation. The constant α in the equation (2) can be used to describe the

influence of initial cell seeding concentration and collagen concentration on the stiffness of

the hydrogel. When α is large, the rate of modulus increase is greater than when α is small.

When α reads zero, the hydrogels will not change in modulus. The application of such

equations is useful in estimating the elastic modulus at time-points which were not directly

measured and predicting when a particular elastic modulus was reached.

In summary, the two non-destructive techniques described here enabled observations of the

reciprocal mechanical influence between the cell and matrix in a corneal stroma wound

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1   healing model for several weeks in culture. This approach greatly enhances our ability to

2   understand corneal tissue remodeling, wound healing behavior and the formation of

3   connective tissue by tissue engineering principles. The mechanical properties of the

4   surrounding environment can affect cell behavior in collagen constructs by regulating cell

5   proliferation (Hadjipanayi et al., 2009), matrix production (Karamichos et al., 2008) and

6   matrix remodeling (Bell et al., 1979, Nirmalanandhan et al., 2006). Hence this study

7   demonstrates a good *in vitro* model revealing cell–matrix interactions over prolonged culture

8   periods.

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1 **Acknowledgements**

6 **Conflict of Interest Statement**

7 The authors whose names are listed on the title page certify that they have NO affiliations

8 with or involvement in any organization or entity with any financial interest (such as

9 honoraria; educational grants; participation in speakers' bureaus; membership, employment,

10 consultancies, stock ownership, or other equity interest; and expert testimony or patent-

11 licensing arrangements), or non-financial interest (such as personal or professional

12 relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed

13 in this manuscript.

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16

1   **Tables**

2   **Table 1.** Values for constant α for different initial collagen and cell concentrations calculated

3   by regression analysis where α defines the rate of change in elastic modulus during the

4   culture period in equation (2) (correlation coefficient R in brackets).

	2.5 mg/ml	3.5 mg/ml	4.5 mg/ml
5×10^5 cells	0.0804 (0.99)	0.0479 (0.97)	0.0142 (0.81)
3×10^5 cells	0.0735 (0.98)	0.0260 (0.90)	0.0115 (0.78)
1×10^5 cells	0.0403 (0.96)	0.0047 (0.55)	0.0000 (0.38)

