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Tailoring the Dependency between Rigidity and Water Uptake of a Microfabricated Hydrogel with the Conformational Rigidity of a Polymer Cross-linker

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Abstract

Many diverse applications utilize hydrogels as carriers, sensors, and actuators, and these applications rely on the refined control of physical properties of the hydrogel, such as elastic modulus and degree of swelling. Often, hydrogel properties are interdependent; For example, when elastic modulus is increased, degree of swelling is decreased. Controlling these inverse dependencies remains a major barrier for broader hydrogel applications. We hypothesized that polymer cross-linkers with varied chain flexibility would allow us to tune the inverse dependency between the elastic modulus and the degree of swelling of the hydrogels. We examined this hypothesis by using alginate and poly(acrylic acid) (PAA) modified with controlled number of methacrylic groups as model inflexible and flexible cross-linkers, respectively. Interestingly, the polyacrylamide hydrogel cross-linked by the inflexible alginate methacrylates exhibited less dependency between the degree of swelling and the elastic modulus than the hydrogel cross-linked by flexible PAA methacrylates. This critical role of the cross-linker's inflexibility was related to the difference of the degree of hydrophobic association between polymer cross-linkers, as confirmed with pyrene probes added in pre-gel solutions. Furthermore, hydrogels cross-linked with alginate methacrylates could tune projection area of adhered cells by solely altering elastic moduli. In contrast, gels cross-linked with PAA methacrylates failed to modulate the cellular adhesion morphology due to lower, and smaller elastic modulus range to be controlled. Overall, the results of this study will significantly advance the controllability of hydrogel properties and greatly enhance the performance of hydrogels in various biological applications.

Keywords: polymer cross-linker, alginate, poly(acrylic acid), elastic modulus, degree of swelling, microfabrication

1. INTRODUCTION

Hydrogels have been extensively studied for use in various devices and products including sensors, actuators, cosmetics, synthetic extracellular matrices, drug delivery, and programmable matrices enabling cell catch and release.[1-8] Hydrogels are often integrated with various microfabrication techniques to assemble pre-defined microstructures and advanced functionalities.[9-10] Successful use of hydrogels in these applications relies on the ability to control several, interdependent physical properties of hydrogels, such as the elastic modulus and the degree of swelling.[11] Specifically, the elastic moduli of hydrogels should be controlled over a wide range to enable the hydrogel with the desired functionality.[12-14] For example, a hydrogel used as a device to catch and release cells should be rigid enough to maintain its structural integrity for a sufficient period of time.[8] Additionally, hydrogels with controlled rigidity should exhibit a minimal difference in the degree of swelling to retain the intended geometry of the hydrogel.

The elastic moduli of hydrogels are commonly controlled by altering the total concentration of the gel-forming precursor or the molar ratio between the crosslinking molecules and the gel-forming precursors. Most crosslinking molecules used to form hydrogels are short molecules with two reactive groups. Inadvertently, the use of divalent, short crosslinking molecules is often plagued by a significant increase of the water uptake with decreasing elastic modulus of the gel.[18] This increase in the degree of swelling results in significant volumetric expansion of the gel and subsequent loss of the desired hydrogel geometry and structure.[15-17] This problem becomes more significant with microfabricated hydrogels, because the decrease in hydrogel size facilitates the osmotically driven flow of water into the hydrogel.

Therefore, this study presents a new strategy to tune the inverse dependency between the degree of swelling and the elastic modulus of a hydrogel. Furthermore, this study demonstrates that this new hydrogel design strategy is useful in assembling microfabricated hydrogels with controlled stiffness but minimal changes to the gel size and pattern, allowing for the refined control of cellular adhesion on the gel. We hypothesized that the chain flexibility of polymer cross-linkers is an important factor to tune the elastic modulus and the degree of swelling of a hydrogel, coupled with a number of reactive groups of the cross-linker in the hydrogel (Scheme 1). This hypothesis was examined using alginate methacrylates and poly(acrylic acid) (PAA) methacrylates as inflexible and flexible polymer cross-linkers, respectively. Alginate with a molecular weight of 100,000 g/mol presents larger conformational rigidity than PAA with an equivalent molecular weight, shown by persistence lengths (P) of 32 and 2 nm, respectively.[19-20] These polymers were modified by varying the number of methacrylic groups, thus controlling the number of cross-links in a polyacrylamide hydrogel. Then, the effect of the polymer chain rigidity on the self-association of the polymers was evaluated with pyrene probes suspended in the pre-gel solutions.[21-22] Elastic moduli and degrees of swelling were measured in neutral media with varying ionic strength. Finally, the hydrogels with controlled elastic moduli were micropatterned with fibronectin, in order to examine the interplay of gel rigidity and swelling on cellular adhesion. Overall, the results of this study will greatly serve to improve the controllability and performance of hydrogels used in various biological applications.

2. MATERIALS AND METHODS

2.1. Synthesis of alginate methacrylates

Alginate was modified with methacrylic groups using carbodiimide chemistry as previously described.[18] In brief, alginate with large fraction of guluronic acid residues (LF20/40, molecular weight ~ 100,000 g/mol, FMC Technologies) was dissolved at a concentration of 1.0 % (w/w) in 0.1 M (2-(N-morpholino) ethanesulfonic acid) (MES) buffer (Sigma-Aldrich). Then, 1-hydroxybenzotriazole (HOBt, Fluka), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC, Thermo Scientific), and 2-aminoethyl methacrylate (AEMA, Sigma Aldrich) were dissolved in the alginate solution at a molar ratio of 2:2:1, respectively, and stirred overnight (Figure S1A). The molar ratio of AEMA to uronic acid moieties was varied to increase the degree of methacrylate substitution to alginate molecules. The solution was dialyzed, sterilized via filtration, and lyophilized. The resulting alginate methacrylates were reconstituted in deionized water at a concentration of 4.0 % (w/w). The conjugation of methacrylate groups onto the alginate was confirmed by ^{1}H-NMR (300 MHz, QE300, General Electric) (Figure S2A).

The degree of substitution of methacrylic groups (DS_{MA}) to alginate was determined by titration with NaOH to calculate the number of free carboxylate groups (Figure S2C).[23] Briefly, 0.2 g of methacrylic alginate was dissolved in 40 mL of distilled water, and the pH of the solution was adjusted to pH 2 by adding HCl. Then, the alginate methacrylate solution was titrated with a 0.1 M aqueous NaOH solution, and the corresponding pH value of the solution was measured with a pH meter (Mettler Toledo). The NaOH solution was continuously added until the pH reached 12 to obtain the titration curve. Then, the number of moles of NaOH at which pH was significantly increased was used to calculate the number of free carboxylate groups. The DS_{MA} was calculated from the following Equation (1),

$$DS_{MA}(\%) = \left(\frac{N_{sub}}{N_{free}+N_{sub}}\right) \times 100 \qquad (1)$$

$$N_{sub} = \left(\frac{m_{alginate} - 176 \times \frac{N_{free}}{N_{avg}}}{287}\right) \times N_{avg} \qquad (2)$$

where N_{free} is the number of free carboxylate groups determined by the titration. N_{sub} is the number of substituted methacrylic groups calculated from the Equation (2); $m_{alginate}$ is the mass of alginate sample (0.2g), N_{avg} is the Avogadro's number, and 176 and 287 are molecular weights of the unmodified uronic acid and uronic acid conjugated with methacrylic group, respectively.

