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Application of limited mixing in the Hele-Shaw geometry in fabrication of Janus hydrogels

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Application of limited mixing in the Hele-Shaw geometry
in fabrication of Janus hydrogels

by

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A THESIS

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It is widely accepted that cells behave differently responding to the stiffness of their extracellular matrix (ECM). Such observations were made by culturing cells on hydrogel substrates of tunable stiffness. However, it was recently proposed that cells may sense how strongly they are tethered to ECM, not the local stiffness of ECM. To investigate both hypotheses, we developed a method to fabricate Janus polyacrylamide (PAAM) gels. We squeeze two drops of different concentrations in the Hele-Shaw geometry to generate radial Stokes flow. When the drops coalesce, limited mixing occurs at the interface due to the narrow confinement, and diffusion normal to the interface generates a gradient of the concentration. To test the first hypothesis, we fabricated Janus gel substrates with varying stiffness by coalescing two acrylamide solution drops of different concentrations and then polymerizing them. Based on the indentation test, we confirmed that the fabricated gel had the zone of varying stiffness. To test the second hypothesis, we mimicked protein-gel tethering variation by varying the degree of functionalization of amine and an amine-reactive-dye. We fabricated PAAM gel of uniform stiffness with varying concentration of primary amines by coalescing two drops of same hydrogel solution with different concentrations of primary amines in the same manner. The gel, containing variable primary amines, was submerged in the solution of a fluorescent dye having amine functional groups. Based on fluorescence imaging, we confirmed that the fabricated gel was functionalized to different degrees
depending on the concentration distribution of the primary amines. We expect that cells cultured on the Janus gel substrates will behave differently responding to changes in the stiffness and matrix tethering density.
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Chapter 1

Introduction

Mechanical signals from cellular microenvironment contribute to the regulation of various important cellular processes that determine cell’s fate and function [1, 2]. Examples of cellular functions regulated by mechanical cues include cell proliferation, migration, spreading, morphology and the differentiation of stem cells [3–5]. Different stiffnesses of porous hydrogels have been reported as the mechanical cue in different cellular behavior [1]. However, a recent finding proposed that these signals are translated through the mechanical feedback of the extracellular matrix (ECM), depending on how strongly cells are tethered with the matrix via anchoring proteins [2]. Substrates of different pore sizes, inversely related to stiffness, offer different ECM-protein anchoring density [2].

To mimic invivo microenvironment for cell culture, it is not enough to focus on hydrogels with constant mechanical properties. Between different types of tissues, ECM rigidity often varies over several orders of magnitude, e.g. brain (260-490 Pa), liver (640 Pa), kidney (2.5 kPa), skeletal muscle (12-100 kPa) and cartilage (950 kPa) [6]. Within the tissue, stiffness can also vary strongly, giving rise to complex rigidity gradients, such as those noted at interfacial tissues [7]. Tissue variation may also be caused by cell-matrix tethering factors, in the microenvironment of uniform stiffness,
by making ECM-protein anchoring density variation [2].

Polyacrylamide (PAAM) hydrogels offer porosity [2] and can easily be tuned with a range of stiffness values by changing either the monomer concentration or the crosslinker-to-monomer ratio [8]. In order to facilitate the study of cellular behavior in gradient micro-mechanical environment, we fabricated Janus PAAM gels either with stiffness variations, or with cell-matrix tethering variations. In our fabrication process, we employed droplet based Hele-Shaw sandwiching device to generate a gradient of concentration. This chapter will review typical cell function and fate in different ECM microenvironment. It will also focus on different techniques available so far in fabricating stiffness gradient hydrogels.

1.1 Cells respond to ECM stiffness

1.1.1 Cell motility and morphology

Cell movement is essential in many physiological processes, including morphogenesis [9], wound healing [10] and tumor metastasis [11]. It is widely accepted that cell migration is guided not only by local gradients in the concentration of chemical factors [12,13], but also by the gradients in matrix stiffness [4,14]. Depending upon the cell type and nature of adhesion receptors and signaling chains, stiffness also regulates cell spreading [15,16].

While migrating, leading edge of a cell expands on to the rigid substrate forming lamellipodia. In Figure 1.1(top row), an NIH/3T3 fibroblast cell, cultured on a polyacrylamide sheet contained a gradient of rigidity, migrated from the soft side of the substrate toward the stiffer side [4]. When a part of the leading edge encountered with higher rigidity, the protrusion (lamellipodia) accelerated and the region expanded until the cell passed through the boundary. Trying to move cell in the opposite sense was not successful (Figure 1(b) in [4]), because protrusion stopped at the
Figure 1.1: (Top row) Time lapse images show 3T3 cell migration from the soft side of the substrate toward the stiffer side. Scale bar: 40 µm. (Bottom row) The projected area of BAECs increases with the substrate stiffness [16].

leading edge while approaching soft region [4]. Changes of the shape of bovine aorta endothelial cells (BAEC), cultured on the polyacrylamide gels of different stiffness, is shown in Figure 1.1(bottom row). After 1 day in culture, changes of cell morphology is found ranging from round to well spread, as the stiffness increases about a few thousands Pa [16].

