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Synthesis of cross-linked poly(aldehyde guluronate) hydrogels

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Abstract

Alginate is an attractive material for controlled drug delivery and cell transplantation applications. However, alginate hydrogels are not degradable, have limited mechanical properties, and lack the functional groups required for cell interaction. To address these limitations of alginate while maintaining their favorable characteristics, we have synthesized new polymers derived from sodium poly(guluronate), the portion of the alginate molecule that is responsible for its gelling behaviour. Sodium poly(guluronate) was isolated, oxidized with sodium periodate, and cross-linked with adipic dihydrazide to yield hydrogels with a wide range of mechanical properties, and with cell adhesion peptides coupled to their backbones. © 1999 Elsevier Science Ltd. All rights reserved.

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1. Introduction

Hydrogels are an important class of biomaterials that have found many pharmaceutical and biomedical applications. Many synthetic and naturally derived materials have been reported to form well-characterized hydrogels. One such material is alginate, a linear polysaccharide isolated from seaweed. Alginate is composed of (1-4)-linked β-D-mannuronic and α-L-guluronic acid units arranged in blocks of poly(mannuronate), poly(guluronate), and alternating units of both sugars. It is well known that the ionic cross-linking of alginate is achieved through the poly(guluronate) portion of the polymer [1]. Alginate has found many biomedical applications including dental impressions, wound dressings [2–4], drug delivery vehicles [5], and cell transplantation matrices [6–9]. Alginate is particularly well suited for the last application due to its gentle gelling behaviour in the presence of divalent cations such as calcium to form hydrogels. Alginate is also considered biocompatible, which is a necessary requirement for biomaterials used for cell transplantation [10,11].

In spite of its many advantageous features, alginate has a number of properties that limit its applicability for drug delivery and tissue engineering applications. The field of tissue engineering addresses the concept of creating natural tissue by transplanting cells onto polymer matrices, where the cells proliferate, synthesize its extracellular matrices, and form natural tissue [12]. In many approaches to engineer tissues, it is desirable to utilize a biodegradable polymer as the cell transplantation matrix. This results in no long-term foreign implant material, and eliminates many potential chronic problems associated with permanently implanted polymers. However, the only enzyme known to degrade alginate is alginase, a bacterial enzyme not synthesized by humans or animals. Mammalian cells cannot attach to alginate, and adhesion is necessary for both long-term viability of cells, and to control cell growth and function on polymers [13]. Moreover, the range of mechanical properties available from alginate hydrogels is quite limited and change in a noncontrollable manner, presumably due to the loss of ionic cross-linking [14]. These limitation have led us to tailor new biomaterials derived from alginate that are biodegradable, possess a wide range of physical and mechanical properties, and exhibit the potential for improved cellular interaction.

To address these limitations of alginate while maintaining the favorable characteristics of alginate, we have synthesized new polymers derived from the portion of the alginate molecule that is responsible for its gelling behaviour. Alginates with high guluronate content were hydrolysed to yield sodium poly(guluronate) segments with low molecular weights. Sodium poly(guluronate) was then partially oxidized with sodium periodate to form poly(aldehyde guluronate) [PAG] which was cross-linked with adipic

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dihydrazide to form hydrogels. Acyl hydrazone bonds are susceptible to hydrolytic cleavage in aqueous conditions [15], therefore these bonds in the cross-linked poly(aldehyde guluronate) hydrogel should provide a degradable linkage. After the breakdown of the hydrogel, the dihydrazides and the poly-(aldehyde guluronate) chains can readily diffuse through tissues and can be cleared by the body due to their low molecular weight. The covalent cross-linking provides an additional level of control, in addition to ionic cross-linking, over the mechanical stability and strength of these hydrogels. In addition, in an attempt to improve upon the cellular interactions with these materials, a cell adhesion peptide, Gly–Arg–Gly–Asp–Tyr (GRGDY) has been successfully coupled to PAG using two different chemistries. These novel alginate-derived hydrogels may potentially provide a family of materials with a wide range of degradation rates, mechanical properties and defined cell interactions.

2. Experimental section

Sodium alginate was purchased from Pronova Biomaterials (Drammen, Norway). Sodium periodate, ethylene glycol, adipic dihydrazide, sodium cyanoborohydride, and anhydrous KBr were purchased from the Aldrich Chemical Company (Milwaukee, WI) and used as received. Ethanol (95%), sodium nitrate, and concentrated hydrochloric acid were purchased from Fisher Scientific Company (Fair Lawn, NJ) and were used as received. Sulfo-*N*-hydroxysuccinimide and 2,4,6-trinitrobenzene sulfonic acid (5%, w/v in methanol) were purchased from Pierce Chemical Company (Rockford, IL). Formaldehyde (37% in water), *t*-butyl carbazate, and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide were purchased from Sigma (St. Louis, MO). The pentapeptide, GRGDY, was synthesized by the Protein and Carbohydrate Structure Facility, The University of Michigan. ^{125}I-GRGDY (0.74 μCi/μg, 360 μCi/μl, $< 2\%$ free ^{125}I) was purchased from Biomedical Technologies, Inc. (Stoughton, MA). ^{1}H and ^{13}C n.m.r. spectra were determined in deuterated water with sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) as the internal standard, on a Bruker AM 360 NMR spectrometer. Chemical shifts are reported in ppm (δ) downfield relative to DSS. Infrared spectra were recorded as % transmittance using a Nicolet 5DX FTIR spectrophotometer and a Hewlett-Packard 7470A plotter. Samples were pressed as KBr pellets using a hydraulic press (Carver). Spectrophotometric measurements were determined using a Perkin-Elmer Lambda 12 UV/VIS spectrophotometer.

