Nondestructively Probing the Cross-Linking Density of Polymeric Hydrogels

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Introduction

Hydrogel-based materials have been increasingly utilized in various applications due to their unique properties.[1] In parallel, a variety of methods both to analyze the microstructure of the gels and to control their physical properties have been developed.[2] The calculation of cross-linking density by coupling theoretical models of structure with bulk physical property measurements[3−5] and analysis of pore structure with a scattering technique[6] or electron microscopy[7] are good examples. However, most methods to analyze gel microstructure require destructive and irreversible deformations of the materials. This may be specifically problematic when one desires in situ monitoring of gel structure during biodegradation or when undergoing a repetitive external stimulation. Therefore, methods that allow one to nondestructively analyze the microstructure of gels, specifically chemically cross-linked gels, would be widely useful.

We propose to utilize a fluorescent excimer/monomer technique to quantify the changing interactions between fluorescent molecules coupled to polymer chains as a measure of cross-linking density of gels. Although this technique has been frequently used to investigate the microenvironment of hydrophobically associated gels to date,[8−11] we propose to extend this technique to characterize the cross-linking density of chemically cross-linked gels. Calcium cross-linked hydrogels prepared with alginates containing covalently coupled pyrene molecules were utilized as a model system in this study. Alginate molecules consist of three different blocks: guluronic acid (G) blocks containing consecutive G residues, mannuronic acid (M) blocks containing consecutive M residues, and alternating MG blocks containing G and M residues in an alternating mode. Alginate forms gels due to an exclusive cross-linking with divalent cations (e.g., Ca^{2+}) between G blocks. The physical properties of these gels can be modulated over a broad range by altering the calcium concentration and fraction of G blocks within the alginate chains,[12] and there have been extensive studies on the properties and applications of alginate gels.[10,12−14] Pyrenes have been known to produce different emission behavior, classified as an excimer or monomer, depending on their intermolecular arrangements.[8,9] Thus, we relate changes in the interactions between pyrenes conjugated to alginate chains to the changes in cross-linking density and physical properties of alginate hydrogels.

Experimental Section

Alginate having different ratios of guluronic acid (G) and mannuronic acid (M) residues—alginates rich in G blocks (MVG; $M_w \sim 269\,100$ g/mol, fraction of M residues:G residues = 0.50:1) and alginates rich in M blocks (MVM; $M_w \sim 280\,000$ g/mol, fraction of M residues:G residues = 1.31:1)—were received from FMC Technologies. Prior to gel preparation, alginate molecules were purified via a repetitive dialysis of the solutions with membranes (Fisher Scientific) in deionized (DI) water for 3 days, followed by freeze-drying. Dried alginate samples were reconstituted to 2% (w/w) with DI water. To label alginate chains with pyrene molecules, 1% (w/w) alginate solutions were first prepared with a 2-[*N*-morpholino]ethanesulfonic acid hydrate (MES, Sigma) buffer at pH 6.5. Then, *N*-hydroxysulfosuccinimide (sulfo-NHS, Pierce Chemical) and 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC, Sigma) were sequentially mixed with alginate solutions. The molar ratio of sulfo-NHS to EDC was 0.5:1.0, and that of EDC to alginate was 0.05:1.0. 8-Aminopyrene-1,3,6-trisulfonic acid trisodium salt (Molecular Probes) stock solutions (0.02 M) were dropped into alginate solutions at a number ratio between pyrenes and alginate sugar residues of 1:130. Reactions were stopped with hydroxylamine (Aldrich) after 24 h, and the solutions were thoroughly dialyzed with membranes (Membrane Technologies) to remove nonreactants. Following the freeze-drying, dried labeled alginate was reconstituted with DI water.