1.1.2 Stem cell differentiation

Adult stem cells, as a part of normal regenerative processes, are believed to egress and circulate away from their niche [17]. Because of local gradients resulting from the complex array of biophysical and biochemical signals from the surrounding ECM, stem cells can migrate through tissues, as shown in Figure 1.2 (top row). When they encounter static stiffness, they differentiate into various anchorage-dependent cell types, including neurons, myoblasts and osteoblasts [5,18–20]. In Figure 1.2 (bottom row), naive mesenchymal stem cells(MSCs) of a standard expression phenotype are seen branched, spindle, or polygonal shapes when grown on matrices respectively in the range typical of $E_{\text{brain}}$ (0.1-1 kPa), $E_{\text{muscle}}$ (8-17 kPa), or stiff cross-linked collagen matrices (25-40 kPa) [5].
1.2 Cells respond to ECM-cell tethering

Stem cells exert mechanical forces via ligated integrins on ECM protein which is covalently attached with the matrix at different anchoring locations. Adhesion strength of this cell-matrix tethering (CMT) depends on number of ECM-protein binding sites, as shown in Figure 1.3(a). A recent discovery proposed that stem cell fate is regulated by CMT, not by the stiffness of the substrate [2]. But obviously, CMT can be controlled with the tuning of stiffness on porous substrates. Fabricating PAAM hydrogels with different stiffnesses makes them porous in different pore sizes which are inversely related to stiffness [2].

Trappman et al. [2] showed that the stiffnesses of PAAM gels play a role in stem cell fate, as previous researchers reported, because ECM-protein anchoring density varies with the stiffness. But to test their hypothesis that CMT regulates stem cell fate, they varied tethering strength on the PAAM hydrogel of same stiffness by using different concentration of a protein-collagen crosslinker. This did not change the amount of
collagen that was bound to the substrate, but it altered cell behavior, as shown in Figure 1.3(b). Epidermal stem cells did not spread on the gel treated with low concentration of ECM-protein crosslinker and they initiated terminal differentiation. However, when gels of the same stiffness were treated with higher concentration of the crosslinker they spreaded and remained undifferentiated [2]

![Figure 1.3](image)

Figure 1.3: (a) Model on collagen binding on soft and stiff PAAM hydrogels. (b) Same stiffness of hydrogel functionalized with different ECM-protein crosslinker concentration shows differences in cell shape (F-actin stained in red), both hydrogels are stained with equal amount of collagen I (green) [2]. Scale bar: 100 µm

Depending on the adhesion strength, cell-ECM experiences ‘force feedback’ (force application by the cell and getting resistance back from the substrate) with different magnitude. Soft feedback fails to assemble a cytoskeleton, rich in polymerized actin [21]. By contrast, stiffer gel offers ‘rigid’ feedback to the cell when force is applied [21], which ultimately makes cell fate decision.

1.3 Hydrogel fabrication with varying stiffness

Polyacrylamide hydrogels can be fabricated by changing either acrylamide (monomer) concentration or bis-acrylamide (crosslinker)-to-acrylamide ratio. With the choice of a proper crosslinking initiator, polymerization of hydrogel can be initiated either chem-
ically or by exposing UV light. Different fabricating techniques have been proposed to generate stiffness gradient in cell mechanics research. This chapter will review some of them.

1.3.1 UV intensity control

![Diagram showing UV intensity control](image)

Figure 1.4: (a) The schematic shows (from top to bottom) a glass coverslip, the hydrogel solution with a dissolved photoinitiator, a treated glass slide, a patterned photomask, a negative photomask, and a UV light source [18]. (b) Hydrogel solution confined between two coverslips. Irradiation gradient is obtained by moving the mask while illuminating the solution [22].

A gradient photomask allows hydrogel solution to be exposed to irradiation with gradual variation of UV intensity, as shown in Figure 1.4(a). Such a photomask with a radial grayscale pattern was used to create gradient stiffness hydrogel via selective activation of the photoinitiator [18]. Although this method is easy to implement, the low resolution of the mask severely limits the precise control of the gradient profile at the micrometer scale. An alternate solution is proposed by sliding a photomask with a uniform speed, as shown in Figure 1.4(b) [22]. However, this method produces non-linear stiffness variation in the high stiffness range. Exposing gel solution with variable intensity of UV light may not complete crosslinking at all locations which eventually could change property over time, or further UV exposure can make the process complicated.
1.3.2 Concentration Control

Figure 1.5: Microfluidic device consists of a patterned PDMS mold attached to an activated glass slide. Inlets are filled with hydrogel solutions with the desired monomer and crosslinker concentration. Reproduced from [14].

