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Controlling Mechanical and Swelling Properties of Alginate Hydrogels Independently by Cross-Linker Type and Cross-Linking Density

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Introduction

There have been many demands for hydrogel systems in various biomedical applications.¹ Among them, hydrogels have been widely used for delivery systems of bioactive reagents, including pharmaceutical drugs, proteins, and genes.^{2,3} Hydrogels are generally considered to be biocompatible due to their high water content and low interfacial tension with the surrounding biological environment, as well as their similarity to the highly hydrated macromolecular-based materials in the body.⁴ One of the most recent and exciting applications of hydrogels is as a delivery vehicle of cells for soft tissue engineering approaches.^{5–8} This approach aims to offer an alternative to the patient who is lacking tissue or organ formation.⁹ However, three-dimensional structures that provide structural integrity while new engineered tissues and organs develop are absolutely necessary for this process. It is also critical to control the mechanical properties of the polymer systems in order to create and maintain a space for new tissue formation¹⁰ as well as control the adhesion and gene expression of the cells.¹¹ Therefore, controlling the mechanical properties of hydrogels easily and precisely is critical in these applications.

Alginate is a naturally occurring polysaccharide that has many attractive features for uses as a hydrogel in tissue engineering. It is typically obtained from brown algae and has been used for many biomedical applications due to its biocompatibility and low toxicity as well as ease of hydrogel formation with divalent cations.^{12–14} However, ionically cross-linked alginate hydrogels lose more than 60% of their initial mechanical strength within 15 h of exposure to physiological buffers.¹⁵ This can be attributed to the loss of divalent ions from the hydrogels by ion exchange with monovalent ions in the surrounding fluid. For this reason, covalent cross-linking has been investigated in order to impart controlled mechanical properties to these hydrogels.^{16,17} Alginate hydrogels covalently cross-linked with poly(ethylene glycol) diamines showed controllable mechanical properties depending on the molecular weight and the weight fraction of the PEG. In addition, as the length of the cross-linking molecules approached the

molecular weight between cross-links (M_c), the properties of hydrogels were significantly affected by the nature of the cross-linking molecules, suggesting an interesting feature of two-component hydrogels.¹⁶ In this context, we investigated whether the generality of this relation would extend to alginate hydrogels covalently cross-linked with various molecules of different size and structure, including adipic dihydrazide, lysine, and poly(ethylene glycol)-diamines. This finding may allow one not only to control the mechanical properties of the alginate hydrogels but also to control simultaneously other hydrogel properties such as the hydrophilic/hydrophobic balance.

Experimental Section

Materials. Sodium alginate with a high content of guluronic acid was purchased from Pronova Biopolymers Inc. (Porthsmouth, NH) and used without further purification. Poly(ethylene glycol) (PEG) with number-average molecular weight of 1000 and 3400 was purchased from Lancaster Synthesis Inc. (Windham, NH) and Aldrich (Milwaukee, WI), respectively. PEGs were dried for 24 h over phosphorus pentoxide in a vacuum and purified by azeotropic distillation with toluene. Methyl ester L-lysine (Lys) and morpholine ethanesulfonic acid (MES) were purchased from Sigma (St. Louis, MO). Adipic dihydrazide (AAD), 4-(dimethylamino)pyridine (DMAP), dicyclohexyl carbodiimide (DCC), 1-ethyl-3-(dimethylaminopropyl) carbodiimide (EDC), and *N*-hydroxysuccinimide (NHS) were purchased from Aldrich (Milwaukee, WI) and used as received. All the chemical reagents were analytical grade.

Covalently Cross-Linked Alginate Hydrogels. Sodium alginate was covalently cross-linked with adipic dihydrazide, methyl ester L-lysine, and PEG-diamines. PEG-diamines were synthesized in a two-step reaction utilizing standard carbodiimide chemistry as previously reported.¹⁶ In brief, PEG (2.5 mmol) was reacted with *N*-*tert*-Boc-glycine (5.5 mmol) in the presence of DMAP (1.25 mmol) and DCC (6 mmol), followed by the removal of *tert*-Boc groups using a $\text{CH}_2\text{Cl}_2/\text{TFA}$ (1/1, v/v) solution, and further purified by extraction and reprecipitation. Synthesis of PEG-diamines was confirmed by TLC, ¹H NMR, and FT-IR.¹⁶ PEG-diamines were denoted as P1000 and P3400 depending on the molecular weight of the starting PEG. A 2% (w/w) sodium alginate solution was prepared in a buffer solution of 0.1 M MES and 0.5 M NaCl, and the pH was adjusted to 6. The molar ratio of EDC:NHS:COO[−] was 2:1:2. The solution was agitated to obtain a homogeneous solution followed by the addition of cross-linking molecules. The solution was cast between glass plates separated by a 2 mm spacer. After 24 h the gel was cut into disks with a diameter of 12.7 mm and placed in double-distilled water to remove unreacted materials and low molecular weight byproducts.