To characterize the structure of labeled alginate, fractions of G and M residues within labeled alginate and nonlabeled alginate chains were compared with a circular dichroism (CD) spectrometer (AVIV 202). The sample concentration was 0.4 mg/mL, and absorbance of the solutions was scanned at wavelengths from 250 to 190 nm at 25 °C. From the CD spectra, molar ellipticity values were acquired in units of deg cm^2/dmol. The ratio between M and G residues (M/G ratio) was calculated by dividing the height of the peak (M residues at 200 nm) by that of the trough (G residues at 220 nm).

Hydrogels were prepared by mixing 2% (w/w) alginate solutions with slurries containing different masses of calcium sulfate ($CaSO_4$, Sigma), varying from 8 to 20% (w/w). The resulting molar ratios between calcium and alginate sugar residues [Ca^{2+}] were thus varied from 0.15 to 0.60. The mixtures were immediately cast between glass plates with 1 or 2 mm thickness after mixing, and after 2 h the gels were cut into disks (12 mm diameter). The gels were stored at 37 °C in α-MEM (Gibco) for a day before testing.

To measure the elastic moduli of the gels, the gel disks were compressed at a rate of 1 mm/min with a mechanical tester (MTS Bionix 100, MTS systems) at 25 °C. Elastic moduli were calculated from the stress vs strain (ϵ) curves limited to first 10% strain. Assuming alginate hydrogels follow an affined network model, the shear modulus (G) was also obtained from the slope of stress vs $-(\epsilon - \epsilon^{-2})$ plot. The degree of swelling (Q) of the gels, defined as the reciprocal of the volume fraction of a polymer in a hydrogel (ν_2), was calculated from weights of incubated gels and dried solids, as follows:

$$Q = \nu_2^{-1} = \rho_p\left[\frac{Q_m}{\rho_s} + \frac{1}{\rho_p}\right] \quad (1)$$

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

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Table 1. Molecular Structure of Alginates and Viscosity and the Excimer Ratios of 2 % (w/w) Alginate Solutions Both before and after Labeling with Pyrenes

	pyrene: alginate sugar residues	M/G ratio	viscosity (Pa s)	fraction of excimer (I_{ex}/I_{mo})
MVG		0.50 ± 0.06	0.7	
pyrene−MVG	1:130	0.60 ± 0.03	0.6	1.3
MVM		1.31 ± 0.07	0.6	
pyrene−MVM	1:130	1.27 ± 0.04	1.0	2.2

Q, the number of cross-links (N) within the gels was calculated on the basis of the rubber elasticity theory[5]

$$N = \frac{GQ^{1/3}}{RT} \tag{2}$$

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

For fluorescent studies with the gels, hydrogels prepared from the labeled alginate were excited at a wavelength (λ) of 330 nm with a fluorescent spectroscope (Fluoromax) at 25 °C. The excitation and emission slits were kept constant at 5 nm. The responding emission peaks at λ of 375 and 420 nm were regarded to represent the emission of monomers, and the emission peak at λ of 500 nm was regarded to represent the emission of excimers, even when the excimer peak was hardly detectable.[7] The excimer ratio (I_{ex}/I_{mo}) was calculated by dividing the height of the excimer emission peak (I_{ex}) by the heights of monomer emission peaks (I_{mo}: sum of peak heights at 375 and 420 nm). All reported values represent the mean, and error bars indicate the standard deviation ($n = 4$).

Results

Alginate molecules were labeled with pyrenes via a reaction between carboxylic groups within the polymer chains and primary amine groups in the pyrene derivatives. The number ratio between pyrenes and alginate sugar residues (1:130) was chosen to avoid interactions between pyrenes conjugated to a single alginate chain. The pyrene conjugated to alginate insignificantly altered the measured M/G ratio of the polymer chains, as illustrated with CD spectra (Table 1). Pyrenes covalently conjugated to alginate molecules had a small effect on the rheological properties of pregelled solutions. Pyrenes conjugated to MVG molecules made little change in the viscosity of the solutions, while pyrenes linked to MVM molecules slightly raised their viscosity (Table 1).