To avoid incomplete UV irradiation (it could be insufficient exposure time or intensity), microfluidic gradient generators can be another alternative to fabricate hydrogels with stiffness gradient. The key idea here is to mix acrylamide and bis-acrylamide solution with a gradient variation of their relative concentration and then initiate polymerization by irradiating UV light. Tree-like patterned PDMS mold (Figure 1.5) attached to the glass slide is used to create microchannels [14]. This technique is time consuming, costly, and linear gradient stiffness profiles are difficult to implement with precision [22]. Another rudimentary method to create a gradient of rigidity can be applied by placing two droplets, which are one containing a soft and the other a stiff acrylamide/bisacrylamide mixture, adjacent to each other and covering them with a common coverslip [4].
1.4 Our approach: Developing a sandwiching device

All the techniques reviewed so far have some limitations in generating well defined profile of gradient stiffness. We found that covering two droplets of different concentration of PAAM solution with the same coverslip [4] is the easiest, less time consuming and cost effective technique while mixing is minimized. The big disadvantage of this method is to generate well defined limited mixing interface because this method does not offer proper controlling over mixing.

Figure 1.6: (a) Schematic shows Hele-Shaw geometry and controlled sandwiching of the droplets. (b) Sandwiched droplets before and after coalescence. Images taken from high speed videography (200 fps).

In our approach, we developed a device to sandwich liquid droplets in the Hele-Shaw geometry, as shown in Figure 1.6(a), to generate radial Stokes flow. By narrowing gap between the wetting surfaces, two squeezed droplets expand radially, meet each other and at some point coalesce together, leaving a well defined mixing interface (as shown in Figure 1.6(b)). Because of concentration difference between the two droplets, diffusion normal to the interface generates a gradient of the concentration.
This device has very general applicability, although we developed it for fabricating Janus gel, which is composed of two different zones (either different stiffnesses, or different cell-matrix tethering strengths) separated by a gradient.

By employing our device, we made concentration gradient by sandwiching two droplets of PAAM gels, one for soft and the other for stiff. This sandwiched gel was then irradiated using UV light to initiate crosslinking between polymers.

Stiffness of fabricated Janus hydrogel was characterized by atomic force microscopy (AFM) using Hertz’ nano indentation model. This measurement was verified with static indentation test using a confocal microscope. The Thickness profile of gradient hydrogel was measured through z-scanning by the confocal microscope. To test gradient functionalization of Janus gel, we fabricated PAAM gel of uniform stiffness added with different allylamine concentration. Allylamine contains primary amine that can be tagged with fluorescent dye to see the gradient functionalization of Janus gel from fluorescent imaging analysis. We expect that, with the proper choice of concentration of two different droplets made from bi-functional ECM-protein cross-linker solution, it will be possible to use constant stiffness hydrogel as a Janus hydrogel with cell-ECM tethering strength variation.
Chapter 2

Fabrication of Janus gel

Polyacrylamide (PAAM) hydrogels are widely used substrates for culturing cells. The modulus of elasticity of the gel can be varied by changing the relative concentration of acrylamide and bis-aryl amide. The surface chemistry of PAAM gel can be kept constant while changing its mechanical properties [23]. The pore sizes of the gel are on the order of 100 nm which is enough to support the culturing cells. Immunofluorescence is made possible at high magnifications because of the thin, translucent quality of PAAM gel [8].

In this chapter, Janus hydrogel fabrication process will be discussed. This process includes preparation of coverslips and glass slides, preparation of hydrogel solution, device design and experimental setup.

2.1 Glass preparation

To attach hydrogel on to the coverslips, it is essential to prepare coverslips accordingly. We coated coverslips through amino-silanization to covalently bind hydrogel on it. Chloro-silanazation of glass slides were made to get uniform attachment and smooth top surface of the gel [8, 24]. It also helps in easy removal of the glass slide after gel fabrication. The following protocol for this preparation is summarized from [25].
2.1.1 Amino-silanated coverslips preparation

Materials used

- 0.1 M NaOH (Sigma-Aldrich)
- Deionized H₂O
- 0.2 M HCl (Fisher Scientific, Cat. No. A144)
- 3-Aminopropyltriethoxysilane (APES) (Sigma-Aldrich, Cat. No. 741442)
- 70% Glutaraldehyde (Fisher Scientific, Cat. No.BP2547-1) in phosphate-buffered saline (PBS)
- 100 mm petri dish (VWR, Cat. No. 25384-302).

Procedure

1. We dispersed coverglasses into a petri dish (100 mm) containing 20 ml of 0.2 M HCl and incubated overnight at room temperature with gentle agitation of 60 rpm on an orbital shaker.