2.2. Synthesis of PAA methacrylates

Poly(acrylic acid) (PAA, molecular weight ~ 100,000 g/mol, Sigma-Aldrich) was modified with methacrylic groups following the same carbodiimide-activated substitution process used to prepare alginate methacrylates. In brief, PAA dissolved in 0.1 M MES buffer at 1.0 % (w/w) was mixed with HOBt, EDC, and AEMA, and then stirred overnight (Figure S1B). The solution was dialyzed, sterilized via filtration, and lyophilized. The PAA methacrylates were reconstituted in deionized water to a concentration of 4.0 % (w/w). The conjugation of methacrylate groups onto the PAA was confirmed by ^{1}H-NMR (300 MHz, QE300, General Electric) (Figure S2B). The degree of substitution of methacrylic groups to PAA was determined with the titration method described above.

2.3. Fluorescent analysis of the self-organization between polymer cross-linkers

The self-organization of polymer cross-linkers was evaluated using a pyrene probe.[24] The pyrene (Sigma-Aldrich) was dissolved in cyclohexane to prepare a stock solution with

concentration of 6.0×10^{-4} M. The pyrene stock solution was mixed with a solution of the polymer cross-linker with varying polymer concentrations. The final concentration of pyrene was kept constant at 6.0×10^{-6} M. Then, the mixture was sonicated for 10 minutes to ensure uniform dispersion of pyrene in the polymer solution and further incubated overnight to allow the pyrene to preferentially associate with the cross-linkers. The mixture loaded in a quartz cuvette was excited at wavelength of 330 nm and the resulting emission spectrum from 350 to 450 nm was obtained using a fluorometer (Fluoromax-4, Jobin Ivon).

2.4. Preparation of hydrogels

Hydrogels were prepared through a free-radical cross-linking reaction between acrylamide (Sigma-Aldrich) and alginate methacrylates or PAA methacrylates. The acrylamide was dissolved in deionized water at a concentration of 7.4 % (w/w). The cross-linker, alginate methacrylates or PAA methacrylates, was added to the acrylamide solution. The molar ratio between the cross-linker and acrylamide was varied from about 0.00017 to 0.00043. A 1.0 % (w/w) ammonium persulfate (APS, Sigma-Aldrich) and 0.1 % (w/w) N,N,N,N-tetramethylethylenediamine (TEMED, Sigma-Aldrich) were added to the pre-gel solution to activate the cross-linking reaction. The mixture was poured between glass plates with a spacer of 1.0 mm thickness. After one hour, the gel was punched out in a form of a disk with diameter of 10 mm. The gel disks were incubated in water until the gel swelling reached equilibrium. For certain experiments to examine effects of ionic strength of the media, the gel disks were incubated in PBS of varied ionic strength. The ionic strength was altered by varying total salt concentration of PBS from 0 (pure DI water) to 170 mM.

2.5. Characterization of hydrogel properties

The hydrogel stiffness was evaluated by measuring the elastic modulus (E) with a mechanical tester (MTS Insight, MTS). At room temperature, the gel disks were compressed at a constant rate of 1.0 mm/s, and the resulting stress was recorded by MTS software (Testworks 4). The elastic modulus was calculated from the slope of the stress-strain curve for the first 10 % strain.

The degree of swelling (Q), defined as the reciprocal of the volume fraction of a polymer in a hydrogel (v_2), was calculated from the following Equation (3),

$$Q = v_2^{-1} = \rho_p \left[\frac{Q_m}{\rho_s} + \frac{1}{\rho_p}\right] \quad (3)$$

where ρ_s is the density of water and ρ_P is the density of polymer, and Q_m is the swelling ratio measured by weighing hydrogel disks swollen to equilibrium and those dried via lyophilization.

Assuming that the hydrogel behaves like an elastic network model; the number of elastic cross-links ($N_{\text{x-link}}$) was calculated from E and Q using Equation (4),[25]

$$N_{X-link} = \frac{SQ^{-1/3}}{RT} \quad (4)$$

where S represents shear modulus, R represents the gas constant (8.314 J mol^{-1} K^{-1}), and T represents the temperature at which the modulus was measured (25 °C). Assuming that the hydrogel follows an affined network model, S was calculated from the slope of the stress vs. - ($\lambda - \lambda^{-2}$) curve, where λ is the ratio of the deformed distance to the initial length.[26]

2.6. Patterning of cell adhesion proteins on a hydrogel surface

A cell adhesion protein, fibronectin (Fn) from bovine plasma (BD Biosciences) was dissolved in phosphate buffered saline (PBS, pH 7.4) at the concentration of 50 μg/mL. 1.0 mg of monoacrylated poly(ethylene glycol)-N-hydroxysuccinimide (acryl PEG-NHS, M_w 3500 g/mol) was added to 1.0 mL of each Fn solution, and stirred at room temperature for 4 hours. Acryl-fibronectin was placed on top of a PDMS stamp which consists of an array of circular posts with a diameter of 500 μm (Figure S3), and incubated at 37 ºC for 2 hours. Excess acryl-fibronectin solution on top of the PDMS was removed, and the PDMS stamp was dried with a stream of N_2 gas. The PDMS stamp was placed on top of a glass slide and gently pressed, followed by incubation at 37 ºC for 1 hour. The PDMS stamp was removed, and the glass slide was kept under vacuum until further use. In parallel, glass coverslips (1.8 × 1.8 cm) were treated with 0.4 % 3-(trimethoxysilyl propyl) methacrylate (pH 3.5, adjusted with glacial acetic acid) for 1 hour, in order to allow covalent bonding of hydrogel to coverslip via co-polymerization.[27-28] A pre-gel solution with the polymer cross-linkers was placed on a micro-sized patterned glass slide, and immediately covered with the pre-treated coverslip. The hydrogel polymerized within one hour, and the coverslip was gently detached from the glass side. The hydrogel was washed with PBS supplemented with 0.05 % Tween 20 three times to remove unconjugated fibronectin, followed by washing with PBS. Then, the hydrogel was detached from the coverslip and further incubated in PBS before seeding cells on the gel surface.