2.1. Size exclusion chromatography (SEC)

SEC analysis was performed on a liquid chromatograph consisting of a Spectra System P1000 pump (Thermal Separation Products), a Rheodyne 7010 manual injector, a dual differential viscometer and right-angle laser light scattering (RALLS) detector (Viscotek T 60, $\lambda = 670$ nm) and a laser refractometer detector (Viscotek LR40, $\lambda = 670$ nm), the detectors being connected in parallel. Aqueous sodium nitrate (0.1 M) at ambient temperature with nominal flow rate of 0.7 ml/min was used as the mobile phase. The separations were carried out on two TSK GMPW$_{XL}$ (TosoHaas, 7.8 × 300 mm) mix bed columns. The chromatograms were analysed using the TriSEC 3.0 GPC software (Viscotek).

2.2. Mechanical analysis

The mechanical properties of cross-linked PAG gels were determined using an MTS Bionix 100 mechanical tester (MTS Systems Corporation, France). Samples were compressed with a load cell at room temperature with a constant deformation rate of 1.00 mm min^{-1}. The diameter of the indentor was 3.15 mm. These samples were measured for each condition in triplicate, and the values given in the figures represent the mean and the standard deviation.

2.3. TNBS assay

Analysis of cross-linked samples with trinitrobenzene sulfonic acid (TNBS) allows both unreacted and partially reacted adipic dihydrazides to be quantified in hydrogels. TNBS is known to react with amines, hydrazines, and hydrazides to form a stable trinitrophenyl complex [16]. The maximum absorbance of the trinitrophenyl complex of adipic dihydrazide has been determined to be at $\lambda = 334$ nm. Excess TNBS is added to the hydrogels and allowed to react. The unreacted adipic dihydrazide in the hydrogel reacts with two equivalents of TNBS and forms a soluble complex. The number of unreacted hydrazides remaining in hydrogels is determined by measuring the absorbance of the solution in the first step of the assay. Partially reacted adipic dihydrazide reacts with one equivalent of TNBS to form a trinitrophenyl complex which is bound to the hydrogel, and is thus not measured in the first step when the absorbance of the solution is measured. The hydrogels are removed from the solution, and excess dihydrazide is added to the filtrates in the second step of the assay. The remaining unreacted TNBS (not reacted in step 1) again forms soluble trinitrophenyl derivatives, and the absorbance of the solution is again measured. The number of bound dihydrazide is obtained by subtracting the number of soluble trinitrophenyl complex (absorbance of solution after step 2) from the total number of TNBS added to the hydrogels in step one. Subtracting the number of unreacted and partially reacted dihydrazides from the total number of adipic dihydrazide in each gel yields the number of cross-links.

Specifically, gels were synthesized in triplicate at 6 wt% PAG under various adipic dihydrazide concentrations. Each condition was allowed to gel for 5 h, frozen with liquid nitrogen (−196°C), and lyophilized for 24 h. The dried products were then wetted with 3.2 ml saturated borate buffer (pH 8) for 5 min. Aqueous TNBS stock solution (25 ml, 4.0 μmol/ml) was added and the tubes were incubated at 37°C for 1 h with stirring. The suspension was then filtered through a 0.22-μm filter. Two aliquots of 0.9 ml of the filtrate were introduced into two 25-ml glass vials. To the first set of vials, 100 μl aqueous trichloroacetic acid (1%) was added and the mixture was diluted with aqueous HCl (12 ml, 0.5 M). The absorption of the solution was measured at 330 nm against a blank treated as above but excluding the cross-linked matrix. This first reading provided us with the number of trinitrophenyl derivatives. This number was divided in half to obtain the amount of unreacted adipic dihydrazides. Aqueous adipic dihydrazide (100 μl, 40 μmol/ml) was added to the second set of vials followed by incubation at 37°C for 1 h, with stirring, to allow all the free TNBS to react and form a trinitrophenyl complex. The resulting solution was diluted with aqueous HCl (12 ml, 0.5 M), and the absorption was measured at 334 nm.

2.4. Isolation of sodium poly(guluronate)

Sodium poly(guluronate) was isolated with a slight modification according to a procedure by Haug et al. [17,18]. Sodium alginate (75 g) was added portion-wise to double-distilled (dd) water (3.5 l) with vigorous stirring using a mechanical stirrer. An aqueous hydrochloric acid solution (390 ml, 3 M) was added and the mixture was refluxed for 5 h. The solution was allowed to cool to room temperature overnight, and the supernatant liquid was decanted. The resultant suspension was centrifuged and the solid collected, combined, and suspended in dd water (5 l). Sodium chloride (29.25 g) was added to the aqueous solution followed by the drop-wise addition of an aqueous sodium hydroxide solution (50 ml, 4 M). The pH was then adjusted to 2.25 with drop-wise addition of concentrated hydrochloric acid (20 ml, 12 M). The precipitate was allowed to settle at ambient temperature and the supernatant was decanted. The suspension was centrifuged and the white solid was washed with dd water (500 ml). The solid was suspended in water (1 l), and sodium chloride (3 g) was added to the suspension. The pH of the solution was adjusted to 7.5 with aqueous sodium hydroxide (4 M) followed by the addition of activated carbon (20 g). The suspension was stirred thoroughly for 2 h, centrifuged, and vacuum filtered to remove the activated carbon. The product was precipitated with the addition of ethanol (2 l, 95%), allowed to settle at room temperature, and the supernatant was decanted. The suspension was centrifuged and the solid was collected, combined, and freeze dried under reduced pressure to yield a white solid (38.0 g): M_w 6200 Da, M_w/M_n 1.14; i.r. (KBr pellet, cm^{-1}) 3430.5, 2931.2, 1616.4, 1412.5, 1320.5, 1126, 1092, 1028; ^1H n.m.r. (360 MHz, D_2O) δ 3.90 (br, s), 3.99 (br, s), 4.07 (br, s), 4.45 (br, s), 5.03 (br, s); ^{13}C n.m.r. (360 MHz, D_2O) δ 67.64, 69.87, 71.73, 82.94, 103.66, 178.29.

