Degradation Behavior of Covalently Cross-Linked Poly(aldehyde guluronate) Hydrogels

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Received August 3, 1999; Revised Manuscript Received October 26, 1999

ABSTRACT: There is a significant need in the biomedical field for hydrogels with controllable mechanical and degradative properties. We now report the degradation behavior of novel hydrogels formed by the cross-linking of poly(aldehyde guluronate) (PAG). PAG was prepared from alginate by acid hydrolysis and oxidation and was covalently cross-linked with adipic acid dihydrazide (AAD) to form hydrogels. These hydrogels were degradable in aqueous media due to the hydrolysis of hydrazone bonds formed between the aldehyde of PAG and the hydrazide of AAD. We confirmed the degradation behavior of PAG hydrogels cross-linked with different concentrations of AAD by monitoring the changes of dry weight, modulus, and degree of swelling. The degradation behavior generally depends on the cross-linking density as calculated from the Flory−Rehner equation. However, hydrogels with many dangling single-end molecules showed a retarded degradation behavior irrespective of their low initial modulus and low degree of cross-linking density. We hypothesize this is due to re-*cross*-linking of dangling single-end molecules during degradation. The mechanical properties and degradation time of hydrogels typically increase or decrease together as the degree of cross-linking is varied. However, the findings of the current study suggest that the mechanical properties and degradation time can be decoupled by utilizing partially bound cross-linking molecules that are capable of reversibly cross-linking the polymer to form the hydrogel. This approach to control the properties of hydrogels may find wide utility in the biomedical field and other applications of these materials.

Introduction

Hydrogels have been widely used in many biomedical applications including contact lenses, wound dressings, artificial organs, and delivery carriers for the bioactive reagents due to their high degree of biocompatibility.[1−3] Their biocompatibility is likely related to their high water content and low interfacial tension with the surrounding biological environment.[4,5] One of the most recent applications of hydrogels is as delivery vehicles of cells for tissue engineering approaches.[6−8] The aim of this approach is the reconstruction of tissues and organs using three-dimensionally designed synthetic matrices which mimic the function of the extracellular matrix, and this offers an alternative to the patient who needs new tissues or organs.[9,10] Hydrogels may be potent materials for soft tissue engineering applications due to their similarity to the highly hydrated macromolecular-based materials in the body.[11] Critical properties of hydrogels utilized in these applications include their degradation time and mechanical properties. One typically desires to time the rate of hydrogel degradation to the rate of new tissue formation,[12] and this time may vary significantly for different tissues. The mechanical properties of these materials are critical to their ability to create and maintain a space for new tissue formation in vivo,[12] and the mechanical properties of the materials to which cells adhere can also regulate the gene expression of the cells.[13]

Alginate is a widely used material to form hydrogels for various biomedical applications[14,15] due to its biocompatibility and low toxicity.[16] Alginate forms hydrogels via ionic cross-linking with divalent cations. However, the molecular weight of alginate typically used in these applications is higher than the limit of renal clearance in our body, and ionically cross-linked alginate hydrogels have uncontrollable mechanical properties and disintegration behavior. Therefore, new hydrogels composed of low molecular weight polymers derived from alginate were recently synthesized in our lab which may be useful for cell transplantation and drug delivery applications.[17,18] In brief, polyguluronate, composed of β-D-guluronate residues, was isolated from alginate by acid hydrolysis, oxidized, and covalently cross-linked with adipic acid dihydrazide to form hydrogels with controllable mechanical strength and drug release behavior. The reaction between the aldehyde and the hydrazide results in the formation of hydrazone bonds that are labile to hydrolysis. Therefore, it was considered that this hydrazone bond enables PAG hydrogels to biodegrade.

In this paper, we report the degradation behavior of poly(aldehyde guluronate) hydrogels covalently cross-linked with adipic acid dihydrazide. We monitored the weight loss and the change of shear modulus of the hydrogels as a function of degradation time. This degradation behavior was also confirmed by FT−IR. The loss of mechanical stiffness during degradation was interpreted in terms of changes in cross-linking density according to the Flory−Rehner equation.

