Novel Hydrogels of Hyaluronic Acid: Synthesis, Surface Morphology, and Solid-State NMR

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Abstract: Hyaluronic acid (HA) is a linear polysaccharide consisting of repeating disaccharide units of *N*-acetyl-D-glucosamine (GlcNAc) and D-glucuronic acid (GlcUA). A convenient methodology was recently developed that allowed the attachment of pendant hydrazido groups to the glucuronate moieties of hyaluronate oligosaccharides. This methodology was extended to high molecular weight hyaluronate (1.5×10^6), and the products were cross-linked with four homobifunctional activated esters to give novel HA hydrogels. Solid-state ^{13}C NMR using cross-polarization and magic angle spinning (CP–MAS) revealed that the lyophilized native HA and hydrazido-HA retained solution-like structures in the solid state. The four HA hydrogels showed significant structural changes relative to native HA, and the carbon resonances of the cross-linkers were clearly evident. The surface morphologies of these cross-linked HA derivatives were examined using scanning electron microscopy (SEM). The electron micrographs of the freeze-dried hydrogels showed the presence of regular sheetlike structures forming pores (20–50 μm). In contrast, native HA showed predominantly fibrous and irregular structures.

Introduction

Hyaluronic acid (Figure 1) is a viscoelastic biomaterial that is a major component of the extracellular matrix and of the synovial fluid in joints.[1] It has unique rheological properties that are unparalleled by any other natural or synthetic polymer. These unique properties indicate the use of hyaluronate as a surgical aid, especially for the replacement of the natural vitreous in ophthalmic procedures.[2] Intraarticular injections of high molecular weight noninflammatory HA (NIF-HA) with or without corticosteroids offer considerable potential for treatment of osteoarthritis in humans.[2b,3,4] NIF-HA is also widely used for viscosupplementation and viscosurgery. Native sodium hyaluronate has been used as a delivery vehicle for the controlled release of growth factors[5] and was shown to serve as a template for nerve regeneration.[6] Unfortunately, even the highest available molecular weight HA (*ca.* 6.0×10^6) does not provide the optimal rheological properties that are required of an efficient viscosurgical tool. Moreover, the HA solutions currently used for viscosupplementation in treating osteoarthritis do not remain in the joint for prolonged periods and thus fail to exert a long-lasting effect.[7]

One potential solution to these problems is the production of covalently cross-linked derivatives of hyaluronate that form "hydrogels". Such a macromolecular network could be swollen in water to several times its original volume and could be expected to have superior rheological and viscoelastic properties compared to solutions of native HA. In addition, these derivatives would be expected to show increased resistance to degradation *in vivo* by hyaluronidases. Given the potential of hyaluronate in biomedical research, many approaches to the chemical modification of HA have been undertaken. For example, HA has been cross-linked using divinylsulfone[8] and diepoxybutane[9] under highly basic conditions that are known to degrade high molecular weight HA to oligomeric fragments. Cross-linking reactions of HA with bishalides[10] and formaldehyde[11] have also been reported. However, none of the final products were unambiguously characterized by spectroscopic methods.

We recently described a convenient methodology for the chemical modification of HA oligosaccharides with dihydrazides to generate pendant hydrazido groups attached to the glucuronic acid residues.[12] We now report an extension of this methodology to high molecular weight HA (1.5×10^6), and we illustrate how the pendant hydrazido group can be employed to introduce covalent cross-links into the modified HA to produce novel hydrogels. These water-insoluble, cross-linked derivatives of HA were lyophilized and then characterized by solid-state ^{13}C NMR using cross-polarization and magic angle spinning (CP–MAS). In addition, the surface morphologies of the lyophilized HA hydrogels were analyzed using scanning electron microscopy (SEM).