2. The next day we decanted acid wash and washed cover glasses five times with 20 ml of di H₂O using gentle agitation on the orbital shaker.

3. We replaced last water wash with 20 ml of 0.1 M NaOH and incubated 1 h at room temperature with gentle agitation.

4. We decanted base wash and washed cover glasses five times with 20 ml of di H₂O using gentle agitation.

5. We replaced last water wash with 20 ml as appropriate of 1:100 dilution of 3-aminopropyltrimethoxysilane (APES) in di H₂O and incubated 1 h at room temperature with gentle agitation.
6. We decanted diluted APES waste and washed coverglasses exhaustively in repeated 20 ml changes of di H$_2$O with gentle agitation for 5 min each. It is important to completely rinse off the unreacted APES, for it will create an orangebrown precipitate with glutaraldehyde (step 8) that fluoresces under UV light and can thus interfere with immune staining techniques.

7. We replaced last water wash with 20 ml, as appropriate of a 0.5% glutaraldehyde in PBS (1:140 dilution of 70% glutaraldehyde in PBS) and incubated 1 h at room temperature with gentle agitation.

8. We decanted diluted glutaraldehyde waste and washed coverglasses exhaustively in repeated 20 ml changes of di H$_2$O with gentle agitation on an orbital shaker for 5 min each to remove the residuum.

9. We dried cover glasses in a desiccant chamber and used them within 24 hours after drying.

2.1.2 Chloro-silanated glass slides preparation

Materials used

- Glass slides (VWR, Cat. No. 16004-368)
- Dichlorodimethylsilane (DCDMS) (Sigma-Aldrich, Cat. No. 40140)
- Kimwipe
- Deionized H$_2$O

Procedure

1. We placed 75 µl of DCDMS onto a glass slide and spread it by placing another glass slide on top of it. We left this in the fume hood for 1 min and then
separated glass slides from each other. Before using these treated glass slides, we left the treated sides up in the fume hood for 5 min to allow DCDMS to be dried completely. During this process, Kimwipe was laid over the bench so that it could absorb the excess DCDMS.

2.2 **Statically compliant hydrogel preparation**

Depending on the crosslinking initiation method, hydrogel solution preparation will vary. To initiate crosslinking between monomers and monomer chains as shown in Figure 2.1, appropriate cross-link initiator is used.

![Figure 2.1](image_url)

**Figure 2.1:** Schematic shows hydrogel formation by crosslinking monomers and monomer chains

Using the device, mentioned in Section 1.4, we generated concentration gradient of mixed acrylamide and bis-acrylamide solution by sandwiching two droplets. Then we irradiated this sandwiched solution with UV light. To verify stiffness measurement, we depended on two different methods, a) static indentation test (data taken from confocal microscopy imaging), b) nano-indentation test (using AFM). We found that we were able to indent hydrogel sufficiently using a 0.67 mm-diameter steel ball for lower stiffness value (<4 kPa) of hydrogel. In this low range, hydrogel fabricated from crosslinking with a chemical crosslinking initiator found uniform. That is why we fabricated hydrogel with both chemical (cross-link initiated immediately after...
adding the initiator) and UV cross-link initiators. In the following subsection both fabrication methods will be discussed.

2.2.1 Preparation of hydrogel solution for UV crosslinking initiation

For gradient hydrogel fabrication, two solutions, which one containing soft and the other stiff with different acrylamide/bisacrylamide mixture, are prepared. This process is adopted from [18] and modified accordingly.

**Materials used**

- Irgacure 2959 (UV cross-link initiator) (BASF- The Chemical Company)
- 1x phosphate-buffered saline (Sigma-Aldrich, P5493-1L)
- 40% (w/v) acrylamide stock solution (Sigma-Aldrich, Cat. No. A4058)
- 2% (w/v) bis-acrylamide stock solution (Sigma-Aldrich, Cat. No. M1533)
- UV lamp (Black Ray, 100W, 365 nm, UVP, Upland, CA)

**Procedure**

1. 1% (w/v) Irgacure stock solution is prepared in PBS. As Irgacure is insoluble in water/PBS, the solution is left in the oven overnight at 65°C and shaken well at next day morning.

2. Acrylamide, bis-acrylamide, PBS and 1% Irgacure to their desired amount are mixed together to make 1 ml solution. Droplet associated with soft hydrogel is made from 7.5% acrylamide and 0.05% bis-acrylamide mixer, while for the other one, it is 8% acrylamide and 0.48% bis-acrylamide mixer.

3. PA gel solution is degassed under strong vacuum for 30 minutes to remove all dissolved gases that may limit the free radical polymerization.
When fluorescent tagging is necessary, we added 0.05% allylamine unless otherwise mentioned. Chemically (molecular weight and chemical structure) allylamine and acrylamide are almost same, except that allylamine has primary amin that can be tagged by fluorescent dye.