Methods. The shear moduli (G) of alginate hydrogels were obtained from a compression test. The stress–strain curve of alginate hydrogels was initially measured by an MTS Bionix 100 mechanical tester (MTS Systems, France) with a deformation rate of 1.0 mm/min. Under the assumption of an affine network model, the shear modulus can be determined 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.¹⁸ The hydrogels were swollen in double-distilled water for 2 days, 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 (v_2):

$$Q = v_2^{-1} = \rho_p[(Q_m/\rho_s) + (1/\rho_p)] \quad (1)$$

where ρ_p is the polymer density (0.8755 g/cm³ for sodium alginate),⁸ ρ_s is the density of water (0.9971 g/cm³ at 25 °C),

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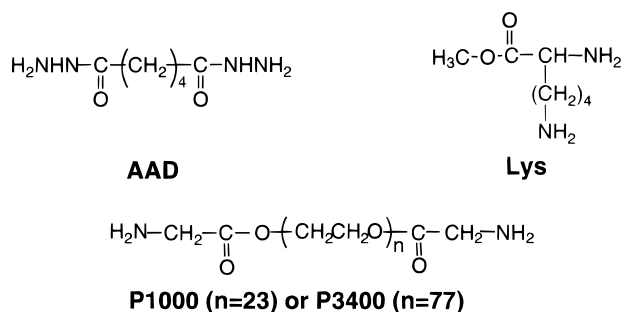


Figure 1. Chemical structure of cross-linking molecules used in this study.

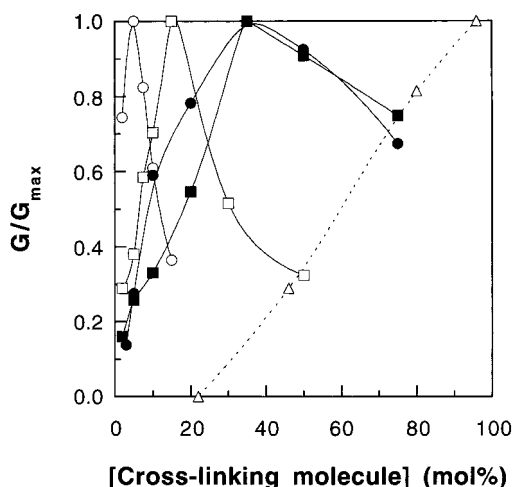


Figure 2. G/G_{\max} vs concentration of cross-linking molecules plot of alginate hydrogels cross-linked with AAD (■), Lys (●), P1000 (□), P3400 (○), and Ca^{2+} (△).

and Q_m is the mass swelling ratio, defined as the mass ratio of absorbed water and the dried gel. The molecular weight between cross-links was calculated from the modulus data according to the rubber elasticity theory using the following equation:^{18,19}

$$M_c = RTc/G \quad (2)$$

where M_c is the molecular weight between cross-links (g/mol) and c is polymer concentration (g/m³).

Results and Discussion

Sodium alginate was ionically cross-linked with Ca^{2+} or covalently cross-linked using EDC chemistry with bifunctional cross-linking molecules of various molecular weights and chemical structures, including adipic dihydrazide (AAD), methyl ester L-lysine (Lys), or PEG-diamines (P1000 and P3400) (Figure 1). All covalent cross-linking reactions were carried out in an MES buffer solution at pH 6.0 as it was considered to be optimum for this coupling reaction.¹⁶ EDC is known to be most reactive at acidic pH and loses its activity as the pH increases above 7.5.²⁰ The reaction with each of the bifunctional cross-linking molecules led to the rapid formation of hydrogels, and hydrogels with widely varying mechanical properties were formed with this method (Figure 2). In the case of alginate hydrogels cross-linked with PEG-diamines, we have previously documented that the majority of cross-linking molecules were covalently bound to alginate with an overall conversion of around 90%.¹⁶ The elastic moduli obtained from compression testing of alginate hydrogels were converted to the shear moduli under an assumption of