To confirm the covalent attachment of fibronectin on the hydrogel surface, fibronectin-conjugated hydrogels were first incubated in PBS mixed with monoclonal anti-fibronectin produced in mouse (Sigma Aldrich) overnight at 4 ºC. The antibody was diluted in PBS at 1:300 before use. The solution was then aspirated and rinsed three times with PBS. Next, the gels were incubated in PBS mixed with Alexa Fluor 488 goat anti-mouse IgG (Life Technologies) for 2 hours at 37 ºC. The fluorescent antibody was diluted in PBS at 1:1000 before use. After

rinsing three times with PBS, the fluorescent image of the hydrogel surface was captured with a fluorescence microscope (DM6000 B, Leica).

Separately, the amount of fibronectin linked to the hydrogel surface was evaluated by running a colorimetric immunoassay.[32] First, the acryl fibronectin-conjugated hydrogel was incubated with mouse anti-fibronectin monoclonal antibody (Sigma Aldrich) for 2 hours at room temperature. Second, the hydrogel was incubated with anti-mouse IgG-biotin (Sigma Aldrich) for 2 hours at room temperature. Third, the hydrogel was incubated in streptavidin-horseradish peroxidase (Sigma Aldrich) for 30 minutes. The hydrogel was then incubated in a substrate solution (mixture of H_2O_2 and tetramethylbenzidine (TMB), R&D Systems) for 30 minutes. After adding stop solution (2N H_2SO_4), the absorbance of the gel at 450 nm was obtained using a spectrophotometer (Synergy HT, BioTek). Varying amounts of fibronectin were separately assayed to obtain a standard curve. The amount of fibronectin was normalized to the area of circular pattern of the acryl fibronectin.

2.7. Cell culture on the hydrogel micro-patterned with fibronectin

Fibroblasts (NIH 3T3, ATCC) were seeded on the hydrogel surface patterned with fibronectin. 1.0×10^4 cells suspended in 100 μL of Dulbecco's Modified Eagle Medium (DMEM) were placed on top of each hydrogel. DMEM was supplemented with 10 % fetal bovine serum and 1 % penicillin/streptomycin. The cells were incubated at 37 ºC with 5 % CO_2 for 1 hour to allow the cells to adhere to the hydrogel surface. Then, the hydrogel was washed with PBS to remove unbound cells, and further incubated in fresh culture media for 24 hours. The cells adhered to the hydrogel were imaged with a CCD camera mounted on an inverted microscope (Leica DMIL). To visualize the actin organization and nuclei of the cells adhered on the hydrogel, the cells were stained with fluorescently labeled phalloidin and 4′,6-diamidino-2-phenylindole (DAPI). The

stained cells were then imaged using a Zeiss LSM 700 confocal microscope. The projected cell area was measured using Image*J* software.

3. RESULTS AND DISCUSSION

3.1. Synthesis of polymer cross-linkers and fluorescent analysis of their self-association

Alginate and poly(acrylic acid) (PAA) were modified with methacrylic groups via chemical reaction between carboxylate groups of polymer and primary amine group of 2-aminoethyl methacrylates (Scheme 1 and Figure S1). Increasing the molar ratio between the 2-aminoethylmethacrylates and the repeating units of the polymers resulted in an increase of the degree of substitution of methacrylic groups (DS_{MA}). Two different numbers of methacrylic groups per polymer chain (N_{MA}) were obtained at 70 and 140 for both alginate and PAA, as confirmed through titration (Figure S2). Alginate methacrylates remained soluble in water even when half of the uronic acid units of alginate were substituted with methacrylic groups. In contrast, the PAA substituted with N_{MA} above 140 became insoluble in water.

The hydrophobic association between the polymers substituted with varying number of methacrylic groups was examined with a ratio of the third-to-first vibrational fine structure (I_3/I_1) in the fluorescence spectrum of pyrene (Figure 1). The I_1 peak arises from the transition that can be enhanced by the distortion of the π-electron cloud. The I_1 peak becomes more notable at the expense of other peaks (I_3) as the microenvironment of pyrene becomes more polar. This I_3/I_1 ratio, therefore, represents the degree of hydrophobic association between methacrylic groups linked to the polymer cross-linkers. I_3/I_1 of the unmodified alginate or PAA solution was independent of polymer concentration. The I_3/I_1 of the unmodified polymer solution was approximately 0.6, which is characteristic value for the pyrene dispersed in water. In contrast, I_3/I_1 of the polymer cross-linker solution increased as the polymer concentration exceeded a

critical concentration termed as the critical aggregation concentration (CAC) (Figure 1B & 1D). The CAC of alginate methacrylates was higher than that of PAA methacrylates by one order of magnitude. In addition, the CAC for PAA methacrylates decreased with increasing N_{MA} while that for alginate methacrylates was independent of the N_{MA}.

Earlier studies reported that the persistence length of alginate is ten times longer than that of PAA for polymer molecules with similar molecular weights of 100,000 g/mol.[19-20] The alginate molecules contain guluronic acid groups, which limit the rotation between the sugar rings. In contrast, PAA consists of ethylene units, allowing for greater rotation (Scheme 1).[29] We suggest that the difference in chain inflexibility between polymers influences the hydrophobic association between methacrylic groups linked to the polymer, according to the pyrene assays shown above. The lower critical aggregation concentration of PAA methacrylates than alginate methacrylates implicates that the inflexible alginate resists the hydrophobic association more strongly than the flexible PAA. We also propose that the hydrophobically associated methacrylic group do not participate in the chemical cross-linking reaction to form a hydrogel, according to previous studies performed using fluorescence resonance energy transfer (FRET).[30] Therefore, the effective number of methacrylic groups available to chemical cross-linking reaction is larger with alginate methacrylates than PAA methacrylates, at the same overall N_{MA}.

3.2. Analysis of degree of swelling and elastic modulus of the hydrogel

Polyacrylamide hydrogels were prepared via *in situ* radical polymerization of acrylamide monomers and cross-linking reaction with either alginate methacrylates or PAA methacrylates. The polyacrylamide did not form a hydrogel without the alginate methacrylates or PAA methacrylates in the pre-gel solution. The mixture of polyacrylamide and unmodified alginate or PAA did not form the hydrogel. Furthermore, in the absence of polyacrylamide in the solution,

alginate methacrylates and PAA methacrylates did not form a hydrogel within the polymer concentration range employed in this study.

The stiffness of polyacrylamide hydrogel was evaluated by measuring elastic modulus (E) of the hydrogel. Increasing the molar ratio between the alginate methacrylates and acrylamide ($M_{X\text{-}linker}$) from 0.00017 to 0.00043 led to the linear increase of E of the hydrogel (Figure 2A and Table 1). Note that the concentrations of polymer cross-linkers in the pre-gel solution were higher than CAC, as characterized in Figure 1. Specifically, at N_{MA} of 140, the inverse dependence of Q and $M_{X\text{-}linker}$ for hydrogels cross-linked by PAA methacrylates was two-fold larger than that for hydrogels cross-linked by alginate methacrylates (Table 1). Therefore, E of the hydrogel cross-linked by alginate methacrylates could be tuned from 27 to 100 kPa. In contrast, increasing $M_{X\text{-}linker}$ with PAA methacrylates resulted in the limited increase of E from 9 to 40 kPa.