2.2.2 Hydrogel fabrication by chemical crosslinking initiation

Materials used

- 10% (w/v) Ammonium persulfate (APS) (Sigma-Aldrich, Cat. No. A3678)
- N,N,N’,N’-Tetramethylethylenediamine (TEMED, cross-link initiator) (Sigma-Aldrich, Cat. No. T22500)
- 40% (w/v) Acrylamide stock solution (Sigma-Aldrich, Cat. No. A4058)
- 2% (w/v) Bis-acrylamide stock solution (Sigma-Aldrich, Cat. No. M1533)

Procedure

1. 3% acrylamide and 0.06% bis-acrylamide is mixed with PBS to prepare 1 ml hydrogel solution.

2. Solution is then degassed under strong vacuum for 30 min to exhaust dissolved oxygen.

3. 1/100 total volume of APS and 1/1000 total volume of TEMED to gel solutions are mixed with the degassed gel solution.

4. 40 µl of the gel solution is pipetted onto the treated side of the chloro-silanated glass slide and the amino-silanated coverslip is placed gently on top of the gel solution. To get the uniform thickness, 250 µm spacers are used. Sandwiched gel is kept in the fume hood for 15 min to allow the polymerization done completely.
5. Bottom glass slide is removed gently with the help of the spacer as the lever.

After washing for 4-5 times, hydrogel is kept submerged in PBS all the time.

2.3 Design of hydrogel sandwiching device

The main objective of designing the sandwiching device is to ensure limited mixing when coalescence happens in the Hele-Shaw geometry (Section 1.4). This device allows us to expand sandwiched droplets uniformly radially. While mixing, two droplets couple together along a straight line to minimize surface energy by reducing total surface area.

Our device consists of two parts: bottom (40 mm x 80 mm x 10 mm) and top (43.5 mm x 60 mm x 25.5 mm) as shown in Figure 2.2. The top part is attached to a micromanipulator which can move vertically up and down with micrometer precision, while the bottom part is fixed on the optical breadboard. The top part is designed so that it can hold a treated coverslip, at the same time it will not block UV light to pass through. The bottom part is used to hold a treated glass slide, and it remains stationary throughout the fabrication. Both parts of the device are fabricated by a 3D printer (Object30 Pro, Stratasys Ltd; material used: Veroblue). Detailed design with actual measurement is attached in Appendix A.

2.4 Experimental setup and working procedure

A treated coverslip is placed in the upper part of the device with the support of a rectangular coverslip. This conjugate (rectangle and circular coverslips shown in Figure 2.3) is placed in the slot suitable for rectangular coverslip. This slot is made so that coverslip can always remain parallel to the bottom glass slide.

Two droplets of different concentrations, soft and stiff, are placed on the treated glass slide, next to one another. The top part of the device is gradually lowered by
Figure 2.2: Experimental setup. The sandwiching device is assembled with an optical breadboard and a micromanipulator.

rotating knob (in the Figure 2.2, the top one). Liquid droplets make a contact with the coverslip and further lowering makes them expand. At some point they coalesce and create a straight mixing interface. After coalescence, molecules diffuse from the higher concentrated zone to the lower concentrated zone. We wait for 1-3 min to get reasonable band of diffusion zone. Then a UV lamp is placed right above the window of the top part. 10 min-long irradiation completes polymerization, and the Janus hydrogel with gradient stiffness is fabricated.

Going over this procedure, it is possible to create variation in cell-matrix tethering strength. This can be made by gradient functionalization of hydrogel with bifunctional cross-linker whose one arm is cross-linked with protein molecules. By generating differential concentration of the cross-linker, protein binding strength with the gel will vary accordingly. To prove this hypothesis, we made gradient of concentration of fluorescent dye (Alexa flour 488, Life Technology), by placing two droplets of hydrogel with constant stiffness containing different concentration of allylamine. For this time, constant stiffness hydrogel (instead of treated coverslip), prepared by
Figure 2.3: 5 µl water is spread on top of a rectangular coverslip (22mm × 50mm × 0.16mm). Because of water, surface tension can fix treated circular coverslip with the rectangular one.

Mixing 8% acrylamide, 0.48% bis-acrylamide, was used. The concentration of allylamine, which is added in the solution, between two droplets differ by fourth times—one containing 0.05% and the other 0.2%. Allylamine contains primary amine which is linked with a functional group of the fluorescent dye. Fabricated hydrogel is then submerged in Alexa Flour dye solution for overnight. Next day morning, samples are washed several times.
Chapter 3

Characterization of Janus gel with stiffness gradient

For mechanical characterization, Janus PAAM hydrogel with gradient stiffness was indented with a sphere at different locations. Because of the spherical indenter being used, the Hertz model was adopted to analyze the data. For static indentation test, by probing with microsphere under gravitational forces, confocal microscopy imaging technique is used to measure indentation depth. Force vs. deflection data were captured from nano indentation test using atomic force microscopy (AFM). Stiffness measurements from these two different methods are compared.