Experimental Section

General Procedures. Animal-derived sodium hyaluronate (Amvisc) was obtained as its sodium salt from MedChem Products, Inc. (Woburn, MA). Fermentation-derived HA (Cristalhyal) was provided by Collaborative Laboratories Inc. (East Setauket, NY). Both sources were employed for chemical modification and cross-linking. Adipic dihydrazide and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) were purchased from Aldrich Chemical Co. Homobifunctional cross-linkers bis-

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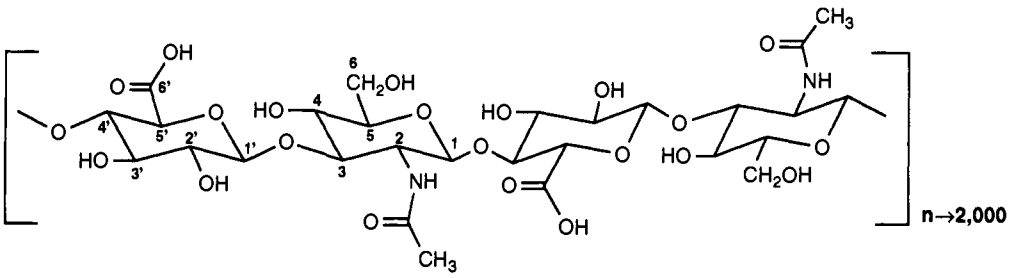

Figure 1. Structure of hyaluronic acid (HA) showing two disaccharide repeat units.

(sulfosuccinimidyl)suberate (BS[3]), 3,3'-dithiobis(sulfosuccinimidyl-propionate) (DTSSP), dimethyl suberimidate·2HCl (DMS), and ethyleneglycolbis(sulfosuccinimidylsuccinate) (sulfo-EGS) were obtained from Pierce Chemical Co. Spectrapor membrane tubing (molecular weight cutoff = 3500) was purchased from Fisher Chemical Co. All water employed was first distilled and then ultrafiltered with a SYBRON/Barnstead Nanopure system.

Preparation of Hydrazido-Functionalized HA (1). Sodium hyaluronate (200 mg, 0.50 mmol) was dissolved in water such that the concentration of the HA solution was approximately 4 mg/mL. To this mixture was added a 30-fold molar excess of adipic dihydrazide (3.5 g, 20 mmol). The pH of the reaction mixture was then adjusted to 4.75 using 0.1 N HCl. Next, EDC (382 mg, 2.0 mmol) was added in solid form. The pH of the reaction mixture was maintained at 4.75 by addition of 0.1 N HCl. The reaction was allowed to proceed for 2 h, or until no further increase in pH was observed, at which time the pH of the reaction mixture was adjusted to 7.0 by addition of 1 N NaOH. Dialysis tubing was prepared by soaking the membrane in water at room temperature for 3–4 h and subsequent rinsing with water. The reaction mixture was then transferred to the pretreated dialysis tubing and dialyzed exhaustively against water. The clear and viscous final mixture was lyophilized for 48 h.

General Procedure for Generating Cross-Linked Derivatives of Hyaluronate (2-5). Hydrazido-functionalized HA (**1**) was dissolved in 0.1 M $NaHCO_3$ buffer at a concentration of 15 mg/mL. A clear and colorless solution was formed. To this solution was added the homobifunctional cross-linker (BS[3], DTSSP, sulfo-EGS, and DMS) in solid form. Polymerization (gelation) was observed 30–90 s after addition of the cross-linker. The gels thus formed were purified by repeated washings with water. The gels were allowed to swell in water for 10 days to 2 weeks; the gels obtained in this manner were clear and colorless. The molar ratios of hydrazido-HA (**1**) and the homobifunctional cross-linkers were as follows: **1**:BS[3], 1:1.4; **1**:DTSSP, 1:1.4; **1**:DMS, 1:1.4; 1:sulfo-EGS, 1:0.36.

Scanning Electron Microscopy (SEM). SEM was performed on a JSM-3500 JEOL scanning electron microscope. Fully hydrated HA hydrogels **2–5** containing 1.4-1.7 mg HA/mL gel were flash-frozen by immersion in liquid N_2, freeze-dried, and then mounted on a metal stub. Selected samples were sputter-coated with gold using a Hummer VIA sputter coater.