Increasing N_{MA} of PAA methacrylates significantly decreased the degree of swelling (Q) of the hydrogel from 110 to 40, coupled with the increase of $M_{X\text{-}linker}$ (Figure 2B and Table 1). In contrast, Q of the hydrogel cross-linked by alginate methacrylates was slightly decreased from 45 to 20 with increasing $M_{X\text{-}linker}$. Specifically, at a given N_{MA}, the inverse dependence of Q and $M_{X\text{-}linker}$ for hydrogels cross-linked by PAA methacrylates was two-fold larger than that for hydrogels cross-linked by alginate methacrylates (Table 1). Accordingly, at given N_{MA}, the number of elastic cross-links ($N_{X\text{-}link}$), calculated using Equation (2), was linearly increased with $M_{X\text{-}linker}$. The linear dependency was more significantly larger with use of the alginate methacrylates as the cross-linker (Figure 2C and Table 1).

Correlating hydrogel elastic modulus to swelling ratio showed a decrease of Q with increasing E, following a power law, but the inverse dependence was smaller with the use of alginate methacrylates as a cross-linker, independent of N_{MA} (Figure 2D). The Q of the hydrogel cross-

linked by PAA methacrylates rapidly decreased from 110 to 40, while limiting the increase of *E* from 9 to 40 kPa. In contrast, the hydrogel cross-linked by alginate methacrylates showed the limited decrease of *Q* from 45 to 20, while *E* being increased from 27 to 100 kPa.

The difference in the inverse relationship between degree of swelling and elastic modulus is likely related to the difference of the number of hydrophobically associated domains in each polymer. Specifically, the larger inverse dependency between *Q* and *E* of the hydrogel cross-linked by flexible PAA methacrylates indicates that the hydrophobically associated methacrylic groups influence *Q* more significantly than *E*. Conversely, the inflexible alginate methacrylates with the smaller number of hydrophobically associated domains could increase the number of elastic networks proportional to its polymer concentration. The smaller decrease of *Q* with increasing *E* of the hydrogel cross-linked by alginate methacrylates is also attributed to the multiple hydroxyl groups of saccharide units, similar to glycosaminoglycan molecules that facilitate water transport in a natural extracellular matrix.[23]

3.3. Tailoring dependencies between degree of swelling and elastic modulus of the hydrogels with ionic strength

The effect of hydrophobic association between polymers on hydrogel properties became more significant by changing the ionic strength of the hydrogel incubation media. In this study, hydrogels cross-linked by alginate methacrylates or PAA methacrylates were incubated in aqueous media with varied ionic strength ([I]) from 0 to 170 mM. Increasing [I] of the incubation media led to the decrease of *E* of the hydrogel cross-linked by alginate methacrylates, specifically at $M_{X\text{-}linker}$ of 0.00034 and 0.00043 (Figure 3A and Figure S4A). In addition, the inverse dependency between *E* and [I], quantified by fitting it to an exponential decay, increased

with increasing $M_{X\text{-linker}}$ (Table 2A). In contrast, hydrogels cross-linked by PAA methacrylates displayed insignificant dependency of *E* on [I] of the incubation media (Figure 3B, Figure S4B, Table 2B).

The [I] of the incubation media also effected hydrogel swelling, but the effect was mediated by the type of polymer cross-linker. The *Q* of the hydrogel was inversely dependent of [I], as expected. However, at a given $M_{X\text{-linker}}$, the dependency between *Q* and [I], quantified by fitting it to an exponential decay, was smaller with the hydrogel cross-linked by alginate methacrylates than that cross-linked by PAA methacrylates (Figure 4, Figure S5, and Table 2).

We interpret that increasing ionic strength of the hydrogel incubation media decrease the electrical double layer thickness of the carboxylic acid units of PAA methacrylates, and the subsequent decrease of the intra- and intermolecular electrostatic repulsion leads to the stronger association between the methacrylic groups of PAA methacrylates. The resulting compact hydrophobic domains likely limited the water entry into the hydrogel, thus resulting in a more significant decrease of *Q* with increasing ionic strength. Accordingly, the smaller dependency of *Q* on the ionic strength for the hydrogel cross-linked by alginate methacrylate is attributed to the smaller number of hydrophobic domains in the hydrogel. In addition, the larger inverse dependency of *E* of the hydrogel cross-linked by alginate methacrylates on the ionic strength implicates that electrostatic repulsion between the carboxylate groups of alginate chains contributes to the elastic response of the hydrogel, concerted with the number of cross-links.[31] Similarly, the minimal dependency of *E* on [I] for the hydrogel cross-linked by PAA methacrylates is related to weak electrostatic interaction between carboxylic groups of PAA. This effect of intermolecular repulsion on the hydrogel stiffness, however, needs to be further investigated in future studies.

3.4. Analysis of cell adhesion to fibronectin-micropatterned hydrogels with controlled rigidity

The dependence of hydrogel properties on cross-linker's inflexibility was further demonstrated by incubating cells on a gel micropatterned with cell adhesion proteins. This *in vitro* platform is often used to understand the interplay of matrix elasticity and spatial organization of cell adhesion ligands in regulating cellular phenotypic activities.[32] In this study, a circular array of fibronectin reacted with acryl PEG-NHS was placed on the polyacrylamide hydrogel cross-linked either by alginate methacrylate or PAA methacrylate (Figure S3). Both diameter and spacing of the circles within which fibronectin molecules were localized were kept constant at 500 μm (Figure 5A). The fibronectin mass per circular pattern was kept constant at 9 ng, as determined with a colorimetric immunoassay (Figure S6). By varying $M_{X\text{-}linker}$ from 0.00017 to 0.00043, the elastic modulus of the hydrogel cross-linked by alginate methacrylate was significantly increased from 27 to 85 kPa, while that of the hydrogel cross-linked by PAA methacrylate was increased only from 9 to 20 kPa, as demonstrated above.

Interestingly, the type of polymer cross-linker influenced the diameter of fibronectin micropatterns on the hydrogels during incubation in cell media. Specifically, circular arrays of fibronectin molecules linked to hydrogels cross-linked by alginate methacrylates exhibited minimal changes in their diameters, independent of its $M_{X\text{-}linker}$ and subsequent elastic modulus (Figure 5B). In contrast, circular array of fibronectin molecules linked to the hydrogel cross-linked by PAA methacrylates, specifically at $M_{X\text{-}linker}$ of 0.00017, displayed two-fold increase of the diameter from 500 to 1,000 μm, due to significant water uptake of the hydrogel (Figure 5B). Accordingly, fibronectin density of the individual circle was significantly decreased as the gel became softer.