Experimental Section

Materials. Sodium alginate with high guluronate content (Protanal LF 20/60) was purchased from Pronova (Drammen, Norway). Sodium periodate and adipic acid dihydrazide were purchased from Aldrich (Milwaukee, WI) and used without

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10.1021/ma991286z CCC: $19.00
Published on Web 12/18/1999

further purification. Dulbecco's modified Eagle's medium (DMEM) was purchased from Life Technologies (Grand Island, NY). All the chemical reagents were analytical grade and used as received.

Synthesis of Poly(aldehyde guluronate) (PAG). PAG was prepared according to a previously reported method.[17,19] In brief, polyguluronate (PG) was isolated from sodium alginate by acid hydrolysis and collected at pH 2.85. For further purification, the precipitate was dissolved in double-distilled water at neutral pH and activated carbon added. The solution was stirred thoroughly, filtered to remove the activated carbon, precipitated by ethanol, and lyophilized. PG was oxidized with 0.25 M sodium periodate solution at room temperature. The ratio between guluronate unit and periodate was 1:1. An equimolar amount of ethylene glycol was added after 19 h to stop the oxidation. The resultant solution was filtered and precipitated by ethanol. The precipitate was collected, redissolved in double-distilled water, and dialyzed (MWCO 1000, Spectra/Por) for 3 days. The solution was then concentrated under reduced pressure and lyophilized.

Aldehyde Assay. The degree of oxidation (%) was defined as the number of oxidized guluronate residues per 100 guluronate units, and determined by measuring the number of aldehyde groups in PAG. An excess amount of *tert*-butyl carbazate was added to the PAG solution, and the amount of unreacted *tert*-butyl carbazates was measured by the addition of trinitrobenzenesulfonic acid (TNBS) solution. The colored complex of *tert*-butyl carbazates and TNBS was quantified spectrophotometrically at 334 nm.[17]

Preparation of PAG Hydrogels. A 20 wt % solution of PAG was mixed with different amounts of adipic acid dihydrazide (AAD), placed into 48-well tissue culture plates, and incubated at room temperature for 4 h to form hydrogels. All the solutions were prepared in DMEM, and the pH was adjusted to 7.4 before mixing. The final concentration of PAG in the hydrogel was fixed at 6 wt %, and the AAD concentration varied from 50 to 250 mM. Covalent cross-linking between PAG and AAD was confirmed by FT−IR. All the hydrogels were immersed in DMEM (pH 7.4) at 37 °C for 24 h to reach complete hydration before testing.

Cross-Linking Efficiency. The TNBS was utilized to determine the extent of effective cross-linking (double-end), pendent groups during the cross-linking reaction (dangling single-end), and unreacted cross-linking molecules.[17] Briefly, hydrogels were synthesized and lyophilized, and this was followed by treatment with a TNBS solution (5.76 mM) for 1 h. The mixture was filtered through a 0.22 μm filter, and the filtrate was used for this assay. The unreacted AAD in the hydrogel reacts with 2 equiv of TNBS and forms a soluble complex.[20] The complex solution was diluted with 0.5 N HCl, and its yield was spectrophotometrically determined at 334 nm. Dangling single-end AAD molecules form a complex with 1 equiv of TNBS, and in this case the complex remains bound to the hydrogel and will not be measured in the previous solution. Therefore, an excess amount of AAD was subsequently added to the filtrate to react with remaining TNBS. The amount of complex bound to the hydrogel was calculated by subtracting the amount of soluble complex from the total amount of TNBS initially added to the hydrogel. Subtracting the amount of unreacted and single-end AAD molecules from the total amount of AAD used yields the cross-linking efficiency.

