Supporting Information for

Non-Invasive Probing of the Spatial Organization of Polymer Chains in Hydrogels Using Fluorescence Resonance Energy Transfer (FRET)

by Hyun Joon Kong[1,2], Chan Joong Kim[1], Nathaniel Huebsch[1,3], David Weitz[1], David J Mooney[* 1]

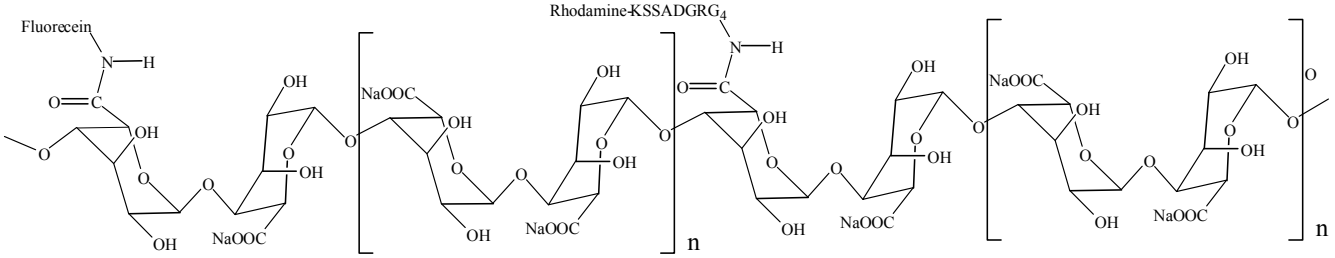
$[Ca^{2+}]$/[Uronic acid]	E (kPa)	S
0.15	23 ± 3	32
0.4	60 ± 4	26
0.6	110 ± 7	24

Supplemental Table 1. Elastic modulus (E) and swelling ratio (S) of the gel varied with molar ratio of calcium to the uronic acids of alginate molecule $[Ca^{2+}]$/[Uronic acid].

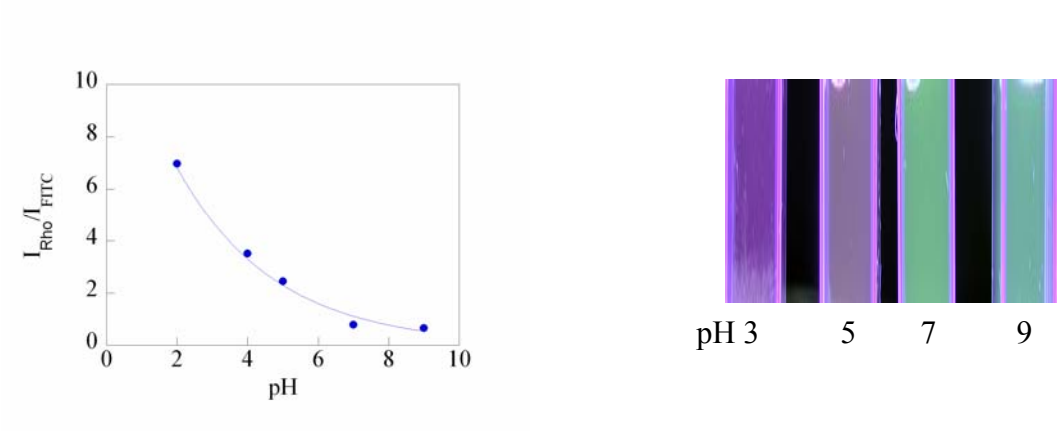
*

pH	Q	$J(\lambda)$ $(M^{-1} nm^4 cm^{-1})$	R_0 (nm)
9.0	0.978± 0.12	$3.046 \times 10^{15} \pm 7.31 \times 10^{12}$	5.58 ±0.11
7.0	0.889 ±0.07	$3.018 \times 10^{15} \pm 1.38 \times 10^{12}$	5.49 ±0.07
5.0	0.585 ±0.02	$2.751 \times 10^{15} \pm 1.06 \times 10^{13}$	5.04 ±0.03
3.0	0.147 ±0.01	$2.22 \times 10^{15} \pm 9.14 \times 10^{12}$	3.55 ±0.04