From fluorescent imaging analysis, thickness of Janus gel is measured at different locations to get the surface profile across the mixing interface.

3.1 Hertz model

The Hertz model approximates the sample as an isotropic and linear elastic solid occupying infinitely extended surface both in $xy$ and $-z$ axis. Furthermore, it is assumed that the indenter is not deformable and that there are no additional interactions between the indenter and the sample [26]. If these conditions are met, Young’s modulus
(\(E\)) of the sample can be calculated or fitted using Hertzian model [27].

The stiffness of PAAM gel is determined with an improved method based on the Hertz theory, applicable for both in atomic force microscopy [28] and in probing hydrogel with microsphere under gravity [4]. The model for a spherical indenter is described as:

\[
F = \frac{4Er^2}{3(1-\nu^2)}\delta^2, \tag{3.1}
\]

where \(F\) is the applied force on the substrate, \(E\) is the Young’s modulus of the substrate, \(r\) is the radius of the sphere, \(\nu\) is Poisson’s ratio for substrate material, and \(\delta\) is the indentation depth.

Figure 3.1: (a) Sketch of the indentation experiment by AFM. The cantilever is moved towards the sample. (b) Indentation, \(\delta\), made by a steel microsphere placed on a substrate at static indentation test.

For static indentation test (Figure 3.1b), force is calculated as weight of the ball. Knowing the density and radius of the sphere, mass can be calculated by multiplying density and volume of the sphere. For evaluating Young’s modulus, Equation 3.1 can be rearranged as the following:

\[
E = \frac{30.82(1-\nu^2)\rho r^5}{\delta^2} \tag{3.2}
\]

where \(\rho\) is buoyancy-corrected density of the sphere. In our case, it was 6720 kg/m\(^3\).

In AFM instrumentation for nano-indentation test, the indentation depth is com-
Figure 3.2: Deflection of the cantilever vs. piezo displacement curve during nano indentation test.

Commonly defined as being the difference between piezo movement and cantilever deflection after the point of contact \((Z_0, d_0)\) as shown in Figure 3.2. While piezo movement and cantilever deflection are both related to this initial point, the indentation is generally described as the difference between the relative changes for the piezo and cantilever,

\[
\delta = (Z - Z_0) - (d - d_0) \tag{3.3}
\]

Indentation force is calculated by multiplying the spring constant and deflection of the cantilever. By rearranging Equation 3.1, we get

\[
d^2 = \left[ \frac{4Er_1^2}{3k(1 - \nu^2)} \right]^{\frac{3}{2}} \delta \tag{3.4}
\]

where \(d\) and \(k\) are the deflection and spring constant of the cantilever.

Plotting \(d^2\) vs \(\delta\) as shown in Figure 3.3, a slope can be drawn. Finally Young’s
modulus can be calculated from the following:

\[ E = \frac{3k(1 - \nu^2)Slope^{\frac{3}{2}}}{4r^{\frac{1}{2}}} \]  \hspace{1cm} (3.5)

Figure 3.3: Slope of this curve is used in Equation 3.5.

If the interaction between the tip and surface is dominated by an elastic indentation rather than by an adhesion between tip and sample, the deformation of sample is described by the Hertz model [28,29].

In theory, if tip-sample interaction remains elastic (no adhesion) during approach towards the surface and retraction from the surface, deflection vs indentation plotting should match together. But, analyzing the retraction curve, we can see strong adhesion between the indenter tip and the sample, in all the cases, while pulling the tip off the surface (it happens strongly for softer gels). That clearly indicates that approach data do not correspond to Hertz model perfectly. Retraction data directly come from the resistance of the material where adhesion does not have any role in the tip-sample interaction. However, around the contact point, adhesion between the tip and the sample is dominant which again does not correspond to the model described
by Hertz. In evaluating stiffness, those data have to be discarded.

For both measurement technique, we assumed Poission’s ratio for PAAM hydrogel to be 0.45 [30].

### 3.2 Static indentation test

A steel ball (0.67 mm diameter, 7.72 g/cm³, NEMB, Norkfolk, CT) is placed on a soft fluorescently tagged polyacrylamide hydrogel (4% acrylamide, 0.15% bis-acrylamide, 0.05% allylamine, cross-link initiated by UV light). The indentation caused by the steel ball was measured by confocal microscopy (Olympus IX 81 inverted microscope) imaging technique. Series of $z$-image were captured, starting from the inside of the gel to above the top surface of the gel (Figure 3.4c). Distance between consecutive $z$-images is calibrated by the known thickness of a coverslip (160 µm).