Mechanical Measurements. The shear modulus (G) of PAG hydrogels was obtained from a compression test. The elastic modulus in compression of PAG hydrogels was measured by an MTS Bionix 100 mechanical tester (MTS Systems, France). The deformation rate was 0.5 mm/min, and the diameter of the indentor was 3.15 mm. Under the assumption of an affine network model, the shear modulus (G) can be obtained from a slope of σ vs $-(\lambda - \lambda^{-2})$ plot, where σ is the stress and λ is the ratio of the deformed length to the undeformed length of the hydrogel.[21]

Swelling Measurements. The hydrogels were swollen in DMEM (pH 7.4) for 24 h at 37 °C, excess water on the hydrogel was removed, and the gels were weighed. Degree of swelling (Q) was defined as the reciprocal of the volume fraction of the polymer in the hydrogel (ν_2)

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

where ρ_p is the polymer density (0.8755 g/cm^3 for sodium alginate),[22] ρ_s is the density of water (0.9971 g/cm^3 at 25 °C), and Q_m is the swelling ratio, defined as the mass ratio of absorbed water and the dried gel.

Degradation of PAG Hydrogels. PAG hydrogels cross-linked with different amounts of AAD were prepared, immersed in DMEM (pH 7.4), and incubated at 37 °C. During degradation, changes of weight loss, shear modulus, and swelling ratio of PAG hydrogels were measured and normalized to their initial values before degradation. Hydrolytic degradation of hydrazone bonds was also monitored by FT−IR. All of the experiments were done under sterile conditions to prevent bacterial and fungal contamination.

Instruments. Size-exclusion chromatographic (SEC) measurements were performed using a system equipped with a triple detector system including a laser refractometer (LR40, Viscotek), a differential viscometer, and RALLS (T60, Viscotek). A 0.1 M $NaNO_3$ buffer solution (pH 6.3) was used as a mobile phase and the flow rate was 0.7 mL/min. A set of two TSK-gel columns (G4000PW$_{XL}$ and G3000PW$_{XL}$) was used for separation. FT−IR spectra were recorded by an Avatar 360 spectrophotometer (Nicolet, WI) using the KBr pellet method. The resolution was 2 cm^{-1} with a repetition of 32 scans. Spectrophotometric measurements were done using a Perkin-Elmer Lambda 12 UV/vis spectrophotometer.

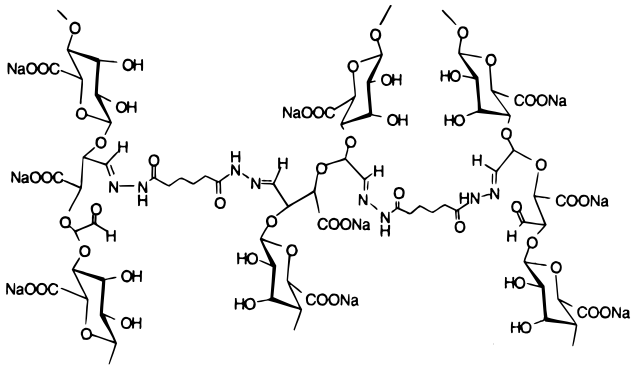

Figure 1. Chemical structure of poly(aldehyde guluronate) hydrogel covalently cross-linked with adipic acid dihydrazide.

Results and Discussion

Synthesis and Characteristics of PAG Hydrogels. Commercially available alginate was hydrolyzed under acidic conditions to break down β-glycosidic linkages between mannuronate and guluronate residues.[17] Polyguluronate (PG) was then isolated at pH 2.85 and the M_w was 7000 (M_w/M_n = 1.60) as determined by SEC. PG was then oxidized with sodium periodate to prepare poly(aldehyde guluronate) (PAG). The M_w of PAG was 5700 (M_w/M_n = 1.64), and its degree of oxidation was determined to be 66.5%. PAG was subsequently cross-linked with adipic acid dihydrazide (AAD), a bifunctional cross-linker, to form hydrogels without the use of any additive or catalyst (Figure 1). Aldehyde groups are much more reactive toward hydrazide groups compared to carboxyl groups and thus react with AAD very rapidly without a catalyst. This coupling reaction was confirmed by disappearance of a symmetric vibrational band of the aldehyde group at 1735 cm^{-1} and appearance of a carbonyl band of the hydrazide at 1658 cm^{-1} from the FT−IR spectra.