Solid-State NMR. NMR experiments were performed on a home-built NMR spectrometer operating at a ^1H frequency of 301.47 MHz, using a magic angle spinning probe incorporating Doty Scientific stators and a novel double-resonance circuit,[13] with ^1H rf field strengths equivalent to 50 KHz. Sample quantities of lyophilized HA derivatives were typically 60 mg, and each sample was equilibrated for several days at 55–65% relative humidity prior to recording the NMR spectrum. No humidity-dependent spectral changes were observed. In a typical experiment, 2000 transients were averaged, with an acquisition delay of 4 s. Spectra were obtained with CP-MAS[14,15] at spinning frequencies of 3.6–10.1 KHz. MAS sidebands were suppressed using the $N = 6$ SELTICS sequence,[16] with proton irradiation turned off during the low-frequency pulse. This sequence was expected to give quantitative spectral intensities for all but the carbonyl signals. Signal losses of approximately 15% were estimated for the carbonyl groups, on the basis of expected chemical shielding anisotropies of 150–200 ppm. These were factored into intensity calculations. Methylene carbons were distinguished from methines using the delayed decoupling pulse sequence.[17,18] Other assignments were deduced by comparison with carbon chemical shifts based on standard substituent parameters. Overlapped signals were deconvolved to obtain spectral intensities. Intensities used for quantification were only from nonoverlapped, noncarbonyl resonances in unsubtracted spectra. Spectra were obtained under carefully controlled conditions of spinning speed and probe-tuning, in order to facilitate clean subtraction of the native hyaluronic acid background in difference spectra. Spectra to be subtracted were scaled using the anomeric carbon peak. This resonance appeared to show the least variation between substituted derivatives, cross-polarized efficiently and quantitatively, and was not overlapped with spacer carbon resonances in any of the spectra examined. All spectra were referenced to external tetramethylsilane.

Results

A new methodology for introduction of a reactive hydrazido functionality into HA oligosaccharides[12] was extended to the chemical modification of high molecular weight HA (Figure 2). Sodium hyaluronate derived from either animal or fermentation sources was dissolved in water to a final concentration of 4 mg/mL. A large excess of adipic dihydrazide was added, and the pH of the mixture was adjusted to 4.75, which had been previously determined to be optimal for the coupling reaction.[19] Addition of the water-soluble carbodiimide EDC caused an increase in pH, and the pH was maintained at 4.75 by addition of 0.1 N HCl for *ca.* 2 h. The pH was then increased to 7.0, and the reaction mixture was purified by dialysis to remove reagents and by-products. The hydrazido-functionalized HA (**1**) was isolated as a white fibrous mass in quantitative yield after lyophilization. No significant differences in the properties of any materials prepared from the different HA sources were observed.

The incorporation of the adipate linker and pendant hydrazido moiety were verified on lyophilized samples by solid-state ^{13}C NMR using CP–MAS. Figure 3 shows a comparison between the CP–MAS spectrum of native HA (Figure 3A)[19] with that of HA derivative **1**, which was functionalized with the six-carbon hydrazido linker (Figure 3B). As expected, the spectrum of derivatized HA superficially resembled that of HA, with additional intensity in the carbonyl region from the carboxyl groups of the pendant moiety and in the aliphatic region between 25 and 35 ppm. The intensity in the aliphatic region had the characteristic behavior of a methylene carbon in the "delay-without-decoupling" (DWD) experiment. Using standard substituent parameters,[20] the chemical shifts of the adipate linker group were predicted (Table 1). The resonance near 34 ppm corresponded closely to