![Figure 3.4: (a) $xz$ image at the center of the indentation. Scale bar: 100 µm. (b) Radius of the steel ball and bottom point of indentation depth is calculated from the fitting curve, (c) Perimeter at cross-section of the ball from each $z$-image is plotted. Unsuccessful radius calculation is discarded.](image-url)
3.2.1 Microsphere diameter and bottom point of indentation depth

The radius of the steel ball is calculated by fitting projected radius of the steel ball at different z-images (Figures 3.4a&b). Once radius and center of the sphere is known, subtracting z-center by the radius will evaluate the bottom point of the indentation depth, where the first image is taken as the reference.

![Image](image1.png)

Figure 3.5: (a) Fluorescent intensity data is taken from outside of the ball area (as shown in red). Above the top surface of the hydrogel is shown in the image. (b) Intensity profile along the z-image is plotted. (c) The first derivative of curve b evaluates the top surface.

3.2.2 Top surface of the hydrogel

To get indentation depth, it is essential to locate the top surface of the hydrogel. Mean fluorescent intensity (in arbitrary unit) of a measurement window as shown in Figure 3.5, which is selected outside of the ball area so that all through it can count.
fluorescent intensity of each image, is plotted against the z-image position, as shown in Figure 3.5. Taking central difference, top surface can be evaluated at the location where the slop is maximum [31].

3.2.3 Result

Indentation depth is calculated by subtracting the bottom point of the indentation depth from the measurement of the top surface. In both cases, reference points are same.

In Equation 3.2, all the parameters on the right hand side are known. The modulus of elasticity of PAAM hydrogel is then calculated and are shown in Table B.1. Mean stiffness of the gel we get as 223 Pa.

Table 3.1: Stiffness measurement of soft hydrogel (4% acrylamide, 0.15% bis-acrylamide, 0.05% allylamine, UV cross-linked)

<table>
<thead>
<tr>
<th>Data point</th>
<th>Sphere diameter (µm)</th>
<th>Indentation Top</th>
<th>Indentation bottom</th>
<th>Indentation depth (µm)</th>
<th>Young’s modulus, E (Pa)</th>
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</thead>
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3.3 Nano indentation test by AFM

To get confidence over Janus hydrogel stiffness measurement by AFM, we went through static indentation test prescribed in the preceding section. We measured stiffness of the same sample by AFM (MFP-3D, Asylum Research, Goleta, CA) instrument. This section will evaluate the measurement and will also describe how we get the result.
3.3.1 Spring constant of the cantilever

In our case, a colloidal probe (V-shape Silicon Nitride cantilever, Novascan) is used as a cantilever. The spring constant of this cantilever (attached with a borosilicate glass bead) is calibrated by measuring the Brownian fluctuations on the lever, popularly known as thermal tune. The equi-partition theorem applied to the first flexural mode in the cantilever relates the mean squared amplitude of the cantilever motion $<A^2>$ and the spring constant $k$ to the absolute temperature $T$ and Boltzmann’s constant $k_B$ by the following relationship:

$$\frac{1}{2}k <A^2> = \frac{1}{2}k_B T$$ (3.6)

To calibrate the mean squared amplitude, it is necessary to calibrate the Inverse Optical Lever Sensitivity (InvOLS) with units of nm/Volt of the cantilever. We do this by performing a force curve with the surface. The expression for the spring constant then becomes:
In this expression, \(< \Delta V^2 >\) is the mean squared voltage fluctuations due to movement in the first flexural mode of the cantilever. Software internally uses the fitting parameters to calculate spring constant \([32]\).

![Figure 3.7: Thermal spectrum of the cantilever in water. Red line shows the fundamental resonance frequency of the cantilever.](image)

For the particular cantilever shown in Figure 3.6, spring constant is measured as 53.84 nN/µm (nominal value is 60 nN/µm, supplied by the manufacturer).

### 3.3.2 \(d^3\) vs \(\delta\) data fitting

From Equation 3.4, Young’s modulus of the hydrogel can be calculated from the slope of \(d^3\) vs \(\delta\) data fitting. While doing indentation test, three sets of data were recorded encompassing approach and retraction curves. Those data sets are respectively z-piezo driving data in nm, deflection data in nm and feedback signal from z-piezo actual response in nm. Experimental analysis was characterized by taking deflection and feedback data for retraction curve (Section 3.1). Contact point is evaluated by drawing ‘zero deflection line’, which we get from the fitting flat region of the curve.
Figure 3.8: Away from the contact point corresponds best fit to hertzian model while cantilever approaching to the gel. In our calculation, thermal drift, possibly caused by liquid medium, is compensated by subtracting zero deflection curve from the whole data set.