5

6

1   **Figure Captions**

2   Fig. 1. (A) Disassembled view of sample holder and hydrogels and (B) recorded image of
3   hydrogel deformation.
4
5   Fig. 2. Cell proliferation after 7 and 25 days in hydrogels with: (A) different initial cells
6   concentrations and an initial collagen concentration of 3.5 mg/ml; (B) different initial
7   collagen concentrations and an initial cell concentration of 5 x 10^5 cells per hydrogel. Cell
8   viability (live cells green, dead cells red) after 25 days in hydrogels with an initial cell
9   concentration of 5 x 10^5 cells per hydrogel and an initial collagen concentration of (C) 2.5
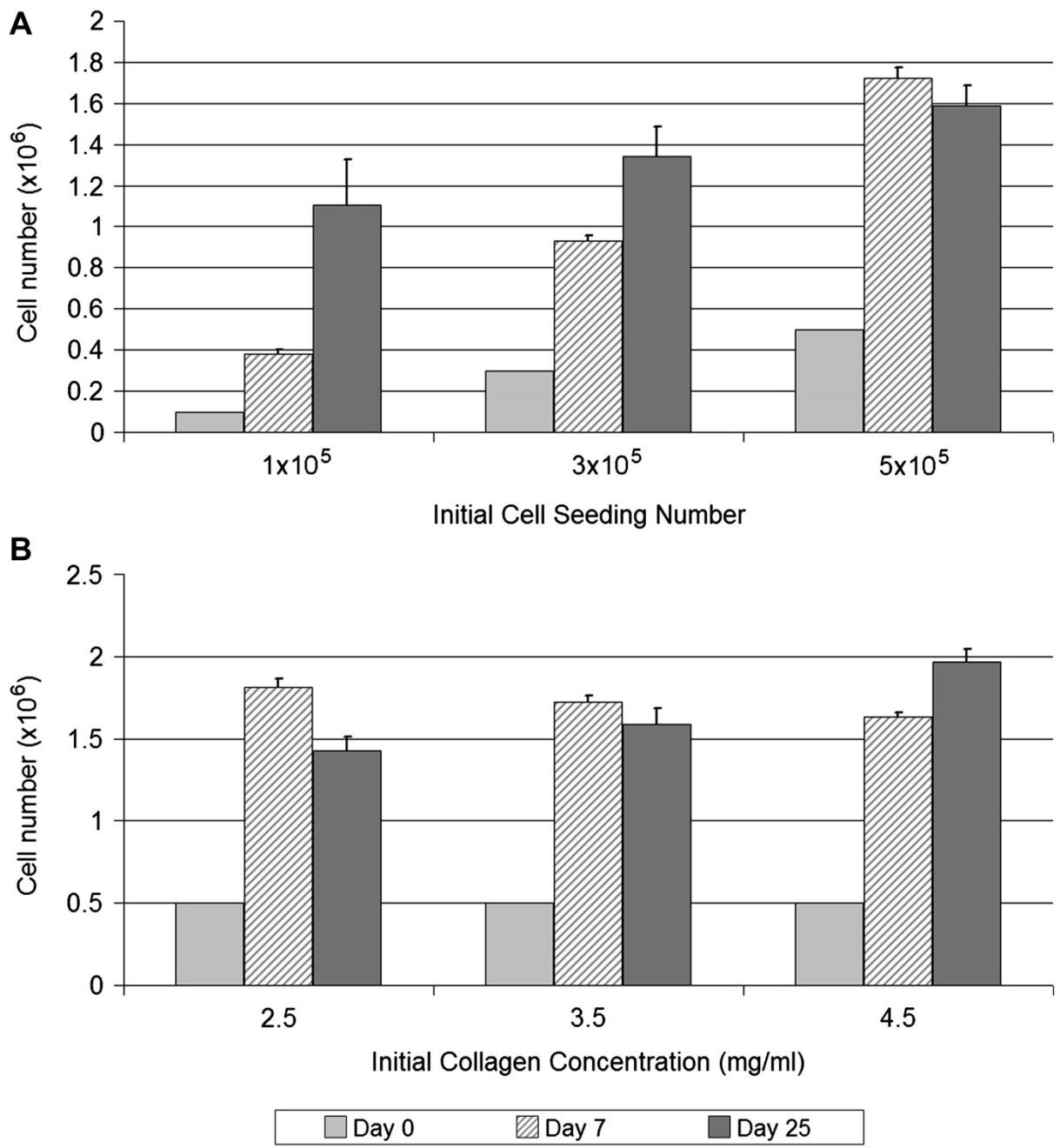10   mg/ml and (D) 4.5 mg/ml (scale bar ¼ 200 mm).
11
12   Fig. 3. Elastic modulus (A–C) and thickness (D–F) of hydrogels with initial collagen
13   concentrations of 2.5 mg/ml (A, D), 3.5 mg/ml (B, E) and 4.5 mg/ml (C, F) ($n = 6$ for each