Hydrogels cross-linked by alginate methacrylate could regulate cell morphology based on the elastic moduli. Fibroblasts seeded on the hydrogel adhered exclusively on the patterned area of fibronectin. These cells spread more rapidly on the hydrogel prepared at $M_{X\text{-}linker}$ of 0.00043 (Figure 5D). In addition, the average projection area of cells adhered to the hydrogel prepared at $M_{X\text{-}linker}$ of 0.00043 (i.e., elastic modulus of 85 kPa) was 1.4 times greater than that of cells adhered to the gel prepared at $M_{X\text{-}linker}$ of 0.00017 (i.e., elastic modulus of 27 kPa) (Figure 5C and 5E). We suggest that this increased extent of cell spreading should be attributed to the increase of an elastic modulus of the gel, because there was a minimal change of the fibronectin density in the circular micropattern.

Separately, cells plated on the hydrogel cross-linked by PAA methacrylates displayed insignificant dependence of cell adhesion morphology on $M_{X\text{-}linker}$, despite the changes in elastic modulus (Figure 5F and 5G). Specifically, average projection area of cells plated on the hydrogel prepared at $M_{X\text{-}linker}$ of 0.00017 (i.e., elastic modulus of 9 kPa) was comparable to those plated on the gel at $M_{X\text{-}linker}$ of 0.00043 (i.e., elastic modulus of 20 kPa) (Figure 5C). Additionally, the circular area where the cells adhered was approximately four times larger with $M_{X\text{-}linker}$ of 0.00017, due to the larger water uptake of the hydrogel. According to previous studies, such fibronectin dilution significantly impacts the dependency of cellular projection area on elastic modulus of the gel. Therefore, we attribute this independency of cellular projection area on elastic modulus of the gel to the lower and smaller elastic modulus range controlled with flexible PAA methacrylates.[33]

These interesting roles of chain flexibility of polymer cross-linkers on tuning hydrogel properties and cellular adhesion may be further developed by changing other cross-linker variables, such as the molecular weight of polymeric cross-linkers. For example, decreasing the

molecular weight of the polymer cross-linker will further alter the persistence length of polymer chains and decrease the total chain flexibility.[34] Separately, chemical conjugation of polar groups onto the polymer cross-linker may counterbalance the hydrophobic association between the flexible cross-linkers and facilitate the intermolecular chemical cross-linker.[35] Conversely, chemical conjugation of hydrophobic groups such as methylene groups, may further amplify the hydrophobic association between the polymers.[36-37]

4. CONCLUSIONS

In conclusion, this study demonstrates an advanced strategy to modulate the inverse dependency between the degree of swelling and the elastic modulus of the polyacrylamide hydrogel using polymer cross-linkers with varied chain inflexibility. The inflexible alginate methacrylate cross-linker allowed the control of elastic modulus over a broad range while limiting the change of the degree of swelling, driven by limited hydrophobic association. In contrast, the flexible PAA methacrylates cross-linker enabled the control of the degree of swelling of the hydrogel over a broad range while limiting change of the elastic modulus, because of their active hydrophobic association. Therefore, hydrogels cross-linked by inflexible alginate methacrylates were advantageous to study and also modulate effects of gel rigidity on cellular adhesion, as demonstrated with hydrogels micropatterned with the circular array of fibronectin. Overall, this study will allow one to move one step forward towards the advanced control of complex hydrogel properties and significant improvement of the hydrogel function.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. + These authors contributed to the work equally (co-first authors).

Notes

The authors declare no competing financial interest.

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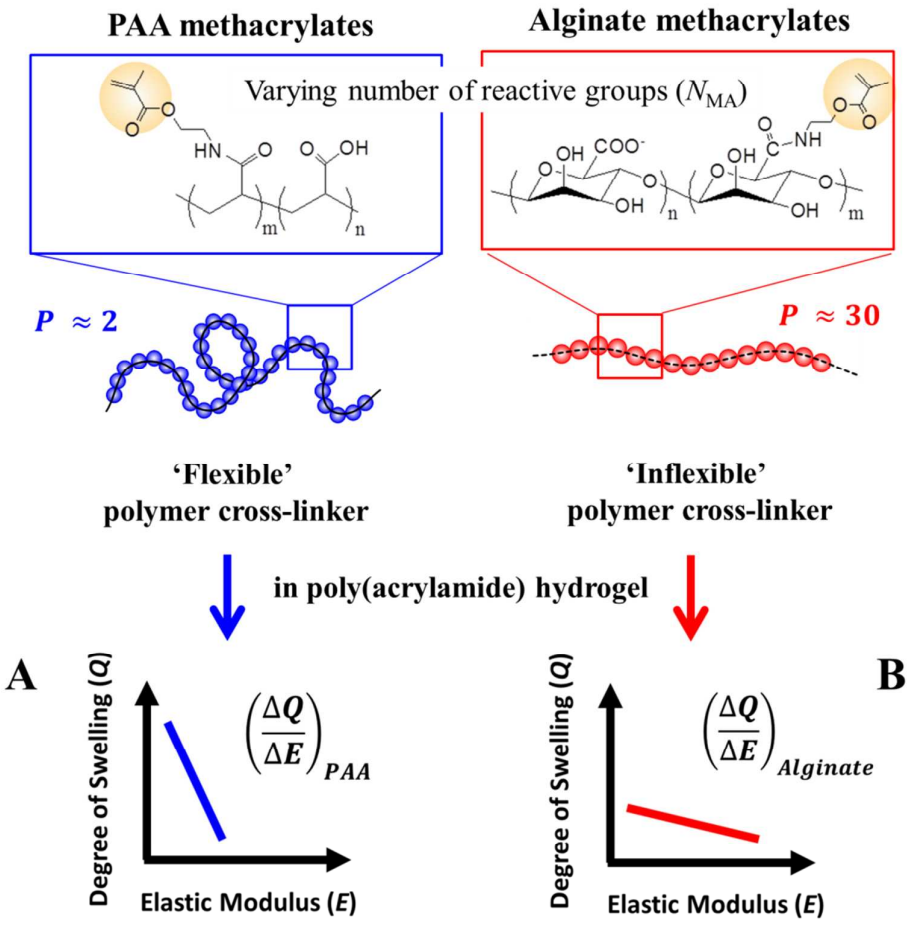
Main Figures



Scheme 1. Schematic diagram of regulating the inverse dependency between the degree of swelling and the elastic modulus of the polyacrylamide hydrogel using polymer cross-linkers with varied chain inflexibility, coupled with the number of reactive methacrylic groups. Alginate and PAA present different persistence lengths (P) of 32 and 2 nm, respectively. Alginate and PAA can be modified with controlled number of methacrylic groups per polymer chain (N_{MA}). (A) The flexible PAA methacrylates cross-linker enables the control of the degree of swelling of the hydrogel over a broader range. (B) In contrast, the inflexible alginate methacrylate cross-linker allows the control of elastic modulus over a broader range.