2.5. Oxidation of sodium poly(guluronate)

In a 1-l Erlenmeyer flask wrapped with aluminum foil, sodium poly(guluronate) (40.0 g) was dissolved, with stirring, in dd water (400 ml). A solution of sodium periodate (0.5 M, 400 ml) was added and the reaction was stirred for 19 h at room temperature. Ethylene glycol (40 ml) was then added to the reaction mixture to reduce any unreacted periodate. The reaction was stirred for 2 h at ambient temperature and the solution was filtered and exhaustively dialysed (Spectra/Por membrane, MWCO 1000) against dd water for 3 days. The water was changed at least three times a day. The solution was then concentrated under reduced pressure to 100 ml, and freeze dried under reduced pressure to yield a white fluffy product (24.8 g, 62%). All the products gave positive tests with Schiff's reagent (pararosaniline chloride); M_w 6000 Da, M_w/M_n 1.4; i.r. (KBr pellet, cm^{-1}) 3336.5, 2942.4, 1724.4, 1616.4, 1405.8, 1321.1, 1159.4, 1117.2, 1025.8.

2.6. Aldehyde assay

The percentage oxidation of sodium poly(guluronate) was quantified by measuring the number of aldehydes in these polymers using *t*-butyl carbazate. Carbazates are well known to react with aldehydes to form stable carbazones in a similar manner to hydrazone formation. Thus, we can determine the aldehyde content of PAG by measuring carbazate consumption when excess carbazate is reacted with the PAG. Unreacted carbazates were determined by adding excess aqueous TNBS solutions and measuring the absorbance of the colored complex (trinitrophenyl derivative) at 334 nm. In brief, solutions of poly(aldehyde guluronate) (0.5 ml, 10 mM) and *t*-butyl carbazate (0.5 ml, 24.75 mM) in aqueous trichloroacetic acid (1%) were mixed, and allowed to react for 24 h at room temperature. A volume of 200 μl was transferred to a disposable scintillation vial containing aqueous TNBS solution (2 ml, 4 mM, 0.1 mM borate buffer, pH 8). The mixture was allowed to react for 30 min at room temperature, diluted with aqueous hydrochloric acid (0.5 M), and the absorbance of the solution was measured at 334 nm. Aqueous *t*-butyl carbazate solutions were used as standards to obtain a calibration curve for determining the unreacted carbazates in experimental samples. The blank which was used comprised of dd H_2O (0.5 ml) and aqueous trichloroacetic acid (0.5 ml, 1%). Aqueous formalin solutions were used to test the efficacy of this procedure and to normalize the experimental results. To eliminate the contribution of the terminal aldehydes when calculating the aldehydes formed from periodate oxidation, the aldehyde content in sodium poly(guluronate) was determined. This number was subtracted from the experimental number of aldehydes found in poly(aldehyde guluronate) to obtain the percentage of guluronate units that were oxidized.

2.7. Peptide coupling via carbodiimide chemistry

A solution of aqueous PAG was prepared by dissolving 1.8 g PAG in MES buffer (60 ml, pH 6.5) containing 0.5 M NaCl. Sulfo-*N*-hydroxysuccinimide (sulfo-NHS, 99 mg, 0.45 mmol) was then dissolved in the PAG solution. The peptide incorporation was monitored by adding ^{125}I-GRGDY (122 μl, 500 μg/ml, 360 μCi/ml) to aqueous peptide (0.528 ml, 12.5 mg/ml) and diluting the final solution with dd H_2O (10 μl). Three 20-ml scintillation vials were charged with aqueous PAG (10 g), aqueous peptide (30 μl), and a stirrer bar. A solution of 100 μl of freshly prepared aqueous solution of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC, 145.7 mg, 0.76 mmol) in MES buffer (1.0 ml, pH 6.5) was added to each vial and the reaction was stirred for 12 h at ambient temperature. Each solution was then dialysed (MWCO 1000) against dd H_2O (1 l). The aqueous solution was changed with a fresh solution every day until its radioactive count reached background. Each solution was freeze dried under reduced pressure, and the resulting solid was dissolved in H_2O at a concentration of 5 mg/ml. The radioactivity of 0.5-ml aliquots was counted and the percentage incorporation was calculated. As a control, the same procedure was used for poly(guluronate) and PAG with the exclusion of aqueous EDC.

2.8. Peptide coupling via reductive amination

A solution of aqueous PAG was prepared by dissolving 1.8 g PAG in aqueous sodium bicarbonate buffer (60 ml, pH 9.6). The peptide solution was prepared by adding ^{125}I-GRGDY (122 μl, 500 μg/ml, 360 μCi/ml) to aqueous peptide (0.528 ml, 12.5 mg/ml) and diluting the final solution with dd H_2O (10 μl). Three 20-ml scintillation vials were charged with aqueous PAG (10 g), aqueous peptide (30 μl), and a stirrer bar. Sodium cyanoborohydride ($NaCNBH_3$, 50 mg, 0.8 mmol) was carefully added to each vial and the reaction was stirred for 24 h at ambient temperature. Each solution was then dialysed (MWCO 1000) against dd H_2O (1 l). The aqueous solution was changed with a fresh solution every day until its radioactive count reached background. Each solution was freeze dried under reduced pressure, and the resulting solid was dissolved in H_2O at a concentration of 5 mg/ml. The radioactivity of 0.5 ml aliquots was counted and the percentage incorporation was calculated. As a control, the same procedure was used for sodium poly(guluronate) and PAG with the exclusion of $NaCNBH_3$.