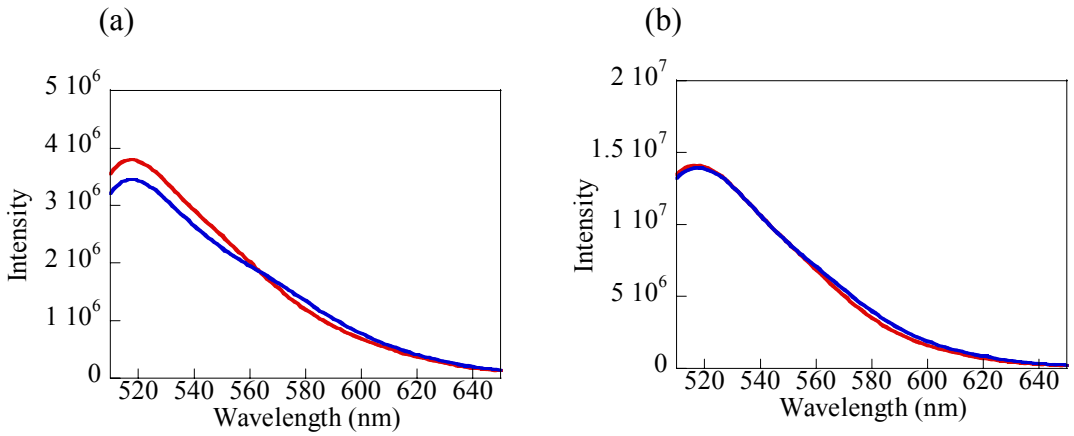
Supplemental Table 2. Dependency of the quantum yield (Q), overlap integral (J), and the critical Forster radius (R_0) of fluorescein-rhodamine pair on pH



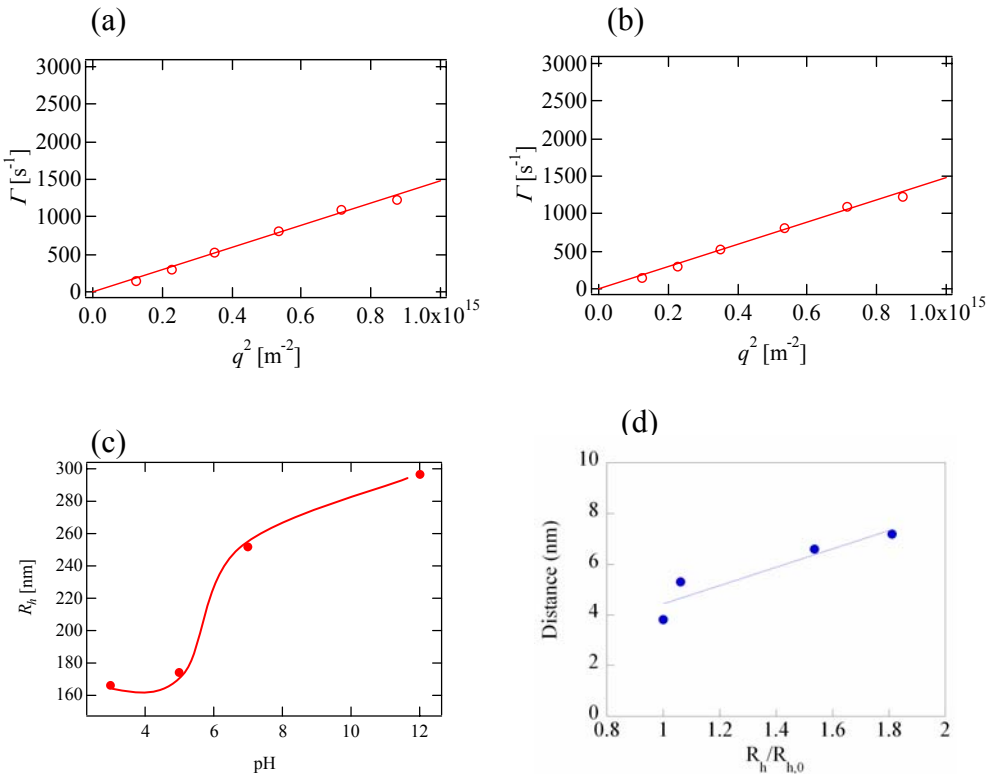
Supplemental Fig. 1. Alginate molecules were covalently linked with both fluorescein and rhodamine-G4RGDASSK-oligopeptides to measure intrachain FRET.



