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

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Hydrogels are increasingly used in a variety of biomedical applications[1−3] owing to their many advantageous features. Physical properties of hydrogels formed from cross-linking between polymer chains are regulated with the cross-linking density, type of cross-linking molecules, and chemistry and molecular weight of polymer chains. It is believed that the spatial conformation and organization of polymer chains in gels are altered by these variables,[4] but this hypothesis has not been examined with gels formed with chemical cross-linking, because of the lack of analytical tools that allow the organization of the polymer chains to be quantified. Several scattering and microscopic techniques[5] have been used to analyze the nano- and microstructure of gels, but these tools do not analyze the spatial intra- and intermolecular arrangements of the single polymer chains in the gel. This study demonstrates a fluorescent resonance energy transfer (FRET)-based technique[5,6] which allows one to noninvasively monitor the conformation of gel-forming polymer chains and evaluate the intermolecular association of polymer chains in the cross-linked network. In this study, the effects of gelling and the number of cross-links in the hydrogel on the spatial organization of polymer chains were specifically analyzed using alginate hydrogels formed with a varied number of ionic cross-links between polymer chains. Single alginate molecules were labeled with both fluorescein (FITC, donor) and rhodamine (Rho, acceptor) to examine the conformational changes within polymer chains both in solution and in a hydrogel (intrachain FRET) (Figure 1 in Supporting Information). Alternatively, fluorescein and rhodamine were coupled to separate alginate molecules in order to evaluate the intermolecular association of polymer chains in the gels (interchain FRET). The number of fluorophores coupled to a single polymer was varied from one to five, and the size of the single polymer chain and association between polymer chains were minimally changed over this range, as confirmed with the gel permeation chromatography and rheological measurements. The results of these studies suggest that gelling processes and the number of cross-links significantly alter the intermolecular association of polymer chains while leading to minimal change in the intramolecular conformation. This study thus provides better understandings of the hydrogel structure on the molecular scale.

In the first study, the ability of the FRET technique to monitor the conformational changes of polymer chains was examined using polymers labeled with both fluorescein and rhodamine (denoted FITC-Rho-alginate). The conformation of the polymer chains in solution was first altered with the pH of the solution, because alginate, which consists of uronic acids, is known to change its hydrodynamic radius in response to pH changes. Polymer solutions were prepared in the dilute regime, at which physical contacts between polymer chains were minimized. Interestingly, at pH 5, the emission intensity of fluorescein (I_{FITC}) in the FITC-Rho-alginate, maximized at a wavelength (λ) of 520 nm, was greatly reduced, as compared with the emission intensity of alginate molecules labeled solely with fluorescein and analyzed under the same condition (Figure 1a). The emission intensity of the coupled rhodamine (I_{Rho}), maximized at λ of 580 nm, was greatly increased, indicating significant energy transfer at this condition. Increasing the pH to 9 led to a smaller decrease of I_{FITC} and a smaller increase of I_{Rho} as compared to pH 5 (Figure 1a). The distance between fluorophores (r) was calculated from the emission intensity of the donor in the absence of the acceptor ($I_{FITC,0}$), the presence of the acceptor (I_{FITC}), and the critical Förster radius (R_0) using the equation $r = R_0[I_{FITC,0}/I_{FITC} - 1]^{-1/6}$. $I_{FITC,0}$ and R_0 were measured in parallel with I_{FITC} at varied pH (SI, Table 2).[6] Interestingly, the distance between fluorophores was linearly related to the pH of the solution (Figure 1b). The ratio between I_{Rho} and I_{FITC} was also linearly related to changes of pH (SI, Figure 2a). The variation of D_{FRET} with pH could also be visually demonstrated by a change of fluorescence when solutions were illuminated with an ultraviolet light (SI, Figure 2b).

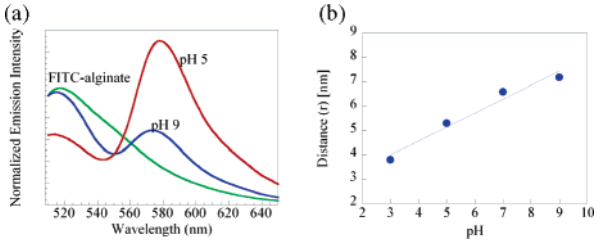

Figure 1. FRET experiment to analyze the conformation of polymers in dilute polymer solutions: (a) emission spectrum from alginate solution with pH varied from 9 to 5. (b) relation of distance between FITC and Rho coupled to a single polymer chain to pH.

Control experiments were also performed in parallel to confirm that the FRET signal in these experiments resulted solely from the intramolecular association of FITC and Rho, and not from intermolecular interactions. Equal volumes of polymers labeled with fluorescein (FITC-alginate) and rhodamine (Rho-alginate) were individually mixed while maintaining solutions in the dilute regime and analyzed. No significant change of I_{FITC} and I_{Rho} was detected with changes of pH (SI, Figure 3). These results clearly indicated that the FRET signal was solely related to the conformational change of single polymer chains in this dilute concentration. However, increasing the polymer concentration would eventually lead to interchain energy transfer because of the increase of physical contacts between polymer molecules.