PAG hydrogels were formed at different concentration of AAD from 50 to 250 mM. Table 1 lists various

Table 1. Characteristics of PAG Hydrogels Cross-linked with Different Concentration of AAD

	cross-linking efficiency (%)						
AAD (mM)	unreacted	single-end	double-end	G_0 (kPa)	Q_m	Q	$(\nu_e)_0$ ($\times 10^5$ mol/cm^3)
100	4.1	11.9	84.0	6.1 ± 0.5	13.2 ± 0.2	12.6 ± 0.2	16.0 ± 0.5
150	4.2	19.9	75.9	7.5 ± 0.8	12.8 ± 0.4	12.2 ± 0.3	17.1 ± 0.9
200	5.9	35.0	59.1	1.4 ± 0.2	16.0 ± 0.6	15.3 ± 0.6	11.1 ± 0.8

characteristics of PAG hydrogels covalently cross-linked with different amounts of AAD. At 50 mM, AAD very weak hydrogels were formed and disintegrated before they reached complete hydration. The elastic moduli obtained from compression testing of stable hydrogels were converted to shear moduli (G) under an assumption of an affine network model. This model suggests that junctions of the network do not fluctuate and transform uniformly with the deformation. Although real networks show a deformation behavior between affine and phantom network models, the PAG hydrogel was assumed to follow the affine network model as it is generally difficult to determine the precise deformation model of a polymer network.[23−25] As the AAD concentration was raised from 50 mM, the interchain cross-links increased with the resulting formation of stable hydrogels that exhibited an increase in the mechanical stiffness. Interchain cross-links act as effective cross-links and mainly contribute to the modulus of the hydrogels.[26] At 150 mM AAD the number of hydrazide and aldehyde groups are almost equivalent. Above 150 mM AAD, however the efficiency of cross-linking decreases as the presence of excess cross-linking molecules induces intrachain cross-links and/or dangling single-end linkages. It is generally considered that the presence of intrachain loops or dangling single-ends causes a decrease in modulus from its expected value.[17,27] Quantification of the AAD reaction confirmed an increased amount of dangling single-end molecules as the amount of AAD used for hydrogel formation increased (Table 1). The amount of cross-linked AAD decreased in parallel as expected, and the amount of unreacted AAD was similar in all cases. At 250 mM AAD hydrogels were not formed, likely due to the high degree of dangling single-end linkages caused by the large excess of cross-linking molecules.

The cross-linking density (ν_e, mol/cm^3) of the PAG hydrogel was subsequently calculated from the Flory−Rehner equation[28,29]

$$\nu_e = -[\ln(1-\nu_2) + \nu_2 + \chi_1\nu_2^2][V_1(\nu_2^{1/3} - 2\nu_2/f)]^{-1} \quad (2)$$

where χ_1 is the interaction parameter, f is the cross-linking functionality, V_1 is the molar volume of water (18.062 cm^3/mol), and ν_2 is the volume fraction of polymer in the hydrogel when it reaches the equilibrium swelling state. We assumed χ_1 to be 0.35, as this value has been previously used to model a similar interaction,[29] and f to be 4. The higher the cross-linking density, the stronger the mechanical properties and the lower the degree of swelling of PAG hydrogels, as expected (Table 1). This calculation is purely based on the degree of swelling of hydrogels, and a comparison with results from elastic moduli measurements[30] was also made to confirm this finding. Values obtained with both models are quite consistent and demonstrate the same trend over time (data not shown).

Degradation Behavior. It is generally considered that the reaction between aldehydes and hydrazides is very fast and the resulting hydrazone bonds will be labile to hydrolysis (Figure 1). This suggests PAG hydrogels should be degradable in aqueous media by a bulk erosion process. The degradation behavior of PAG hydrogels was monitored initially by the hydrogel dry weight loss as a function of degradation time (Figure 2). The higher the AAD concentration used to form hydrogels, the slower the degradation rate. PAG hydrogels cross-linked with 100 and 150 mM AAD degraded within 2 weeks, but those cross-linked with 200 mM AAD exhibited minimal weight loss even after 6 weeks incubation.