Table 1. Characteristics of Alginate Hydrogels Ionically or Covalently Cross-Linked with Various Cross-Linking Molecules

cross-linking molecule	M_w	c_{\max}^a (mol %)	G_{\max}^b (kPa)	Q_{\max}^c	M_c^d (g/mol)
Ca^{2+}	40	96	24.4 ± 0.6^e	n.a. ^f	
AAD	174	35	36.9 ± 3.7	43.3 ± 3.4	527
Lys	233	35	31.1 ± 1.8	78.1 ± 3.5	527
P1000	1000	15	36.5 ± 3.2	78.3 ± 3.0	1320
P3400	3400	5	29.8 ± 2.1	197.7 ± 14.7	3690

^a Concentration of cross-linking molecules at which the hydrogel showed the maximum shear modulus. ^b Maximum shear modulus. ^c Swelling degree of the hydrogel in water at the maximum shear modulus. ^d The theoretical molecular weight between cross-links which was calculated based on the molecular weight of alginate ($M_n = 1.7 \times 10^5$) and the molecular weight of a repeating unit ($M_0 = 198$). ^e The gels were swollen in Dulbecco's Modified Eagle's Medium (DMEM). ^f Not applicable in water.

an affine network model. This model assumes that junctions of the network do not fluctuate and transform uniformly with the deformation. Although real networks generally show a deformation behavior between affine and phantom network models, the alginate hydrogel was assumed to follow the affine network model because it is generally difficult to determine the precise deformation model of a polymer network.^{21,22} As the amount of cross-linking molecules initially increased, the shear modulus of the hydrogel increased in parallel likely due to the increasing number of interchain cross-links (i.e., true cross-links). It is generally considered that interchain cross-links mainly contribute to the mechanical properties of the network.^{23,24} However, above a certain concentration of cross-linking molecules the moduli of the gels began to decrease irrespective of their high conversion. This is likely due to the feature of two-component hydrogels as the nature of the cross-linking molecules affects the properties of hydrogels above a certain amount of cross-linking.¹⁶

A striking finding of this study is that the point (inflection point) at which the mechanical properties decreased with increasing extent of cross-linking strongly depended on the molecular weight of the cross-linking molecule. All of the covalently cross-linked hydrogels showed inflection points, and the value of the inflection point decreased with an increasing molecular weight of cross-linking molecules (Figure 2 and Table 1). In the case of alginate hydrogels cross-linked with AAD and Lys, they showed the inflection points at a similar concentration of cross-linking molecules (35 mol %), likely due to the similarity of the molecular weights of these two cross-linking molecules. However, the maximum shear moduli of hydrogels cross-linked with AAD were slightly higher than those with Lys (Table 1). This might be due to the nonlinear structure and heterofunctional reactivity of amino groups of Lys. The inflection points of hydrogels cross-linked with PEG derivatives also depended on the molecular weight of PEG and were at 15 and 5 mol % for P1000 and P3400, respectively. In contrast, ionically cross-linked hydrogels showed no inflection point within this concentration range of cross-linking molecules as Ca^{2+} can be considered as a zero-length cross-linking molecule. The shear moduli of Ca^{2+} cross-linked hydrogels were lower (24.4 ± 0.6 kPa) than those of covalently cross-linked hydrogels.

The molecular weight between cross-links (M_c) is an important parameter to characterize the structure-property relationship of hydrogels, and it can be calcu-

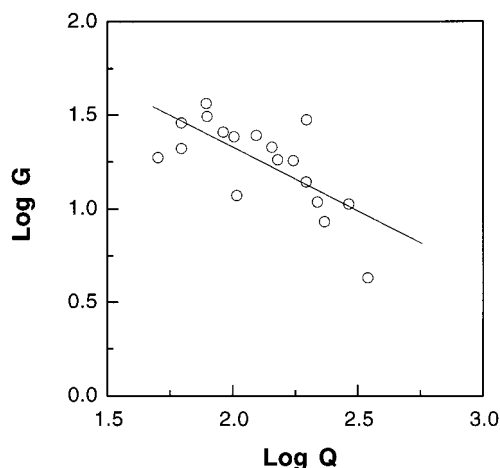


Figure 3. Log G vs log Q plot of alginate hydrogels covalently cross-linked with various cross-linking molecules.