14   data point).
15
16   Fig. 4. Corneal fibroblasts in collagen hydrogels stained for f-actin: (A–C) hydrogel cultured
17   for 28 days with 3.5 mg/ml initial collagen concentration and initial cell concentrations of 1 $\times$
18   10^5 cells (A), 3 $\times$ 10^5 cells (B) and 5 $\times$ 10^5 cells (C); (D–F) hydrogel cultured for 28 days
19   with 5 $\times$ 105 cells initial concentration and initial collagen concentrations of 2.5 (D), 3.5 (E)
20   and 4.5 mg/ml (F); (G–I) hydrogels with 2.5 mg/ml and 5 $\times$ 10^5 initial collagen and cell
21   concentration cultured for 7 days (G), 14 days (H) and 28 days (I); (J–L) hydrogels with 4.5
22   mg/ml and 1 $\times$ 10^5 initial collagen and cell concentration cultured for 7 days (J), 14 days (K)
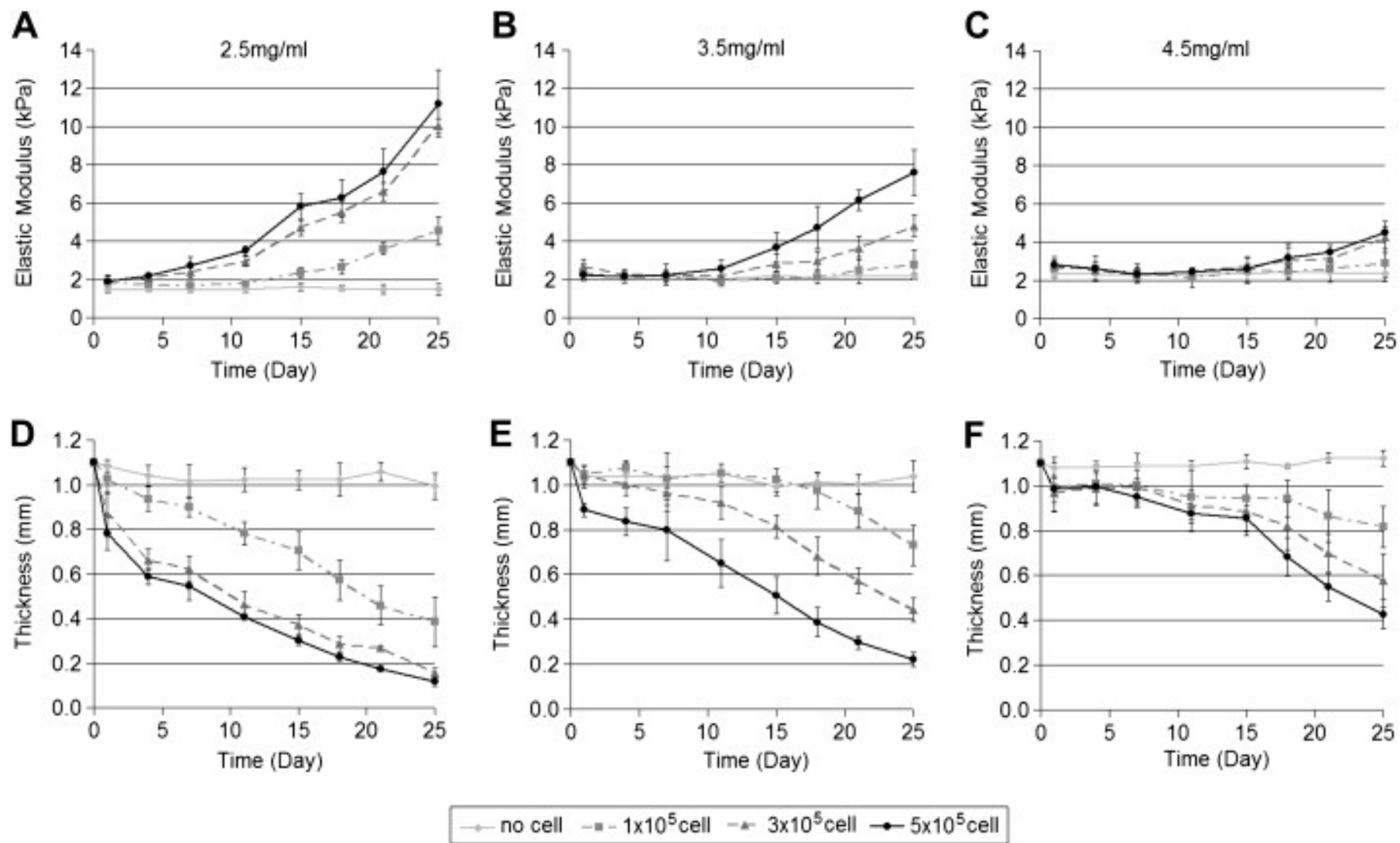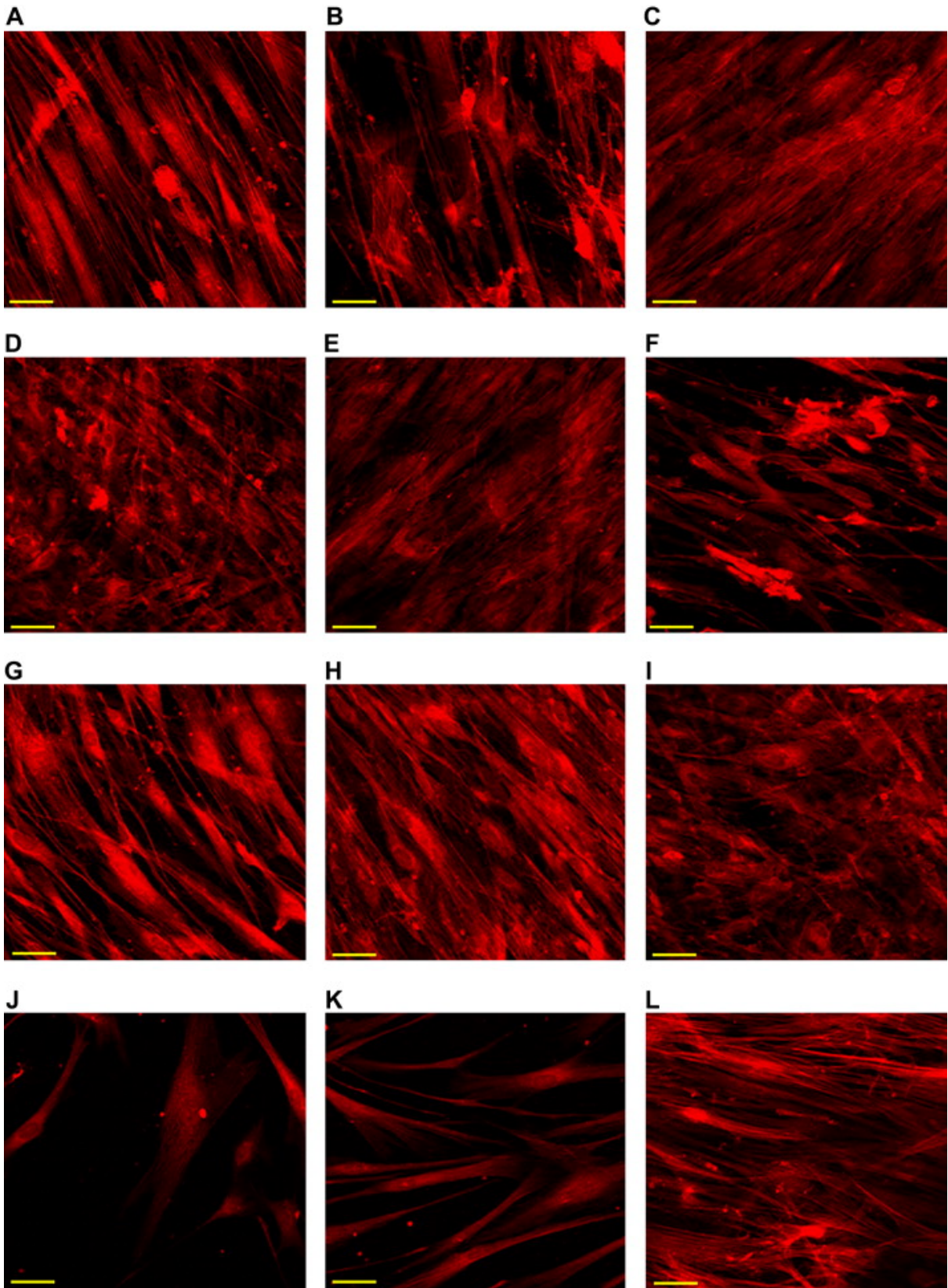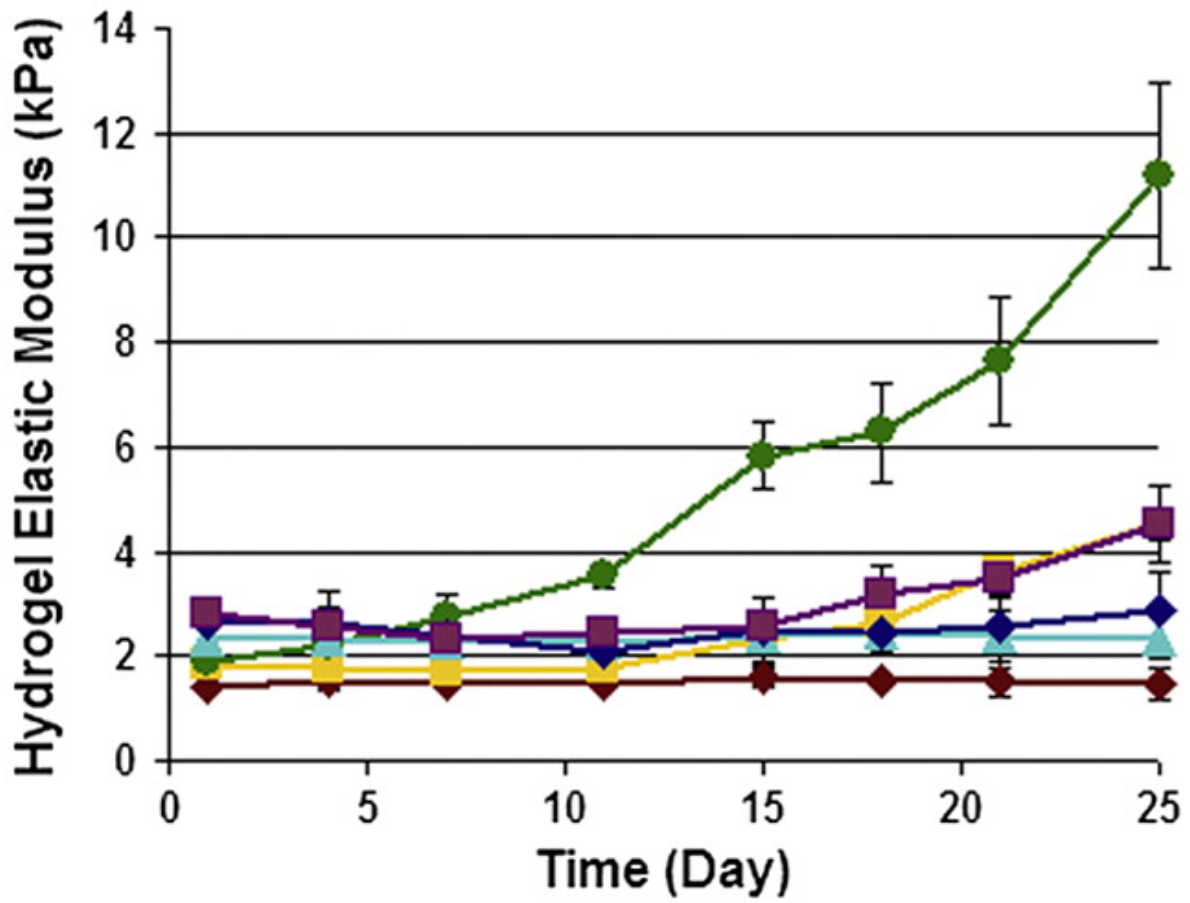23   and 28 days (L). The scale bar is 50 μm.