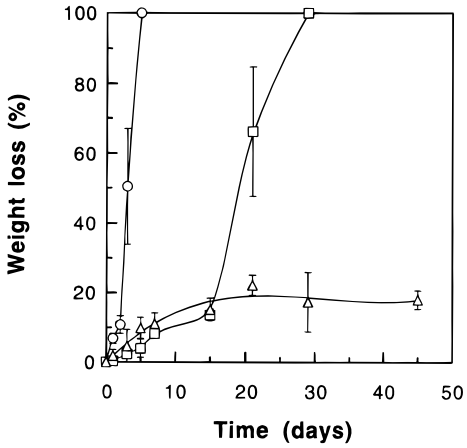

Figure 2. Weight loss of PAG hydrogels cross-linked with 100 (○), 150 (□), and 200 mM (△) AAD. Hydrogels were incubated in DMEM (pH 7.4) at 37 °C.

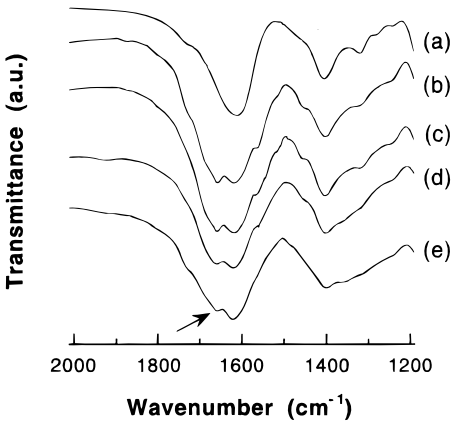

Figure 3. Characteristic infrared absorption bands of (a) PAG, (b) PAG hydrogel, (c) degraded PAG hydrogel after 5 days, (d) degraded PAG hydrogel after 15 days, (e) degraded PAG hydrogel after 29 days. The arrow indicates the carbonyl band for the hydrazide at 1658 cm^{-1}, and PAG was cross-linked with 150 mM AAD.

The degradation of PAG hydrogels can be monitored by FT−IR due to the characteristic absorption band at 1658 cm^{-1} corresponding to the carbonyl group of the hydrazide (Figure 3). The shoulder peak of the symmetric vibrational band for the aldehyde group of PAG is shown at 1735 cm^{-1} (Figure 3a). This peak disappears after covalent cross-linking with AAD, and the carbonyl

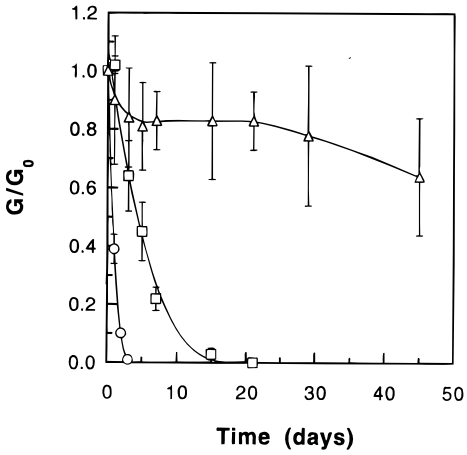

Figure 4. Change of shear modulus of PAG hydrogels cross-linked with 100 (○), 150 (□), and 200 mM (△) AAD. Hydrogels were incubated in DMEM (pH 7.4) at 37 °C.