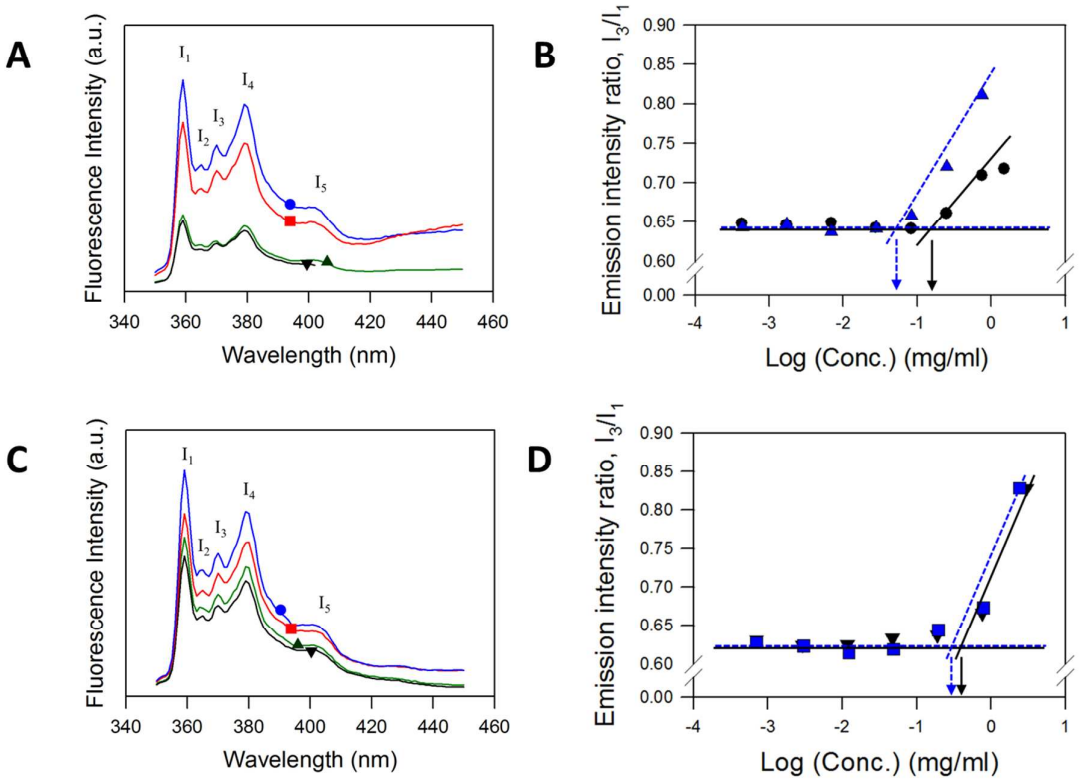


Figure 1. Fluorescent analysis of pyrenes in the pre-gel solutions. (A) Fluorescence emission spectra of pyrenes suspended in PAA-methacrylates. Polymer concentration was increased from 0.125 (▼) to 0.5 (▲), 2.0 (■) and 4.0 mg/mL (●). Number of methacrylic groups linked to a single polymer (N_{MA}) to PAA was kept constant at 70. (B) Dependency of I_3/I_1, the ratio between emission intensity at 370 nm and that at 360 nm, on the concentration of PAA methacrylates with N_{MA} at 70 (●) and 140 (▲). (C) Fluorescence emission spectra of pyrenes suspended in alginate methacrylate with N_{MA} at 70. The polymer concentration was increased from 0.0007 (▼) to 0.012 (▲), 0.19 (■), and 3.00 mg/mL (●). (D) Dependency of I_3/I_1 on the concentration of alginate methacrylates with N_{MA} at 70 (▼) and 140 (■).

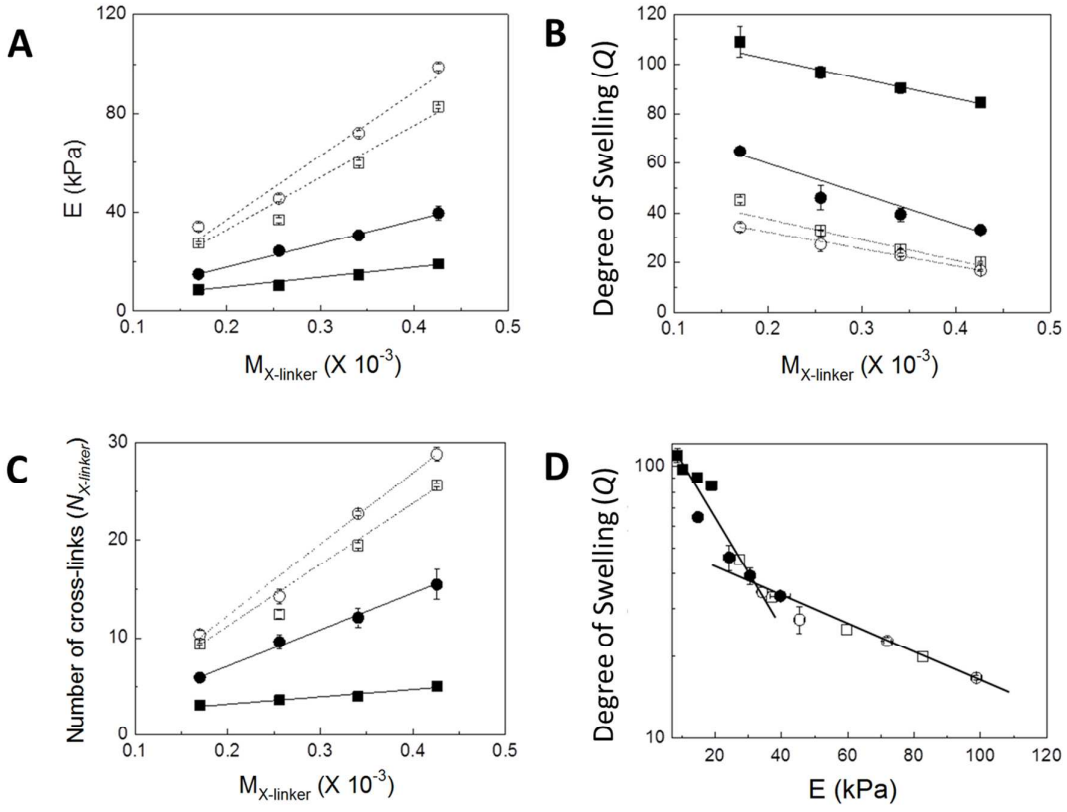


Figure 2. Analysis of the elastic modulus (*E*) and the degree of swelling (*Q*) of the polyacrylamide hydrogel. (A) Dependency of *E* of the hydrogel on the molar ratio between cross-linker and acrylamide ($M_{X\text{-linker}}$). (B) Inverse dependency of *Q* of the hydrogel on $M_{X\text{-linker}}$. (C) Dependency of the number of cross-links ($N_{x\text{-link}}$) on $M_{X\text{-linker}}$. (D) Correlation of *Q* to *E* of the hydrogel. ■ and ● represent hydrogels cross-linked by PAA methacrylates with N_{MA} of 70 and 140, respectively. □ and ○ represent hydrogels cross-linked by alginate methacrylates with N_{MA} of 70 and 140, respectively. Data points and error bars represent average of four different values and standard deviation, respectively. The gels were incubated in deionized water for 24 hours before characterization.