2.9. Representative procedure for cross-linking PAG (as a function of PAG concentration)

In a 24-well plate, solutions of 50, 100, 150, 200 and 250 μl aqueous PAG (20 wt%) were pipetted in three wells each. Aqueous adipic dihydrazide (150 μl, 0.5 M) was then added to each well and the final volume was adjusted to 0.5 ml with dd H_2O. The contents of each well were mixed and allowed to gel for 5 h at ambient temperatures on a mechanical shaker. The thickness of each gel was then measured using a micrometer gauge and the compressive modulus was determined with a mechanical tester. Afterwards, the compressive modulus from each condition (three samples) was averaged and plotted against weight percentage of PAG in each gel. I.r. (KBr pellet, cm^{-1}) 3554.4, 3472.1, 3413.7, 3236.1, 1638.9, 1617.9, 1405.7, 1153.7, 1095.5, 1032.6.

2.10. Hydrogel degradation

Aqueous solutions of poly(aldehyde guluronate) (125 μl, 20 wt%) and adipic dihydrazide (125 μl, 0.5 M) with calcium chloride (80 mM) were mixed in 15-ml conical tubes (in quadruplicates), and allowed to gel for 5 h. Solutions of Dulbecco's modified Eagles medium (DMEM, 10 ml) or phosphate-buffered saline (PBS, 10 ml, without calcium) were added and the tubes were incubated at 37°C. All samples were monitored until complete dissolution of the hydrogels was noted.

3. Results and discussion

3.1. Synthesis of poly(aldehyde guluronate) hydrogels

Alginate was hydrolysed under acidic conditions to yield sodium poly(guluronate), **1**, as a low-molecular weight polysaccharide (6000 Da), following a modified procedure by Haug et al. [17,18]. The purity of this product was accessed by both ^{1}H and ^{13}C n.m.r., and both analyses indicated the complete absence of mannuronic acid in the product, as previously reported with this procedure [19–21]. Sodium poly(guluronate) was then oxidized by sodium periodate to form the poly(aldehyde guluronate), PAG, **2** (Fig. 1). This reaction was monitored by the appearance of the aldehyde symmetric vibrational band (carbonyl) at 1735 cm^{-1} via FT-i.r. (Fig. 2). The degree of oxidation was controlled by the mole equivalent of sodium periodate used in each reaction. The extent of oxidation in this reaction could be estimated by quantifying the consumption of periodate [22]. However, sodium periodate can also be consumed by the depolymerization of alginates [22]. In addition, since the final PAG products were purified in this study by exhaustive dialysis against dd H_2O, it was more appropriate to determine the degree of oxidation of PAG by measuring the number of aldehydes in the final product. To quantitate the aldehydes, aqueous PAG was allowed to react with excess *t*-butyl carbazate at room temperature for 24 h. The unreacted carbazates were then allowed to react with TNBS to form the trinitrophenyl derivative. Photometric measurements of the resulting derivative were then carried out at a wavelength of 334 nm. By subtracting the number of unreacted carbazates from the

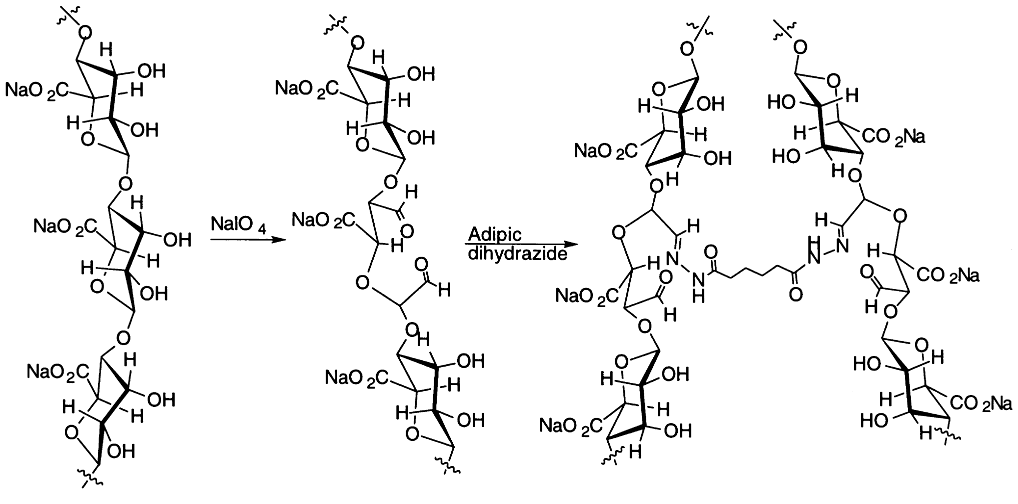

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Fig. 1. Scheme for the synthesis and cross-linking of poly(aldehyde guluronate).

total number of added carbazates, the number of aldehydes in each solution was determined. The degree of oxidation of the polymer was then calculated based on the number of guluronates in each solution. As expected, the degree of oxidation of poly(aldehyde guluronate) increased as the amount of added periodate was increased (Fig. 3). The poly(aldehyde guluronate) intermediate was then cross-linked with a homobifunctional cross-linker, adipic dihydrazide, to form hydrogels. This coupling reaction was followed by the disappearance of the aldehyde symmetric vibrational band and the appearance of a carbonyl band for the hydrazide at 1639 cm^{-1} (Fig. 2).