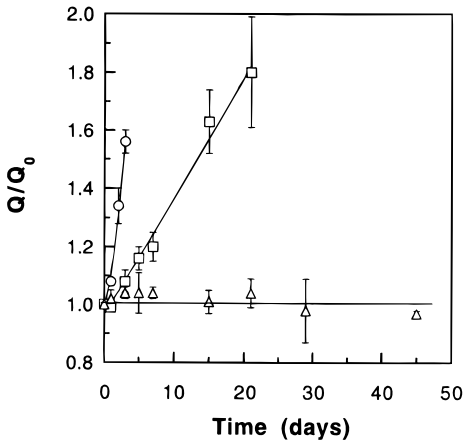

Figure 5. Change of degree of swelling of PAG hydrogels cross-linked with 100 (○), 150 (□), and 200 mM (△) AAD. Hydrogels were incubated in DMEM (pH 7.4) at 37 °C.

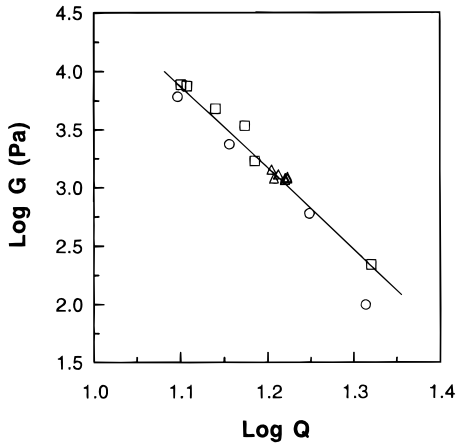

Figure 6. Plot of shear modulus vs degree of swelling of PAG hydrogels cross-linked with 100 (○), 150 (□), and 200 mM (△) AAD after degradation.

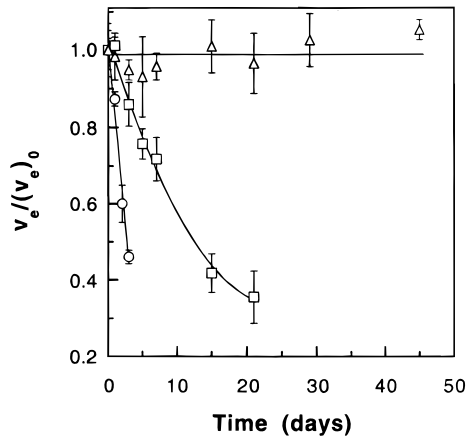

Figure 7. Plot of cross-linking density vs degradation time of PAG hydrogels cross-linked with 100 (○), 150 (□), and 200 mM (△) AAD.

band of the hydrazide appears at 1658 cm^{-1} (Figure 3b). During degradation, this characteristic band becomes weaker and weaker (Figure 3c−e).

As the mechanical properties of the polymer carrier are critical to many tissue engineering approaches, the change in mechanical properties of PAG hydrogels was also followed during degradation (Figure 4). The mechanical strength of PAG hydrogels cross-linked with 100 and 150 mM AAD was lost much faster than the weight. Since PAG has a very low molecular weight (M_w = 5700), the mechanical properties of these hydrogels are dominantly controlled by the cross-linking density. Thus, a small decrease in the cross-linking density may lead to a dramatic loss of mechanical strength before a mass loss is noted. Therefore, the correlation between the loss of mechanical properties and the actual weight loss in biodegradable hydrogels should be considered prior to application of these types of materials. PAG hydrogels cross-linked with 200 mM AAD, in contrast, showed very retarded degradation behavior as shown in Figures 2 and 4, irrespective of their low cross-linking density (Table 1). This very interesting finding will be discussed later.

The changes in the degree of swelling and cross-linking density of PAG hydrogels were determined to more fully characterize the degradation of these hydrogels (Figure 5). PAG hydrogels cross-linked with 100 and 150 mM AAD had an increased extent of swelling with increasing degradation time, but those with 200 mM AAD showed no significant change. The degree of swelling is strongly related to the mechanical properties (i.e., the modulus) and reflects the cross-linking density of the hydrogel.[28] To calculate the cross-linking density of the degraded PAG hydrogels using the Flory−Rehner equation, the Gaussian elasticity of this system must be demonstrated. The modulus is expected to decrease with increasing Q within a certain range of Q for ionic hydrogels. However, at high Q the modulus abruptly increases because of the non-Gaussian elasticity of highly stretched network chains.[31−33] The relation between shear modulus (G) and the degree of swelling (Q) for these hydrogels indicates a linear relationship (Figure 6). Therefore, the cross-linking density of the degraded PAG hydrogels could be calculated on the basis of the Gaussian elasticity network, as shown in Figure 7. These results are quite consistent with those determined for the shear modulus, degree of swelling, and weight loss.