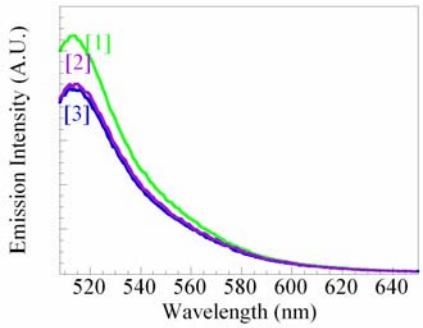
Supplemental Fig. 2. The emission intensity ratio between rhodamine and fluorescein (I_{Rho}/I_{FITC}) (a) and the fluorescent color of the solution under UV light (b) were highly dependent on pH



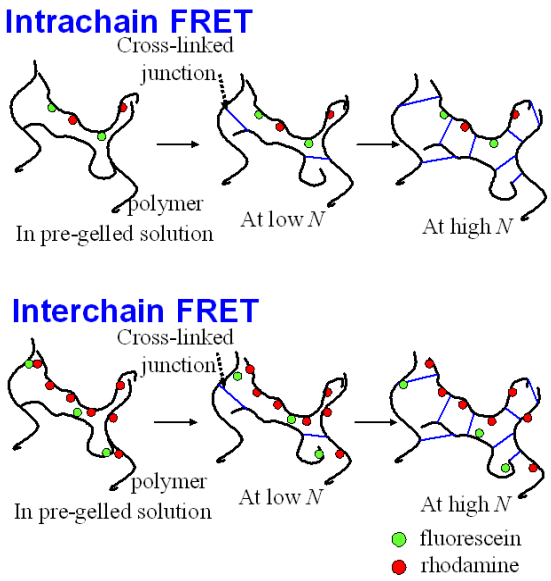
Supplemental Fig. 3. FRET measurement to analyze the interaction between alginate molecules separately labeled with fluorescein and rhodamine in the dilute solution regime. The volume fraction of polymer was kept constant at 0.001. At pH 5 (a) and pH 9 (b), no significant change in the emission intensity of fluorescein was noted from the solutions. In both (a) and (b), red curves are the emission curves of FITC-alginate solution and blue curves are the emission curves of mixture of FITC-alginate and Rho-alginate. Volume fractions of polymer in the solution were kept constant at 0.001 and the volume ratio between FITC-alginate and Rho-alginate was 1:1. The mixtures were excited at a wavelength of 488 nm**.**



Supplemental Fig. 4. Measurement of the hydrodynamic radius (R_h) of alginate molecules using a dynamic laser scattering technique, as pH was varied. The diffusion coefficient (*D*) which corresponds to the slope of the curve of decay rates (Γ) versus the magnitude of scattering waver vectors (q2) was changed as pH was increased from 5 (a) to 9 (b). R_h calculated from *D* was subsequently dependent on the pH of the alginate solution (c). Intrachain distance between fluorescein and rhodamine was also inversely related to the hydrodynamic radius (R_h) of the polymer chains normalized to the value of R_h at pH 3 [$R_{h,0}$] (d).



Supplemental Fig. 5. Intrachain FRET was analyzed as the number of cross-links (*N*) of hydrogels was varied from 1 x 10^{20} (curve 2) to 6 x 10^{20} cm^{-3} (curve 3), and thus led to minimal changes in the emission intensity of fluorescein. Curve 1 is the emission of a hydrogel containing alginates labeled solely with fluorescein. The ratio between fluorescent alginate and unlabeled alginate in the gels was kept constant at a ratio of 1:20, at a total volume fraction of polymer of 0.02.



Supplemental Fig. 6. Gelling and an increase of *N* does not alter the average conformation of polymer chains, but does modulate the number of close contacts between fluorescently labeled segments of different chains, likely due to reduced mobility of uncross-linked segments and a parallel increase in porosity of the gels. For the intramolecular FRET measurement, two fluorescein and two rhodamine molecules were coupled to a single polymer chain. These fluorescently labeled polymer chains were diluted with non-labeled polymer chains. For the intermolecular FRET measurement, two fluorescein molecules were coupled to one group of polymer chain and five rhodamine molecules were coupled to the other group of polymer chains. Each black curve indicates a single polymer chain and the blue line indicates the cross-linking junction.

Experimental

Fluorescent labeling of alginate molecules

Alginate molecules rich in guluronic acid blocks (LF 20/40, FMC polymers) were labeled with fluorescent molecules (FITC-alginate). First, alginate molecules dissolved in 2-(*N*-morpholino)ethanesulfonic acid (MES, Sigma) buffer at pH 6.5 were labeled with aminofluorescein hydrochloride (Invitrogen) using aqueous carbodiimide chemistry. N-hydroxysulfosuccinimide (sulfo-NHS, Pierce), 1-ethyl-3-(dimethylaminopropyl) carbodiimide (EDC, Sigma), and fluoresceins were sequentially added to alginate solution. The molar ratio between fluorescein and uronic acids in alginate molecules was 1:600, so two fluorescein molecules were coupled on average to a single alginate chain. The molar ratio of uronic acids/sulfo-NHS/EDC was kept constant at 1/0.16/0.32. The number of fluoresceins coupled to polymers was evaluated with the emission intensity at a wavelength (λ) of 520 nm with a fluorometer (Fluoromax-3, Jobin Yvon) when the solution was excited at λ of 488 nm.