1   Fig 1

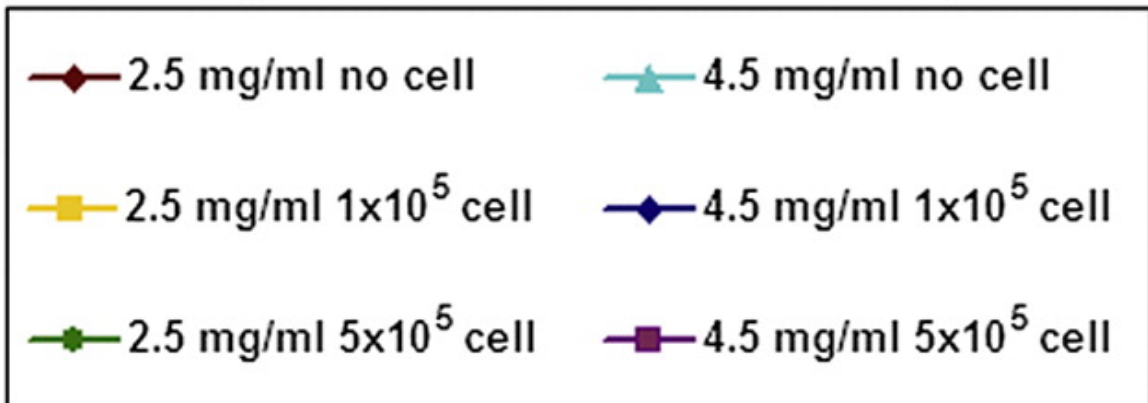1   **Fig 2**

A

B

C   D

Fig 3

1   **Fig 4**

2
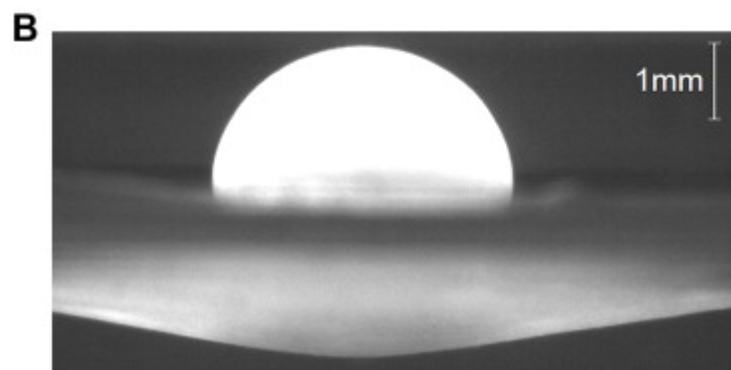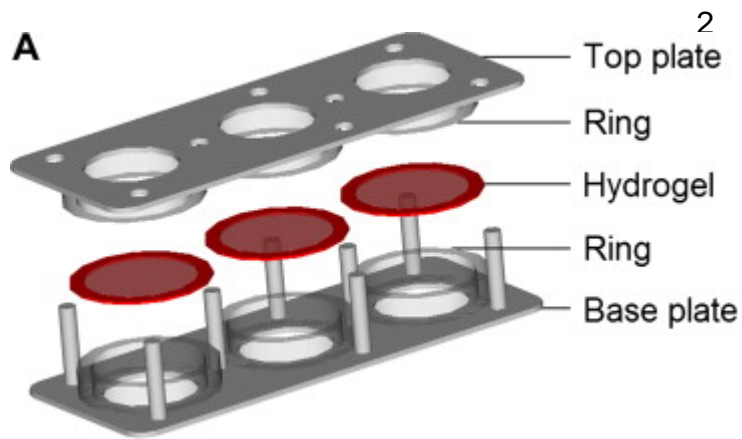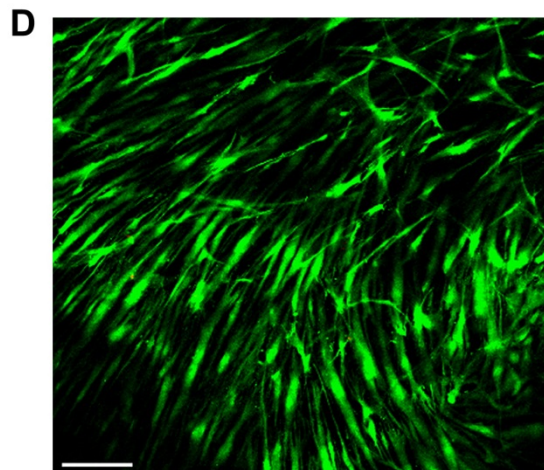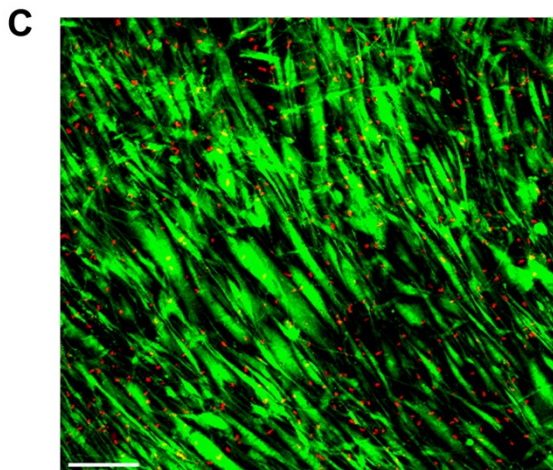
1   Graphical Abstract

Initial collagen concentration and cell number

- ◆ 2.5 mg/ml no cell
- ▲ 4.5 mg/ml no cell
- ■ 2.5 mg/ml 1×10^5 cell
- ◆ 4.5 mg/ml 1×10^5 cell
- ✱ 2.5 mg/ml 5×10^5 cell
- ■ 4.5 mg/ml 5×10^5 cell

2

3