A striking finding of this study is that PAG hydrogels cross-linked with 200 mM AAD showed a retarded degradation behavior, irrespective of their low cross-linking density (Table 1; Figures 2, 4, and 5). These hydrogels contain significant network defects in terms of dangling single-end AAD molecules due to the high concentration of cross-linking molecules, resulting in low moduli. We hypothesize that this large number of single-

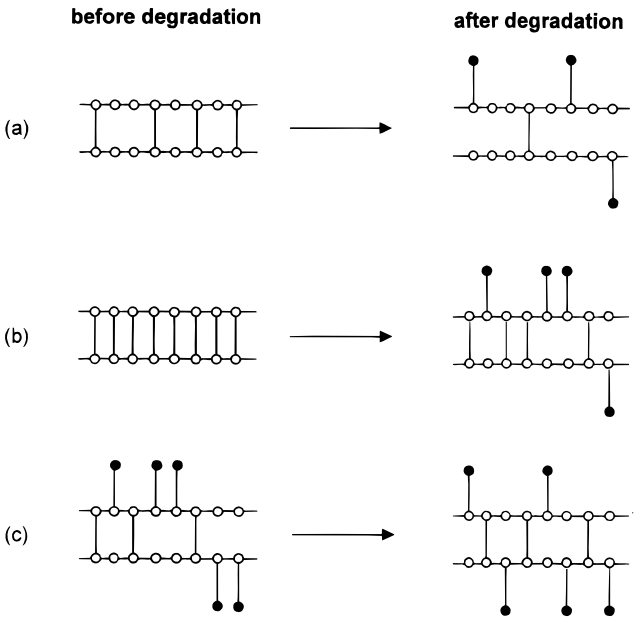

Figure 8. Schematic description of PAG hydrogel degradation: (a) low cross-linking density with few dangling single-end AAD; (b) high cross-linking density with few dangling single-end AAD; (c) low cross-linking density with many dangling single-end AAD. Key: ○, cross-linkable site in PAG; ●, dangling single-end; —, AAD).

end AAD molecules allows re-*cross*-linking of PAG strand following hydrolysis of the initial hydrazone bond (Figure 8c). The degradation behavior of hydrogels with few dangling single-end molecules purely depends on the change of net cross-linking density (parts a and b of Figure 8). However, the hydrogel with many dangling single-end molecules may have no significant change in net cross-linking density during degradation due to re-*cross*-linking of dangling single-end molecules (Figure 8c).

It has been generally considered that the degradation behavior of the hydrogel depends on the cross-linking density. Although a high cross-linking density can retard the degradation of the hydrogel, this also means an increase in the mechanical stiffness of the hydrogel (i.e., the modulus). However, the results of the current study suggest that the degradation and mechanical properties of hydrogels can be decoupled. This approach can potentially be applied to various hydrogel systems with degradable cross-linking sites. This type of controllable degradation, independent of mechanical properties, may have significant advantages for biomedical applications of hydrogels.

Conclusion

We have developed a novel polymer derived from alginate that forms biodegradable hydrogels by covalent cross-linking. We now confirm the degradation of these hydrogels by means of weight loss, decrease of modulus, and increase in the degree of swelling. The degradation time and mechanical properties are typically coupled, but hydrogels with a high content of dangling single-end molecules show a retarded degradation behavior irrespective of its low cross-linking density.

Acknowledgment. The authors would like to thank Reprogenesis and the National Institutes of Health (Grant No. R01 DE13033) for their financial support of this research.

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MA991286Z