Following the membrane-based dialysis for purification and drying process, FITC-alginate molecules were linked with synthetic oligopeptides containing lysine residues (i.e., G_4RGDASSK, Commonwealth Biotech Inc.). The sulfo-NHS, EDC, and oligopeptides were sequentially added in 1% (w/w) FITC-alginate solution reconstituted with MES buffer (pH 6.5). The molar ratios between oligopeptides and uronic acids were varied from 1:600 to 1:250, so the average number of oligopeptides coupled to a single polymer chain was varied from 2 to 5. The molar ratio of uronic acid/sulfo-NHS/EDC was varied from 1/0.16/0.32 to 1/0.4/0.8 depending on the molar ratio between

oligopeptides and uronic acids. Following the dialysis, drying and reconstitution with $NaHCO_3$ buffer (pH 8.5), rhodamine succinimidyl ester (Invitrogen) was added to the solution to couple to lysine residues in oligopeptides. The molar ratio between oligopeptides and rhodamine was kept constant at 1:1, so the average number of rhodamine coupled to a single polymer chain was varied from 2 to 5. Alginate molecules labeled both with fluorescein and rhodamine (denoted FITC-Rho-Alginate) were again purified with dialysis membrane, lyophilized and finally reconstituted to a 2 % (w/w) alginate solution with minimum essential medium α medium (α MEM, Invitrogen). The number of rhodamines coupled to alginates was also evaluated with the emission intensity at λ of 600 nm with a fluorometer when the solution was excited at λ of 560 nm.

In certain experiments analyzing the interaction between polymer chains, alginate molecules were chemically linked either with fluorescein (FITC-alginate) or rhodamine-oligopeptides (Rho-alginate) following the same carbodiimide chemistry and purification procedures described above.

FRET experiment with fluorescent alginate solution

In experiments analyzing the conformation of polymer chains (Intrachain FRET measurement), 2 % (w/w) FITC-Rho-alginate solutions, in which both two fluorescein molecules and two rhodamine molecules were coupled to a single polymer chain, were diluted with MES or $NaHCO_3$ buffer at a ratio of 1:20. For experiments analyzing the spacing between polymer chains (Interchain FRET measurement), 2 % (w/w) FITC-alginate solutions, in which two FITC molecules were coupled to a single polymer chain, and Rho-alginate solutions, in which two rhodamine molecules were coupled to a single

polymer chain, were mixed at the same volume ratio, and the mixture was further diluted with buffer at a ratio of 1:20. The pHs of MES buffer were varied from 3 to 5, and the pHs of $NaHCO_3$ were varied from 7 to 9. The solution in the quartz curette was excited at λ of 488 nm and the resulting fluorescent emission from 500 nm to 650 nm was collected using a fluorometer. Distance between fluorescein and rhodamine coupled to the same polymer chains was calculated from changes in the emission intensity of fluorescein in the presence (I_{FITC}) and absence of rhodamine ($I_{FITC,\,0}$), and critical Förster radius (R_0) using Eq. [1].

$$\frac{I_{FITC,0} - I_{FITC}}{I_{FITC,0}} = \frac{R_0^6}{R_0^6 + r^6}$$

$$r = R_0\left[\frac{I_{FITC,0}}{I_{FITC}} - 1\right]^{1/6} \qquad [1]$$

The emission intensity of fluorescein in the absence of rhodamine ($I_{FITC,\,0}$) and R_0 were measured at varied pHs.

R_0 for the carboxyfluorescein-tetramethylrhodamine pair used in this study were determined experimentally through direct measurement of the quantum yield and overlap integral. Quantum yields (Q) were determined using a standard fluorescein (Sigma) in 0.1M NaOH at pH 13. For quantum yield measurements, emission spectra at excitation of 488 nm and absorption at 488nm were measured using a Fluorometer (Fluoromax-3, Jobin Ivon) and Spectrophotometer (DU530, Beckman Coulter). To obtain accurate absorption measurements, a concentrated solution with an optical density (OD) between 0.005-0.02 was prepared initially and fluorescence measurements were prepared on dilutions of this solution. Quantum yields of the fluorescently labeled alginate samples

were determined from the slope of the curve of optical density vs. corrected emission using equation [2].