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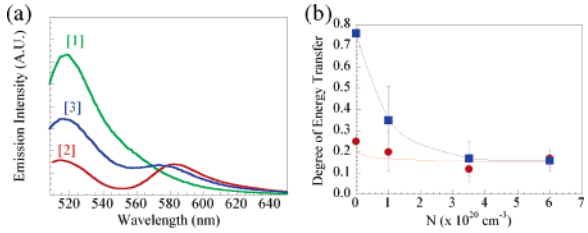

Figure 2. FRET experiments to analyze the spatial organization of polymers in Ca^{2+} cross-linked hydrogels. (a) Alteration of interchain FRET as N was increased from 0 (curve 2) to 1×10^{20} cm^{-3} (curve 3). Curve 1 is the emission of a hydrogel containing alginates labeled solely with fluorescein. (b) Dependency of intrachain D_{FRET} (●) and interchain D_{FRET} (■) upon N.

The hydrodynamic radii of alginate molecules (R_h) was also examined using a dynamic laser light scattering (DLS) system to confirm the FRET results. R_h calculated from the diffusion coefficient of a single polymer chain also linearly increased with pH, as pH was raised above 5 (SI, Figure 4). Measured R_h values were higher than the previously determined radius of gyration of this polymer, likely because R_h is evaluated here by the molecular diffusivity. Interestingly, when R_h values were normalized to R_h at pH 3, denoted as $R_{h,0}$, they could be linearly related to the intrachain distance (r) (SI, Figure 4d).

The relationship between the number of cross-links (N) and conformation of polymer chains in a gel was next examined using FITC-Rho-alginates. Fluorescent polymers were diluted with unmodified polymers at a ratio of 1:20 in order to minimize interchain fluorophore interactions. Increasing the concentration of cross-linking calcium increased the elastic modulus (E) of the gel from 20 to 110 kPa, while slightly decreasing the swelling ratio (S) which is typical with these Ca^{2+} cross-linked gels[7] (SI, Table 1). The mechanism underlying the small dependency of S on E remains to be examined. N calculated from E and S (see SI) was thus varied by almost 1 order of magnitude. Interestingly, the increase of N led to minimal changes of I_{FITC} (SI, Figure 5). In addition, no significant difference in the value of efficiency of energy transfer (D_{FRET}) calculated using the equation $D_{FRET} = 1 - I_{FITC}/I_{FITC,0}$, was noted between FITC-Rho-alginates in solution and those in the gel.

The relevance of N to the distance between polymer chains in the hydrogel was also examined using a mixture of FITC-alginate and Rho-alginate. The concentration of fluorescent polymers in the pregelled solution was in the concentrated regime to allow physical contacts between polymer chains and promote interchain FRET. A high level of D_{FRET} was detected from the pregelled solution (Figure 2a), in contrast to the dilute polymer solution (Figure 1a), as expected. Interestingly, energy transfer decreased as gels were cross-linked and N was raised (Figure 2a). Thus, inter-D_{FRET} was inversely related to N and fitted to a power law $D_{FRET} \propto (N)^{-0.5}$, while intra-$D_{FRET}$ was independent of N. (Figure 2b). This result indicated that increasing N led to a larger average spacing between fluorophores on different polymer chains.

Altogether, the results of this study demonstrate a previously undescribed technique to analyze the intra- and intermolecular organization of polymer chains in both solution and in a hydrogel, in a noninvasive manner. Specifically, the conformation of polymer chains and spacing between polymer chains could be evaluated using polymer chains differentially labeled with a pair of fluorophores. The minimal difference in the value of intrachain D_{FRET} between polymers in solution and in the gel implied that cross-linking junctions along a single polymer chain did not lead to significant conformational changes of the polymer chain with gelling, although some local conformational changes likely occur during cross-linking (SI, Figure 6). The limited conformational change in these gels contrasts with physically cross-linked gels, which form owing to large-scale conformational changes of the polymer chains.[4] In contrast to the studies examining single-chain conformation with intrachain FRET, studies examining interchain FRET suggest polymer chain spacing was altered with gel formation. It is apparent that polymer chains physically interact in the concentrated solution as illustrated with a strong interchain FRET signal. A significant decrease in the FRET signal as N increased infers that cross-linking greatly decreases the mobility of polymer chains, and subsequently inhibits close contact between the polymer segments coupled to fluorophores (SI, Figure 6). It has been previously suggested that increasing N in these gels minimizes the ability of polymer segments to collapse into nanopores in the gels, and the FRET data are consistent with this proposed mechanism, as this would decrease D_{FRET}.[7]

Overall, this study demonstrates a novel method to analyze the spatial organization of polymer chains in gels and its relevance to physical properties of the gels. This FRET-based technique may be broadly used to analyze the structure of gels composed of a wide array of polymers and cross-linking molecules and to understand the structure−property and structure−function relationship. This tool could also be used to monitor structural changes in gels caused by degradation or external stimuli and to understand the diffusion/migration behavior of molecules and cells encapsulated in gel matrices.[9]

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Supporting Information Available: Description of the experimental procedures and additional data. This material is available free of charge via the Internet at http://pubs.acs.org.

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