The appropriateness of this approach to generate biomedically useful polymers is supported by previously described, similar, approaches to stabilize proteins by partial oxidation of their carbohydrate residues, followed by cross-linking with homobifunctional cross-linkers [23–25]. Proteins cross-linked through their partially oxidized carbohydrate residues have found many biomedical applications, including biosoluble surgical materials [26] and blood substitutes [27,28]. Moreover, molecular probes and proteins have been extensively immobilized onto partially oxidized proteoglycans derived from starch, dextran, and cellulose [29–34]. In addition, poly(aldehyde) dextran had been reported to couple the acid hydrazide derivatives of two antineoplastic agents [35].

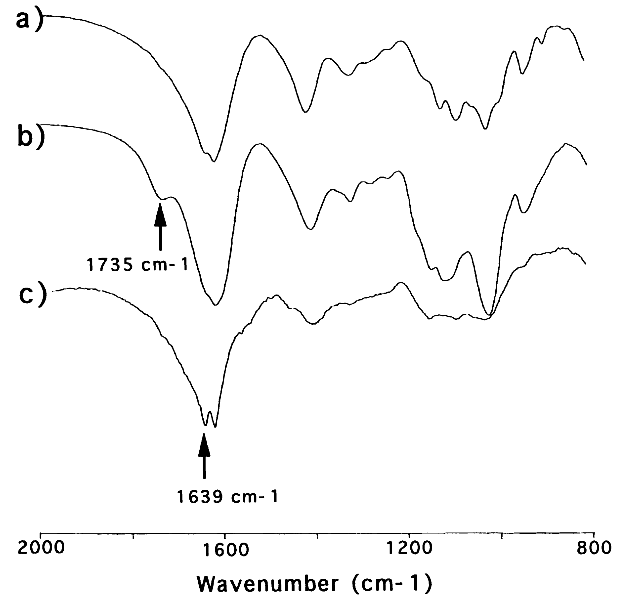

Fig. 2. FT-i.r. of (a) sodium poly(guluronate); (b) PAG; and (c) cross-linked PAG hydrogels. Arrows point to the carbonyl bands of the aldehyde and hydrazide groups.

3.2. Control of extent of cross-linking

Poly(aldehyde guluronate) was next cross-linked with adipic dihydrazide in a series of experiments as the PAG, adipic dihydrazide, calcium ion, and sodium ion concentration were varied. In certain experiments, the pH and

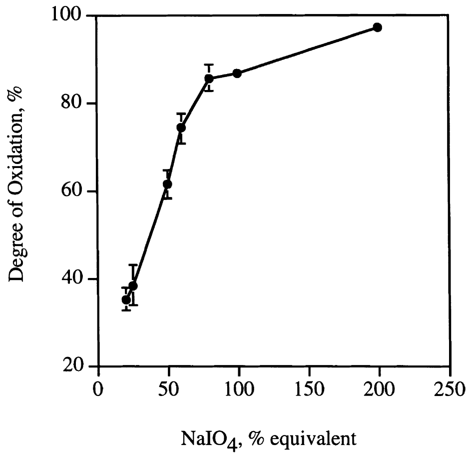

Fig. 3. The experimentally determined degree of oxidation of PAG as a function of the percentage of added NaIO$_4$.

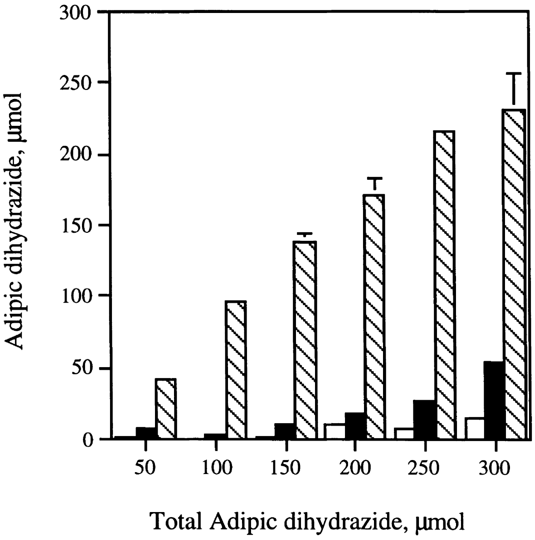

Fig. 4. The number of free (empty bars), partially reacted (filled bars), and cross-linked adipic dihydrazides (striped bars) as a function of the total number of adipic dihydrazide. Values represent the mean ($n = 3$) and standard deviation. All hydrogels (1 ml) were prepared in triplicate with PAG (6 wt%) in dd water.

percentage oxidation of sodium poly(guluronate) were varied as well.

To determine the efficiency of the reaction between the hydrazide and the aldehyde groups, the number of unreacted adipic dihydrazides was measured spectrophotometrically as a function of the concentration of the cross-linker. In addition, the number of adipic dihydrazides that are coupled to PAG through only one hydrazide group was also indirectly determined. Gels were cross-linked (6 wt% PAG) at various concentrations of adipic dihydrazide, and the number of both unreacted and partially reacted dihydrazides were measured. The number of cross-links was found to increase as a function of the concentration of the cross-linker (Fig. 4). The efficiency of the cross-linking reaction ranged between 84 and 96% at all reaction conditions, and was maximal at 300 mM adipic dihydrazide.