To get a best fit with the Hertz model, derivative of $R^2$ value, calculated from $d^2 \delta$ vs $\delta$ fitting curve with the Hertz model, is plotted by changing ‘starting point’ gradually from the contact point to a half of the whole retraction curve, while the end point is fixed at maximum indentation point (Figure 3.8). Curve starting from the contact point does not fit well. Range of data analysis is selected by taking measurement starting from a point where error is minimum and constant (almost zero), which matches very well with the Hertz model (Figure 3.9). By knowing the fitting range and slope of the curve, as shown in Figure 3.9, we can measure stiffness of hydrogel according to Equation 3.4.
3.3.3 Result

The results of stiffness measurement of the same sample which was characterized by confocal imaging are shown in Figure 3.10. Comparing two measurement techniques, we can see that the mean value from AFM instrument matches well with the technique used by confocal imaging.

3.4 Stiffness of Janus gel

From the preceding section, we are familiar with the process of fabricating Janus hydrogel with gradient stiffness. Going over AFM measurement technique, stiffness of Janus hydrogel can be characterized. Deflection vs. indentation data were collected at 26 different locations on the Janus gel along three different lines with 1 mm interval, while distance between the two neighboring lines were 2mm. Lines cross the mixing interface almost perpendicularly. Stiffness measurement of Janus hydrogel is shown
Figure 3.10: Hydrogel (4% acrylamide, 0.15% bis-acrylamide, 0.05% allylamine, UV cross-linked) stiffness measurement by AFM.

in Figure 3.11, detailed data table can be found in Appendix B.

Figure 3.11: Janus Hydrogel stiffness measurement by AFM

3.5 Thickness profile of Janus gel

We hypothesize that surface profile should not be uniform because of stiffness variation. Soft gel should swell more than stiff gel. So, we measured the thickness profile of
hydrogel at 15 different locations covering the mixing interface (Figure 3.11). Fluorescent intensity as a function of z was generated by a convolution of gel intensity profile. Edge was detected by taking derivative of the convolution of intensity profile [31].

Figure 3.12: Janus PAAM Hydrogel surface profile
Chapter 4

Characterization of Janus gel with gradient functionalization

As mentioned in the preceding sections, employing our device and method it is possible to generate gradient functionalization of hydrogel with ECM-protein crosslinker, which will eventually generate cell-matrix tethering strength variation. To prove this hypothesis we fabricated gel of constant stiffness with different concentration of allylamine to tag fluorescent dye accordingly (Figure 4.1a and Section 2.4) and then measured fluorescent intensity along the X-axis at different locations of the gel. There is a direct relationship between dye concentration and fluorescent intensity.

In Figure 4.1b, fluorescent intensity inside the gel is plotted at different location starting from one static zone along the gradient. All the images were taken inside the gel maintaining the same height. In Figure 4.2, hydrogel solution of same stiffness is sandwiched where one droplet contains allylamine, but the other one does not. Fluorescent image from confocal microscope shows concentration gradient along the interface.
Figure 4.1: (a) Schematic shows the hydrogel fabrication and gradient functionalization of fluorescent dye, (b) Intensity profile of Janus hydrogel with gradient functionalization.

Figure 4.2: Concentration of fluorescent dye changes across the mixing interface.
Chapter 5

Conclusion

To understand cellular behavior and function at \textit{in vivo} context, it is necessary to fabricate gradient biomaterials including cell culture medium to study cell culture. We mainly focused on PAAM hydrogel fabrication process where two approaches, gradient stiffness and gradient functionalization, are highlighted. Our fabrication method offers simplicity as well as accuracy.

Application of our device, in cell culture research, will facilitate researchers to culture cells at two different static stiffness (as well as static tethering strength) and at a gradient zone. It will help them to work with multiple approaches in the same cell culturing environment. That means, it saves cost, time and materials as well.

In our case, we have shown 3 mm gradient zone where the approximate width (perpendicular to the interface) of the gel is 12 mm, where stiffness changes 7.8 kPa/mm (Figure 3.11). From the experience, it is possible to generate different band of gradient zone by changing distance among the droplets.

Hydrogel thickness changes with the change of stiffness. Softer gel swells more than the stiffer one. As shown in Figure 3.12, thickness varies between soft (4 kPa) and stiff (25 kPa) gels around 150 µm where the thickness of the stiff gel is around 550 µm.
Bibliography


[27] *Determining the elastic modulus of biological samples using atomic force microscopy*. JPK instruments, Berlin, Germany.


[32] R. Proksch, Thermal Noise Spring Constant Cantilever Calibration Technique with the MFP-3D AFM. Asylum Research, Santa Barbara, CA.
Appendices
Appendix A

Details of the device design

Figure A.1: TOP, FRONT and RHS views of top part of the device. Dimensions are in mm
Figure A.2: TOP, FRONT, RHS and BOTTOM views of bottom part of the device. Dimensions are in mm
Appendix B

Data Table

Table B.1: Janus Gel stiffness measurement

<table>
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