$$Q = Q_R \frac{I}{I_R} \frac{OD_R}{OD} \frac{n^2}{n_R^2} \quad [2]$$

Where Q_R is the reference quantum yield, I_R and I are integrated intensities (corrected to solvent and spectral dependence of instrument optics) from the standard and sample respectively, *OD* are optical density values calculated in the spectrophotometer at the wavelength used for excitation (488 nm) and *n* of biomacromolecules in aqueous solution is typically assumed to be 1.4. The overlap integral (*J*) was evaluated from excitation spectrum of Rho-alginate and emission spectrum of FITC-alginate at different pH values. The excitation spectra were converted to wavelength-dependent extinction coefficient spectra by multiplying with the extinction coefficient of tetramethylrhodamine, 65,000 cm^{-1} M^{-1} at the absorption peak of 555 nm. *J* was calculated using equation [3].

$$J(\lambda) = \int_0^\infty I_{FITC}(\lambda)\varepsilon_A(\lambda)\lambda^4 d\lambda \quad [3]$$

Where λ is the wavelength (nm), I_{FITC} (λ) the donor emission (normalized so that the area under the emission curve is one) as a function of λ, $\varepsilon_A(\lambda)$ the extinction coefficient of the acceptor. The measured *J* and *Q* were used to estimate Förster radius as a function of pH, assuming n = 1.4 and κ = 2/3 (for randomly oriented dipoles), using equation [4].

$$R = \left(\left(\frac{9000 \ln(10)}{128 \pi^5 N n^4} \right) J(\lambda) Q \kappa^2 \right)^{1/6} \quad [4]$$

Measurement of hydrodynamic radius of alginate molecules

The hydrodynamic radius of alginate chains (R_h) varied with pH was measured using a dynamic laser light scattering system (ALV 5000). Briefly, the diffusion coefficient of alginate chain (D) was calculated from the slope of the decay rates (Γ) versus magnitude of scattering vectors (q^2). Then, R_h was calculated using Stokes-Einstein equation, $D = k_BT/6\pi\eta R_h$, where k_B was Boltzmann's constant, T was the temperature, and η was the viscosity of the solvent.

Preparation and FRET measurement of hydrogels

Fluorescently labeled alginate molecules were mixed with a calcium sulfate slurry to prepare gels. For the intrachain FRET measurements, 2 % (w/w) FITC-Rho-alginate solutions, in which both two fluorescein molecules and two rhodamine molecules were coupled to a single polymer chain, were mixed with 2 % (w/w) unmodified alginate solutions at a volume ratio of 1:9. The total volume fraction of polymer was kept constant at 0.02. In parallel, FITC-alginates were diluted with unmodified alginate molecules at the same volume ratio. For the interchain FRET measurements, 2 % (w/w) FITC-alginate solutions, in which two FITC molecules were coupled per chain to one population of polymers, and Rho-alginate solutions, in which five rhodamine molecules were coupled per chain to another population of polymers, were mixed at the same volume ratio. The molar ratio between calcium and uronic acids was varied from 0.15 to 0.60 to modulate the number of cross-links. Mixtures of alginate solutions and calcium were injected onto the glass plates with a spacer of 1 mm and after 20 minutes of curing, gel disks with

diameter of 10 mm were punched out. Gel disks were incubated at 37 °C in the α MEM until the measurement.

The fluorescent emission from 500 to 650 nm after an excitation at 488 nm was collected with a fluorometer. Degree of energy transfer (D_{FRET}) was calculated using Eq. [5]

$$D_{FRET} = 1 - \frac{I_{FITC}}{I_{FITC,0}} \quad [5]$$

Quantification of the number of cross-links from the elastic moduli and swelling ratio of hydrogels

Elastic moduli of gels were measured by compressing gel disks at a rate of 1 mm/min with a mechanical tester (Instron) at 25 °C. Elastic moduli were calculated from the slope of a linear curve relating the stress to the first 10 % of strain (υ). Assuming alginate gels follow an affined network model, the shear modulus (G) was calculated from the slope of stress versus $-(\upsilon-\upsilon^{-2})$ curve. Degree of swelling (S) of the gels, defined as the reciprocal of the volume fractions of polymer in the hydrogels (v_2), were calculated from weights of incubated gels and dried solids, as follows;

$$S = v_2^{-1} = \rho_p \left[\frac{S_m}{\rho_s} + \frac{1}{\rho_p} \right]$$

where ρ_p was the polymer density (0.8755 g cm^{-3}), ρ_s was the density of water, and S_m was the swelling ratio, defined as the mass ratio of absorbed water to the dried gel. The

number of cross-links (*N*) within the gels was calculated from G and S based on the rubber elasticity theory

$$N = \frac{GS^{1/3}}{RT}$$

where R was the gas constant (8.314 J mol^{-1} K^{-1}), and T was the temperature at which the modulus was measured.