Pyrenes conjugated to both polymer chains in solution formed a large fraction of excimers—as measured from the complete overlap between two pyrenes, as illustrated with larger emission curves maximized at a wavelength (λ) of 500 nm (I_{ex}) than emission curves maximized at 375 and 420 nm (I_{mo}) (Figure 1). The emission peak at 420 nm, in this study, was regarded to represent the monomers, since it resulted from intermolecular arrangements distorted from complete overlap between pyrenes.[8] Calculating the excimer ratios (I_{ex}/I_{mo}), defined as the ratio between peak excimer emission (I_{ex}) and monomer emission (I_{mo}), revealed that a larger number of excimers were formed within MVM solutions as compared with MVG solutions. The higher fraction of excimers within MVM solutions indicated a stronger interaction between pyrenes conjugated to MVM chains, as compared to their interactions when coupled to MVG chains.

In order for the pyrene probes to be a useful reporter, it should not alter the gel structure. Conjugation of pyrenes to alginate chains did not significantly alter the physical properties of nonlabeled MVG and MVM gels. Raising the calcium concentration [Ca^{2+}] increased the elastic moduli (E) of both MVG and labeled MVG gels, following a power law, $E \propto [Ca^{2+}]^{\alpha}$ (Figure 2). Values of E at individual M and the value of α (~0.86) were not altered by pyrene coupling to the MVG molecules. Raising [Ca^{2+}] also increased E of MVM and labeled MVM gels in a linear manner, within a comparable magnitude. Increasing [Ca^{2+}] also linearly decreased the swelling ratios (Q) of the nonlabeled and labeled gels in a similar manner (Figure 3). Unlike the significant effect of the fraction of G blocks [Φ(G)] on E of the gels, differences in Φ(G) made little change in the Q of the gels. This result inferred that a larger number of intramolecular cross-links, which did not contribute to the elastic response of the gels, were formed within MVM gels as compared with MVG gels. Shear moduli (G) calculated from E were linearly related to Q of the gels on a log scale, irrespective of Φ(G) and conjugation

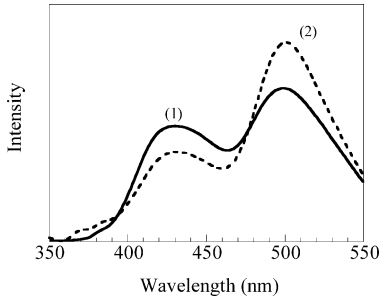

Figure 1. Excitation of solutions of alginate labeled with pyrenes resulted in larger fraction of excimers than monomers. MVM solutions (2) showed a higher fraction of excimer emissions than MVG solutions (1).

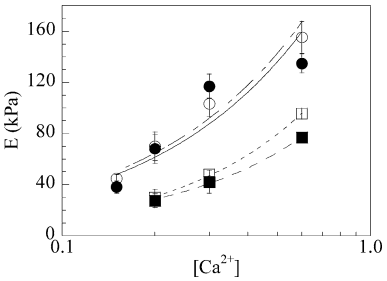

Figure 2. Raising the concentration of calcium [Ca^{2+}] increased the elastic moduli (E) of MVG (●) and labeled MVG (○) gels following a power law. In contrast, increasing [Ca^{2+}] linearly increased E of MVM (■) and labeled MVM (□) gels. [Ca^{2+}] represents the molar ratio between $CaSO_4$ and alginate sugar residues.

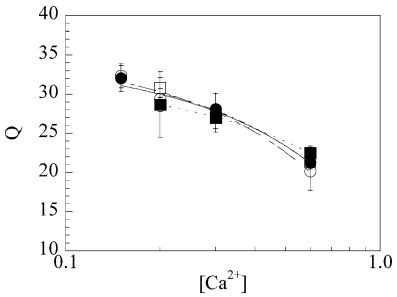

Figure 3. Increasing the concentration of calcium [Ca^{2+}] linearly decreased the swelling ratio (Q) of MVG (●), labeled MVG (○), MVM (■), and labeled MVM (□) gels. [Ca^{2+}] represents the molar ratio between $CaSO_4$ and alginate sugar residues.