This analysis, however, does not differentiate between intermolecular and intramolecular cross-linking. It is well known that the compressive modulus of hydrogels is directly proportional to the intermolecular cross-link density [36]. We therefore quantified the compressive modulus in all experiments as a measure of the extent of intermolecular cross-linking. We found that increasing the concentration of adipic dihydrazide resulted in an increase in the compressive modulus of cross-linked PAG hydrogels (Fig. 5). An optimal value of 560 kPa was obtained for the compressive modulus of the hydrogels at a concentration of 150 mM adipic dihydrazide. As the concentration of adipic dihydrazide was increased further, the modulus decreased to 350 kPa at a concentration of 300 mM of cross-linker (Fig. 5). At this concentration, the number of hydrazide and aldehyde groups are almost equivalent. Above this concentration, we expect the efficiency of cross-linking to

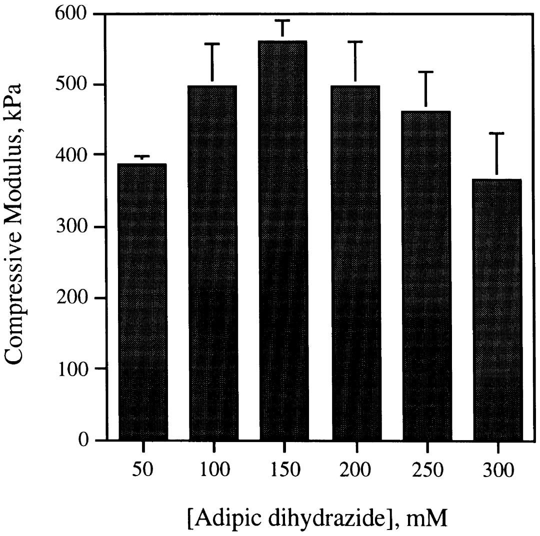

Fig. 5. The compressive modulus of cross-linked PAG hydrogels as a function of the cross-linker concentration. All hydrogels were prepared with PAG (6 wt%) in dd water.

decrease dramatically as the amount of hydrazide groups exceeds the number of aldehydes in the polymer. The fact that the compressive modulus of the hydrogels was optimal at 150 mM dihydrazide suggests that above this concentration intramolecular cross-linking may be occurring at a greater frequency.

In the next set of experiments, gels were cross-linked at various concentrations of PAG. No hydrogels were formed with 4 wt% PAG and below, even after 48 h of reaction. Hydrogels did form starting at 5 wt% PAG, and these hydrogels had a compressive modulus of 82 kPa (Fig. 6). The compressive modulus increased with the PAG content in the gels to a value of 880 kPa at 10 wt% PAG. The number of aldehydes available for cross-linking increases with the

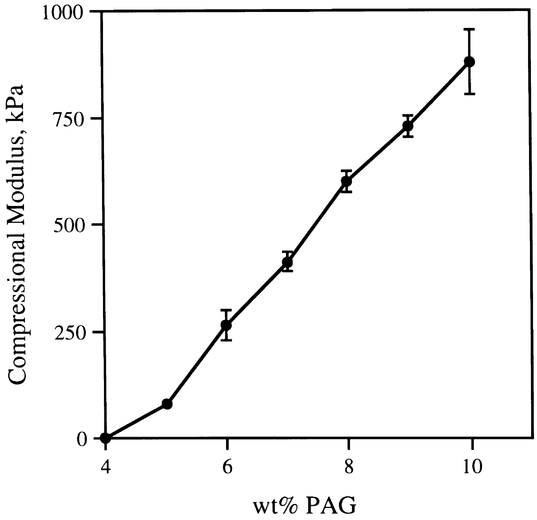

Fig. 6. The compressive modulus of cross-linked PAG hydrogels as a function of the PAG concentration. All hydrogels were prepared with adipic dihydrazide (150 mM) in dd water.

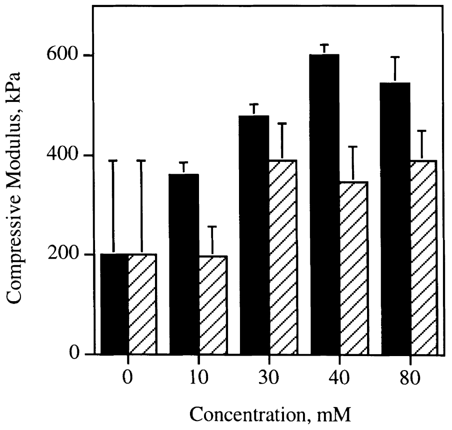

Fig. 7. The compressive modulus of cross-linked PAG hydrogels as a function of the concentration of Ca^{+2} (filled bars), and Na^{+} (striped bars). All hydrogels were prepared with PAG (6 wt%), and adipic dihydrazide (150 mM) in dd water.

PAG concentration, so it is not surprising that the number of cross-links increased with the PAG concentration.

The mechanism of gelation in unmodified alginate is ionic cross-linking of the poly(guluronate) blocks by divalent cations (e.g., calcium, barium, and strontium). To test whether the gelling properties of covalently cross-linked PAG could be controlled in a similar manner, PAG (6 wt%) was cross-linked with adipic dihydrazide (150 mM) at various concentrations of calcium chloride (10–100 mM). The compressive modulus of the resulting gels increased with increasing calcium concentrations to an optimal value of 600 kPa at 40 mM calcium chloride (Fig. 7). Above this concentration, there was no statistical differences in the compressive modulus. To ensure that this result was not due to a non-specific effect of increasing the ionic strength, PAG was cross-linked in the presence of sodium chloride. A slight increase in the modulus was observed initially as the sodium ion content increased, but the value of the modulus leveled out at 390 kPa (Fig. 7). There was a significant difference of 210 kPa between the optimal modulus obtained in the presence of calcium and sodium ions, respectively. These findings suggest that ionic cross-linking still occurs in these gels, and can be utilized to provide another level of control over their mechanical properties.