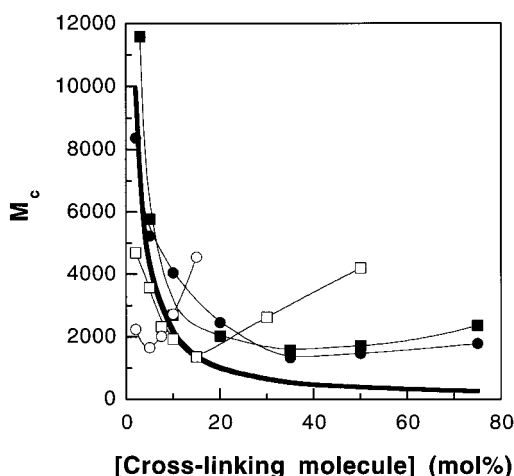


Figure 4. Molecular weight between cross-links (M_c) vs concentration of cross-linking molecules plot of alginate hydrogels covalently cross-linked with AAD (■), Lys (●), P1000 (□), and P3400 (○). The solid line indicates theoretical M_c of alginate hydrogels, which was calculated from the molecular weight of alginate ($M_n = 1.7 \times 10^5$) and the molecular weight of a repeating unit ($M_0 = 198$).

lated from the modulus data. To apply eq 2 to alginate hydrogels, it should be demonstrated that these hydrogels follow the Gaussian statistics model. The shear moduli (G) of networks are expected to decrease with an increase of swelling degree (Q) at low Q because network chains are weakly stretched and follow the Gaussian statistics model. Most hydrogels used in this study followed the Gaussian statistics model (Figure 3), irrespective of cross-linking molecules and cross-linking densities. However, the alginate hydrogel cross-linked with 2 mol % P3400 showed a large amount of water absorption ($Q = 696 \pm 25$), and this hydrogel did not follow the Gaussian statistics model (not shown). M_c of alginate hydrogels cross-linked with P1000 or P3400 decreased as the concentration of cross-linking molecules initially increased, which is in accordance with the rubber elasticity theory. However, M_c subsequently exhibited an increase after it reached a minimum value (Figure 4). This inflection point corresponds to the point where the theoretical M_c becomes close to the molecular weight of the cross-linking molecule (Table 1).¹⁶ The large deviation of the experimental M_c from the theoretical value even at a low concentration of P3400 came from the high degree of swelling, resulting in the non-

Gaussian behavior of networks. M_c of the hydrogels cross-linked with AAD or Lys showed a similar behavior. However, they showed inflection points before they got reached the theoretical values (Table 1). This may be caused by the formation of small loops (i.e., cyclization) due to the low molecular weight of cross-linking molecules which reduce the intermolecular cross-linking efficiency.^{16,25}

While there was a strong dependency of the shear moduli on the concentration of cross-linking molecules (Figure 2), the maximum shear moduli at each inflection point were not significantly affected by the type of cross-linking molecules (Table 1). However, the swelling degrees of hydrogels, in contrast to the shear moduli, were significantly affected by the nature of cross-linking molecules. Hydrogels cross-linked with P3400 had a very high value of swelling degree (198 ± 15) compared to those of other hydrogels in this study (Table 1). This can be attributed to the inherent hydrophilic nature of PEG. The hydrogels cross-linked with AAD demonstrated the least amount of swelling, likely due to the hydrophobic nature of the main chain.

In conclusion, the mechanical properties and swelling degrees of alginate hydrogels can be tightly regulated by using different kinds of cross-linking molecules and controlling the cross-linking densities. The mechanical properties of the hydrogels are mainly controlled by the cross-linking density but are also moderately dependent on the type of cross-linking molecules. The chemistry of cross-linking molecules exerts a significant effect on hydrogel swelling. The introduction of hydrophilic cross-linking molecules as a second macromolecule (e.g., PEG) compensates the loss of hydrophilic character of the hydrogel during the cross-linking reaction between hydrophilic groups in the backbone. This approach to control the properties of hydrogels with various combination of cross-linking densities and kinds of cross-linking molecules may find wide utilities in the biomedical field as well as other applications.

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