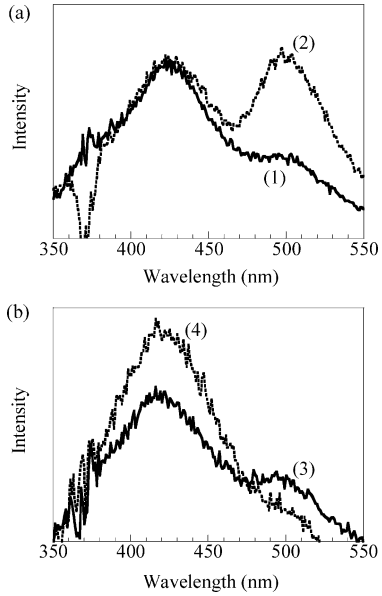

Figure 4. Emission of pyrenes within the ionically cross-linked hydrogels was varied by calcium concentrations [Ca^{2+}] and fraction of G blocks within alginate. (a) MVG gels (1) have much smaller emission of excimers (at a wavelength of 500 nm) than MVM gels (2). (b) Increasing [Ca^{2+}] from 0.24 (3) to 0.60 (4) decreased the emission of excimers within MVG gels, while it increased the emission of monomers. [Ca^{2+}] represents the molar ratio between $CaSO_4$ and alginate sugar residues.

of pyrenes (not shown here). Therefore, both nonlabeled and labeled gels were proposed to follow the Gaussian affined network model. On the basis of this model, the cross-linking density (N) of the alginate gels was calculated from G and Q.[5] This analysis demonstrated that raising [Ca^{2+}] linearly increased N of MVG and MVM gels (not shown here). The dependence of N on [Ca^{2+}] was higher with MVG gels (∼5.1) than MVM gels (∼2.3), also irrespective of conjugation of fluorophores. Together, these results indicated that conjugation of pyrenes did not alter the microstructure of the gels.

Changes in the molecular arrangements within the gels, as varied by calcium concentrations [Ca^{2+}] and fraction of G blocks [Φ(G)], were next investigated by monitoring changes in the interactions between pyrenes conjugated to the alginate molecules. Cross-linking alginate molecules to form the gels significantly reduced I_{ex}/I_{mo}, as compared with the value of I_{ex}/I_{mo} in solutions. Strikingly, the degree of reduction was dependent on [Ca^{2+}] and Φ(G). For example, at a calcium concentration of 0.3 M, I_{ex}/I_{mo} of MVG gels was 0.33 ± 0.09, while I_{ex}/I_{mo} of MVM gels was 1.27 ± 0.38 (Figure 4a). Increasing [Ca^{2+}] from 0.24 to 0.60 M gradually decreased the emission of excimers, while increasing the emission of monomers. In the case of MVG, I_{ex}/I_{mo} of MVG gels was decreased from 0.41 ± 0.07 to 0.26 ± 0.03 over this range of [Ca^{2+}] (Figure 4b).

Finally, the I_{ex}/I_{mo} were related to the number of cross-links of the gels (N), which was varied by altering Φ(G) and [Ca^{2+}]. Interestingly, both MVM and MVG gels showed a reduction in I_{ex}/I_{mo} with increasing N, following a power law, $I_{ex}/I_{mo} \propto N^{\gamma}$ (Figure 5). MVM gels showed the stronger dependence of I_{ex}/I_{mo} on N than MVG gels, as quantified with the larger value of γ (i.e., −1.3 for MVM gels and −0.6 for MVG gels). However, when the values of I_{ex}/I_{mo} of the gels were normalized with I_{ex}/I_{mo} of the solutions, the two curves in Figure 5 were merged into one universal curve. This result

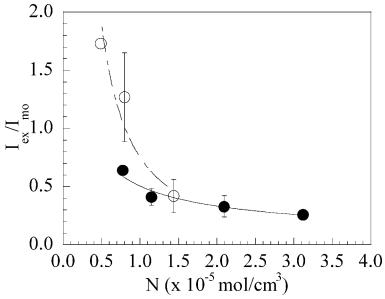

Figure 5. Increasing the number of cross-links (N) of MVG gels (●) and MVM gels (○) led to reductions in the excimer ratio (I_{ex}/I_{mo}), following a power law.