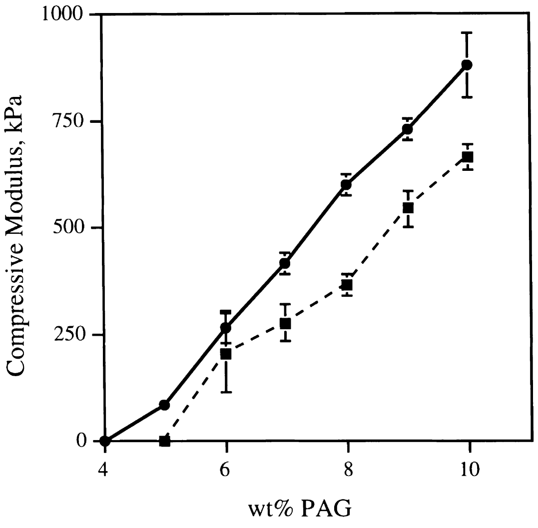

Fig. 8. The compressive modulus of cross-linked PAG hydrogels formed at pH 4.5 (circles), and pH 7.4 (squares). All hydrogels were prepared with PAG (6 wt%), adipic dihydrazide (150 mM), and $CaCl_2$ (40 mM) in dd water.

In certain potential applications for these materials (e.g., cell transplantation) one may want to cross-link these polymers in the body. In this situation the reaction conditions (e.g., pH) must not be harmful to surrounding tissue. To determine if the pH affected the cross-linking, the mechanical properties of PAG cross-linked at pH 7.4 were determined. Adjusting the pH of the aqueous poly(aldehyde guluronate) solution to 7.4 led to a slight decrease in the compressive modulus of hydrogels formed at all concentrations of PAG (Fig. 8). This was expected since it is well known that the reactivity of hydrazide groups with aldehydes is optimal at lower pH values. Under acidic conditions, aldehydes are protonated and are more susceptible to nucleophilic attack by the hydrazide groups. At neutral to basic conditions, however, slower kinetics are in effect and a longer time interval is required for the completion of the reaction, which results in a lower degree of functional cross-linking.

Finally, we have also varied the number of aldehyde units on the poly(guluronate) strand that are available for cross-linking to allow another level of control over the properties of these materials [37–39]. Sodium poly(guluronate) was oxidized using various amounts of sodium periodate and cross-linked at 10 wt% PAG with adipic dihydrazide (150 mM). The compressive modulus increased with the percentage of oxidation of poly(guluronate) to reach a maximum value of 1000 kPa at 91% theoretical oxidation (Fig. 9). As the percentage oxidation of sodium poly(guluronate) is increased, the number of aldehyde groups

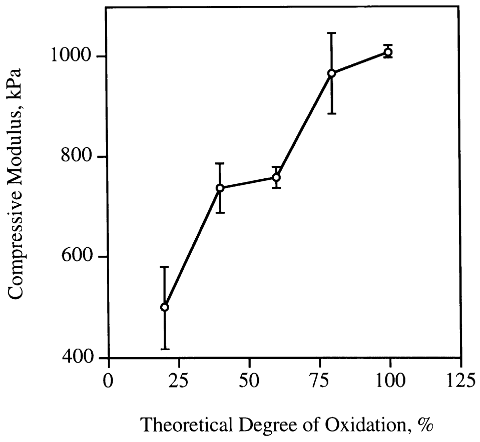

Fig. 9. The compressive modulus of cross-linked PAG hydrogels as a function of the degree of PAG oxidation. All hydrogels were prepared with PAG (6 wt%) and adipic dihydrazide (150 mM) in dd water.

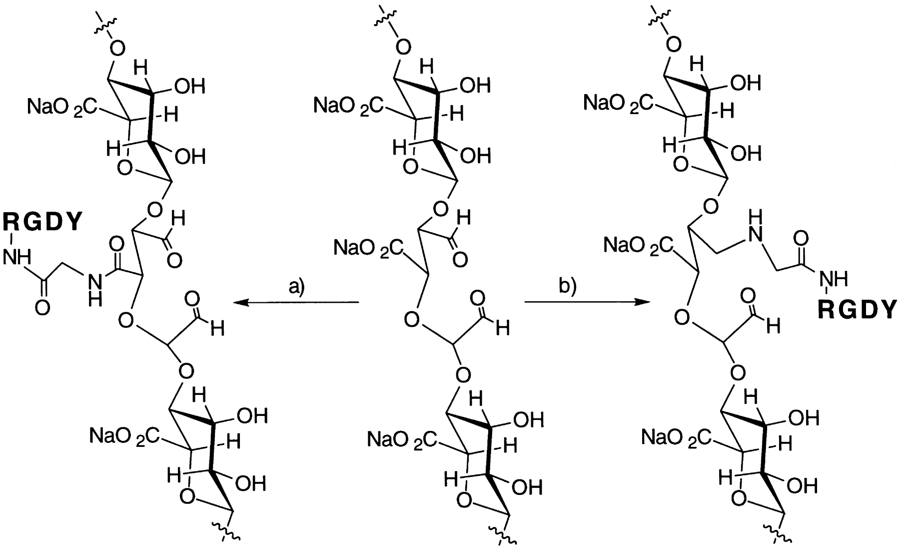

Fig. 10. Coupling cell adhesion peptides to PAG using (a) EDC, *N*-hydroxysuccinimide, or (b) $NaCNBH_3$.

available for cross-linking also increases. This increase in the concentration of aldehyde groups likely results in a higher degree of functional cross-linking, which in turn would result in an increase in the compressive modulus of the gels. Interestingly, whereas cross-linked 20% oxidized PAG hydrogels were relatively malleable and deformed considerably before cracking, 85% oxidized PAG hydrogels were brittle.