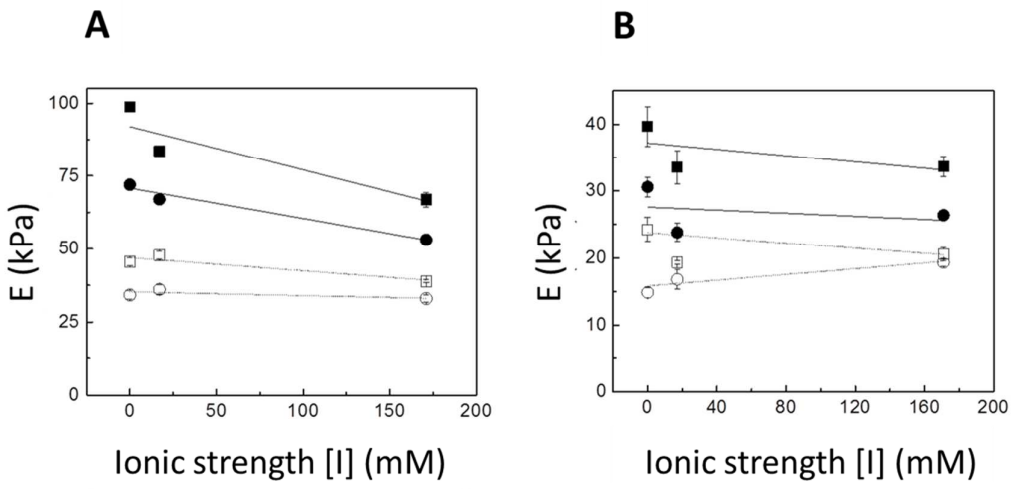


Figure 3. Analysis of effects of ionic strength ([I]) and $M_{X\text{-}linker}$ on elastic modulus (*E*) of the polyacrylamide hydrogel. (A) Dependency of *E* of the hydrogel cross-linked by alginate methacrylates on [I]. (B) Dependency of *E* of the hydrogel cross-linked by PAA methacrylates on [I]. In these experiments, hydrogels were prepared at varied $M_{X\text{-}linker}$ of 0.00017 (○), 0.00026 (□), 0.00034 (●), and 0.00043 (■) for each. N_{MA} to both alginate and PAA were kept constant at 140. Data points and error bars represent average of four different values and standard deviation, respectively.

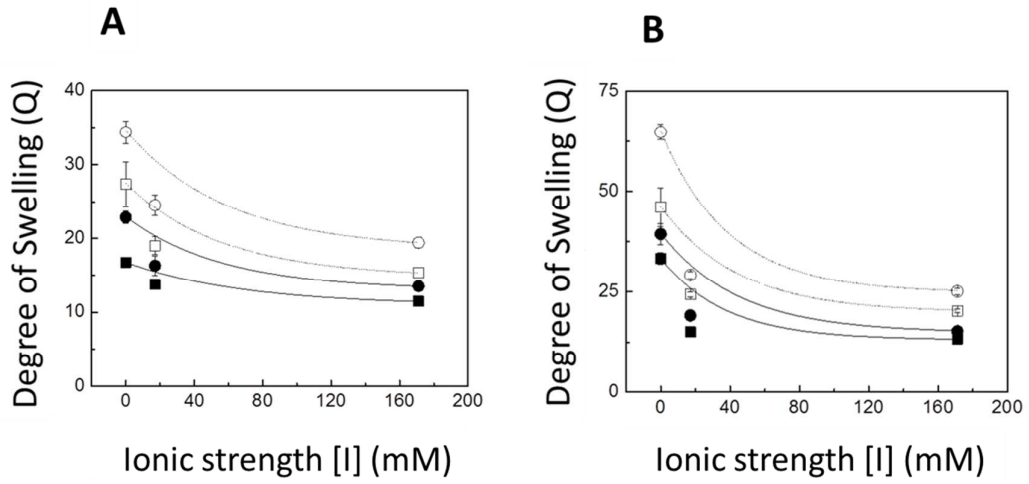


Figure 4. Analysis of effects of ionic strength ([I]) and $M_{X\text{-}linker}$ on the degree of swelling (Q) of the polyacrylamide hydrogel. (A) Inverse dependency of Q of the hydrogel cross-linked by alginate methacrylates on [I]. (B) Inverse dependency of Q of the hydrogel cross-linked by PAA methacrylates on [I]. In these experiments, hydrogels were prepared at varied $M_{X\text{-}linker}$ of 0.00017 (○), 0.00026 (□), 0.00034 (●), and 0.00043 (■) for each. N_{MA} for alginate and PAA were kept constant at 140. Data points and error bars represent average of four different values and standard deviation, respectively.