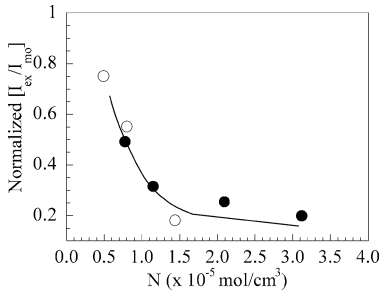

Figure 6. Normalization of the fraction of excimers (I_{ex}/I_{mo}) of hydrogels to I_{ex}/I_{mo} of the corresponding pregelled solution led to a universal curve, described as (normalized I_{ex}/I_{mo}) = $0.38N_0^{-0.8}$. ● represents MVG gels, and ○ represents MVM gels.

indicates that the normalized value of I_{ex}/I_{mo} of the gels could be solely related to the changes in N of the gels following a power law [normalized $I_{ex}/I_{mo} = 0.38N^{-0.8}$] (Figure 6).

Discussion

The results of this study demonstrate that the fluorescent emissions of excimers and monomers within cross-linked hydrogels labeled with pyrene could be utilized as a tool to directly monitor the cross-linking density of the gels. Pregelled alginate solutions [2% (w/w)] exhibited low viscosity and weak shear-thinning behavior, indicating that neither strong physical contacts nor entanglements between polymer chains were present. Therefore, arrangements between polymer chains in the solution likely were driven by hydrophobic interactions between pyrenes, thus leading to the formation of excimers. Although the pyrenes used herein possess sulfonate groups which may cause electrostatic repulsion between the fluorophores, the active excimer formation within the solution indicates that attractions between pyrene moieties dominate the electrostatic effect between sulfonate groups. Overlap between pyrene molecules occurred to an increasing extent with the more flexible MVM chains. G blocks have less flexibility than M blocks; thus, alginates rich in G blocks are regarded to be stiffer than high-M alginate molecules.[15]

The mobility the polymer molecules exhibited in solution was greatly limited when gels were formed via cross-linking between G blocks, as expected. Raising the number of cross-linking G blocks likely decreased intermolecular rearrangements of polymer chains required to form excimers. This possibility is supported by results demonstrating that excimer formation was decreased with increasing calcium concentration and fraction of G blocks. It has been reported that increasing the hydrophobicity of polymer chains that gel via

hydrophobic aggregations led to an increase in the fraction of excimers.[8,10] However, within chemically cross-linked gels such as those used in the present study, cross-linking junctions inhibit physical association between hydrophobic fluorophores by an amount proportional to the number of cross-linking junctions (N). The dependence of I_{ex}/I_{mo} on N decreased with increasing N to 2×10^{-5} mol/cm^3, implying that there may be a critical N at which no further decreases in I_{ex}/I_{mo} can be observed. However, this characterization covers quite a broad range of N from 0 (i.e., pregelled solution) to at least 4×10^{-5} mol/cm^3, which is the maximum N prepared with calcium cross-linked alginate hydrogels. Thus, we propose that the universal curve between normalized excimer fraction and cross-linking density of the gels, described with the power law, indicated that excimer formation within chemically cross-linked gels can be utilized as an index of the cross-linking density of the gels in this range.

This study demonstrated a novel method to nondestructively investigate the cross-linking density of polymeric hydrogels. This method may be very useful to examining the changes of cross-linked structure in situ with time (e.g., gels designed to degrade or gelation). This may be particularly useful when the gels encapsulate sensitive species (e.g., cells), and this method also has the potential to speedily screen out undesirable gels prepared on a larger scale.

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