Preliminary studies with cross-linked PAG hydrogels have demonstrated that these hydrogels are degradable in aqueous solutions (e.g., PBS and DMEM). Gels ($n = 4$) were formed at 10 wt% PAG, 150 mM adipic dihydrazide, and 40 mM $CaCl_2$ concentrations. These gels were then incubated in both PBS or DMEM at 37°C. Gels incubated with PBS degraded and were completely dissolved after 4 months. In contrast, the hydrogels that were incubated with DMEM took only 3.5 months to degrade. The more rapid degradation in DMEM may be due to the reaction of amines present on amino acids in this solution with the aldehydes of the PAG (no amino acids are present in PBS). This reaction would consume aldehyde groups created by the hydrolysis of the hydrazone linkage. This shifts the equilibrium process to favour the dissolution of the hydrogels more quickly.

3.3. Coupling cell adhesion ligands

Hydrogels provide a three-dimensional architecture for cells to proliferate and form natural tissues in tissue engineering applications [10]. However, to survive and multiply

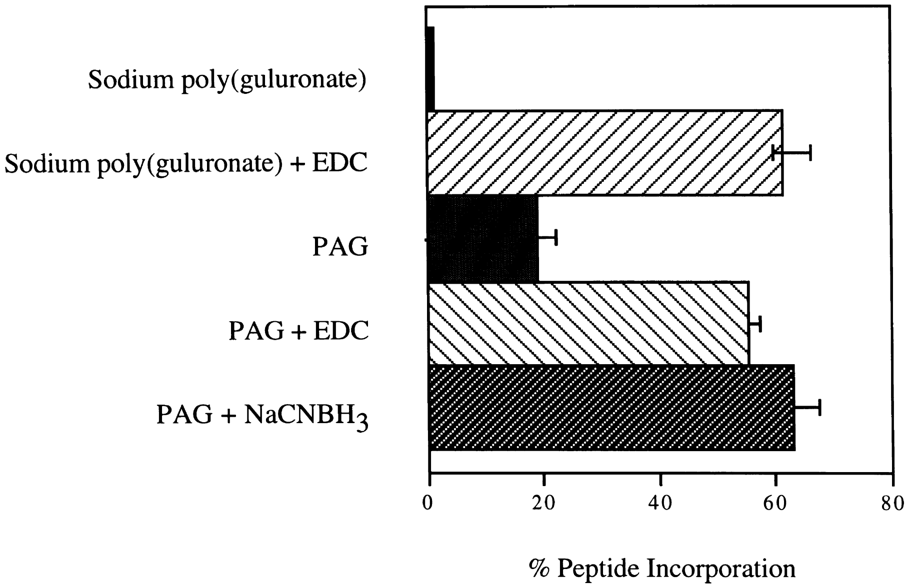

Fig. 11. Percentage peptide incorporation in sodium poly(guluronate) and PAG under various reaction conditions. Values represent the mean ($n = 3$), and standard deviation.

in hydrogels, most cell types need to adhere to the polymer [11]. Cell attachment and interaction lead to the synthesis of a natural extracellular matrix (e.g., elastin and collagen) which eventually replaces the synthetic polymer implant [40]. To improve upon the ability of mammalian cells to attach and interact with cross-linked PAG hydrogels, a cell adhesion peptide, GRGDY, has been successfully coupled to PAG using two different approaches. The peptide was first coupled via its terminal amine with the carboxylic acids on the PAG backbone. Carbodiimide chemistry was utilized to activate the carboxyl group which was reacted with the peptide to form a covalent amide bond (Fig. 10). The second approach was via the reductive amination of the aldehydes of PAG with the terminal amine on GRGDY. The amines of the peptides reacted with the aldehydes of PAG to form imine bonds which were selectively reduced with sodium cyanoborohydride to form amine linkages (Fig. 10).

The degree of incorporation of the peptide in both approaches was followed by adding trace ^{125}I-labeled peptide to each reaction. Sodium poly(guluronate) was used as a control, where 60% incorporation of labeled peptide was obtained with carbodiimide chemistry (Fig. 11). Note that in the absence of EDC, less than 1% of the peptide was incorporated in sodium poly(guluronate). Similar results were obtained upon reacting PAG with ^{125}I-labeled peptide, where a 58% incorporation was achieved with carbodiimide chemistry. However, in the absence of EDC a 20% peptide incorporation in the PAG material was found. The most likely mechanism for this finding is reaction of the amine terminal of the peptide with the aldehyde group in PAG to form a Schiff base. Reductive alkylation of the peptide with PAG in the presence of $NaCNBH_3$ resulted in a 68% peptide incorporation. This finding supports the possibility that an imine bond formed between the peptide and PAG which was then reduced to form a stable amine group while consuming one aldehyde per peptide coupled to PAG. Hence, two efficient methods had been successfully developed to couple cell adhesion ligands to PAG.

4. Conclusions

The limited range of physical properties and lack of degradation of alginates limit their biomedical applications. We have now developed a methodology to synthesize guluronate-based biomaterials with a wide range of mechanical properties, and with cell adhesion peptides coupled to their backbones. We expect these materials to be biodegradable and well suited for both engineering a variety of soft tissues and drug delivery applications.

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