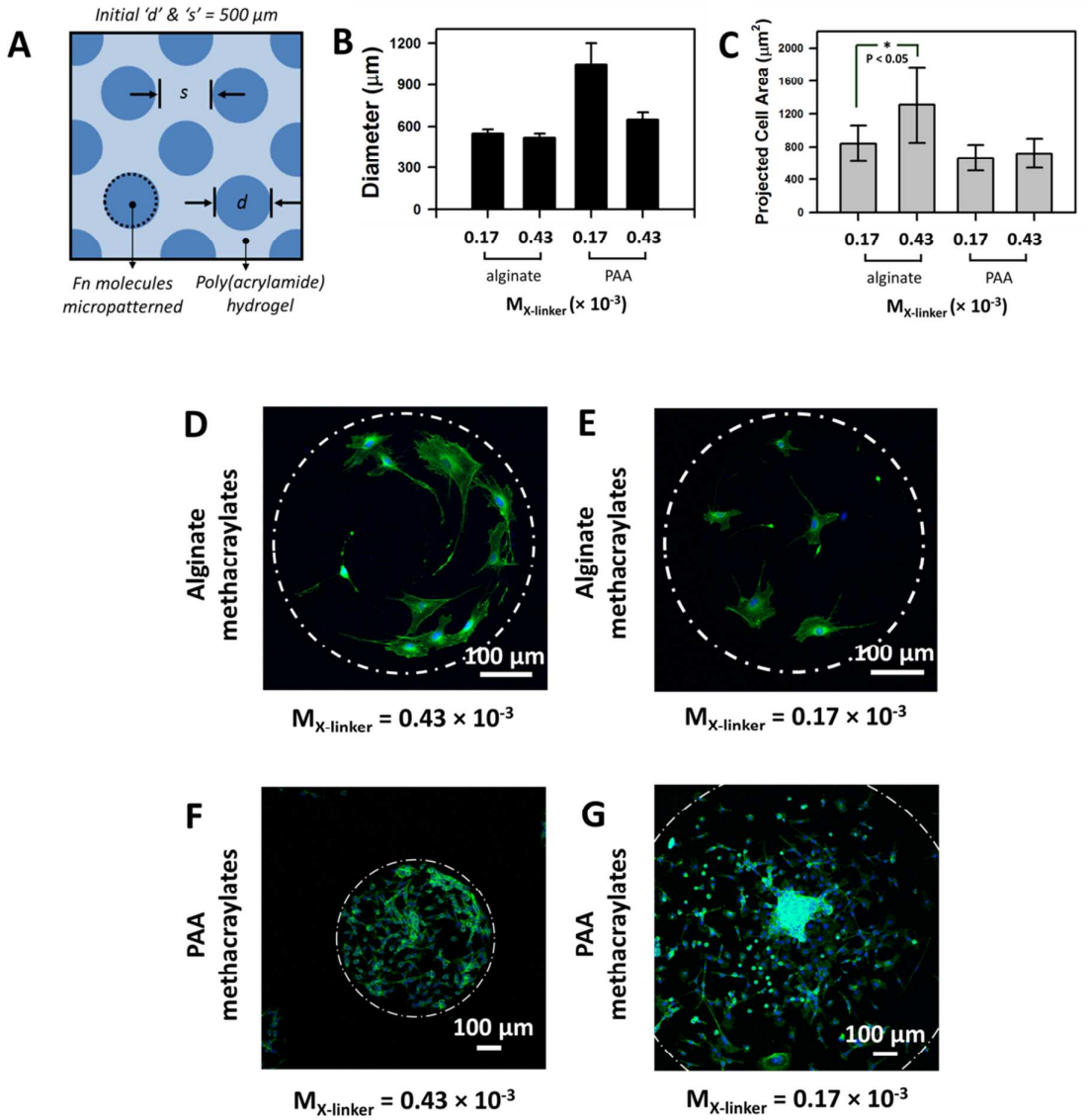

A
Initial 'd' & 's' = 500 µm
s
d
Fn molecules micropatterned
Poly(acrylamide) hydrogel
B
Diameter (µm)
1200
900
600
300
0
0.17 0.43 0.17 0.43
alginate
PAA
M X-linker (× 10-3)
C
Projected Cell Area (µm2)
2000
1600
1200
800
400
0
* P < 0.05
0.17 0.43 0.17 0.43
alginate
PAA
M X-linker (× 10-3)
D
Alginate methacraylates
100 µm
M X-linker = 0.43 × 10-3
E
Alginate methacraylates
100 µm
M X-linker = 0.17 × 10-3
F
PAA methacraylates
100 µm
M X-linker = 0.43 × 10-3
G
PAA methacraylates
100 µm
M X-linker = 0.17 × 10-3

Figure 5. Analysis of the cell adhesion on polyacrylamide hydrogels micropatterned with fibronectin. (A) Schematic depicting initial circular array of fibronectin (Fn) on a hydrogel. Diameter (*d*) and spacing (*s*) of circular fibronectin islands were kept constant at 500 μm. (B) Average diameter of the circular fibronectin islands on the hydrogels incubated in DMEM over 24 hours. (C) Average projection area of cells adhered to the hydrogels at 24 hours after culture. (D & E) Fluorescent images of fibroblasts adhered to the circular fibronectin micropattern on hydrogels cross-linked by alginate methacrylates at $M_{X\text{-}linker}$ of 0.00043 (D) and 0.00017 (E). (F & G) Fluorescence images of fibroblasts adhered to the circular fibronectin micropattern on hydrogels cross-linked by PAA methacrylates at $M_{X\text{-}linker}$ of 0.00043 (F) and 0.00017 (G). N_{MA} to both alginate and PAA were kept constant at 70. In (D) – (G), cell nucleus and intracellular actin were stained with DAPI (blue) and phalloidin (green), respectively. A dot circle in each image represents the boundary of circular fibronectin micropattern. Scale bars represent 100 μm. In (B), the value and error bar of the each bar represent the average and standard deviation of diameters of ten different circular micropatterns per condition.

Tables

Table 1. Dependencies of elastic modulus (*E*), degree of swelling (*Q*), and number of cross-links (*N*) of the polyacrylamide hydrogels on $M_{x\text{-}linker}$. These dependencies were tuned with polymer cross-linker of varying chain inflexibility and N_{MA}.

Cross-linker Type	DS_{MA} (mol %)	Number of methacrylates per polymer chain (N_{MA})	Dependency between *E* and $M_{X\text{-}linker}$ ($\times 10^{-3}$)	Dependency between *Q* and $M_{X\text{-}linker}$ ($\times 10^{-3}$)	Dependency between *N* and $M_{X\text{-}linker}$ ($\times 10^{-3}$)
PAA methacrylates	5	70	41.9	-95.4	7.6
	10	140	96.7	-121.0	37.4
Alginate Methacrylates	12.5	70	220.8	-83.6	63.0
	25.0	140	258.0	-67.2	73.5

Table 2. Dependencies of elastic modulus (*E*) and degree of swelling (*Q*) of the polyacrylamide hydrogels on ionic strength [I] of the hydrogel incubation media. (A) Hydrogels cross-linked by alginate methacrylates with N_{MA} at 140. (B) Hydrogels cross-linked by PAA methacrylates with N_{MA} at 140.

(A)

Cross-linker Type	$M_{X\text{-}linker}$ ($\times 10^{-3}$)	N_{MA}	Dependency between *E* and [I]	Dependency between *Q* and [I]
Alginate methacrylates	0.17	140	-12.3	15.0
	0.26	140	-46.1	12.1
	0.34	140	-101.9	9.4
	0.43	140	-156.1	5.3

(B)

Cross-linker Type	$M_{X\text{-}linker}$ ($\times 10^{-3}$)	N_{MA}	Dependency between *E* and [I]	Dependency between *Q* and [I]
PAA methacrylates	0.17	140	22.4	39.8
	0.26	140	-9.8	25.9
	0.34	140	-8.4	24.2
	0.43	140	-20.9	20.0

TABLE OF CONTENTS GRAPHIC (TOC)

Tailoring the Dependency between Rigidity and Water Uptake of a Microfabricated Hydrogel with the Conformational Rigidity of a Polymer Cross-linker

John J. Schmidt, Jae Hyun Jeong, Vincent Chan, Chaenyung Cha, Kwanghyun Baek, Mei-Hsiu Lai, , Rashid Bashir, Hyunjoon Kong

"In this study, we present an advanced strategy to modulate the inverse dependency between the degree of swelling and the elastic modulus of the polyacrylamide hydrogel using polymer cross-linkers with varied chain inflexibility, alginate methacrylates and poly(acrylic acid) (PAA) methacrylates ."