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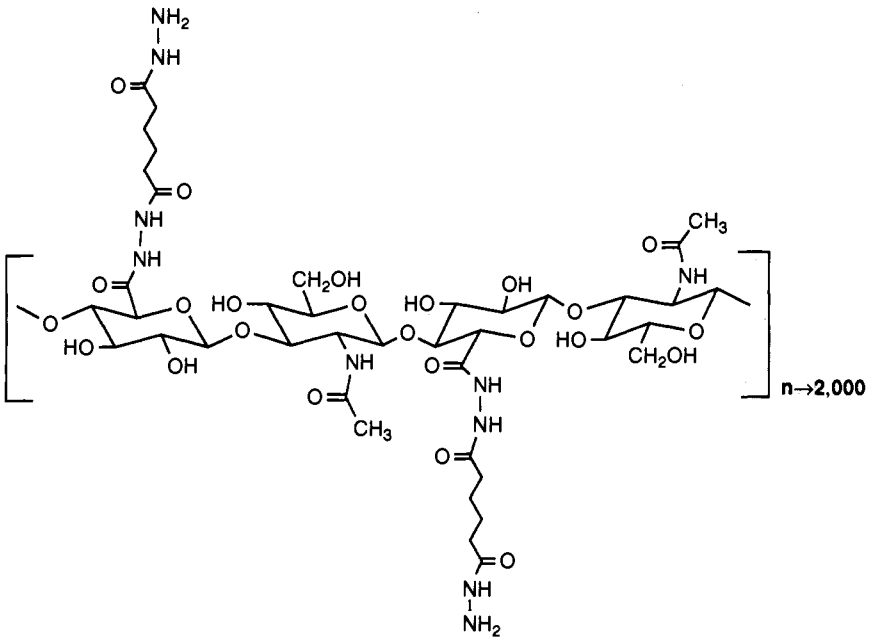

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Figure 2. Hydrazido-modified HA (**1**) shown arbitrarily as a repeating tetrasaccharide unit. Not all carboxylates are modified in preparations of **1**.

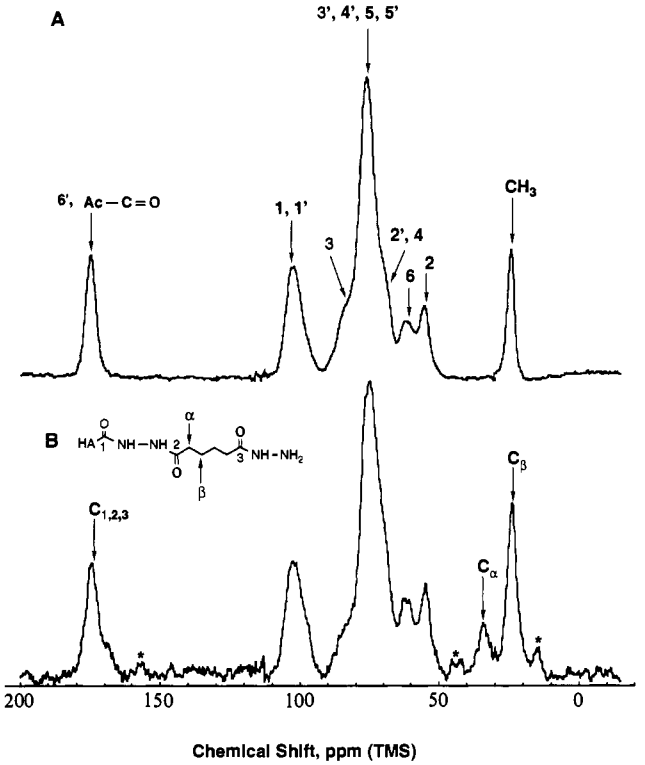


Figure 3. Solid-state CP–MAS ^{13}C NMR spectra of (A) native hyaluronate and (B) hydrazido-hyaluronate (**1**). Asterisked peaks are due to the *N*-acylurea adduct of HA and EDC.[19]

the calculated value for the adipate α-carbons, and the resonance at 25 ppm corresponded to the value calculated for the β-carbons. Subtraction of the two spectra was accomplished by empirical adjustment of a normalization constant, and the residual intensity in the aliphatic region was deduced by integration. The residual intensity corresponded to that expected for functionalization of 17.5–21.5% of the glucuronate carboxyl groups of HA.

Cross-linking of the chemically modified HA derivative **1** could be achieved under mild, aqueous conditions using commercially available homobifunctional reagents (Scheme 1). Both non-cleavable and chemically labile linkers were employed. Thus,

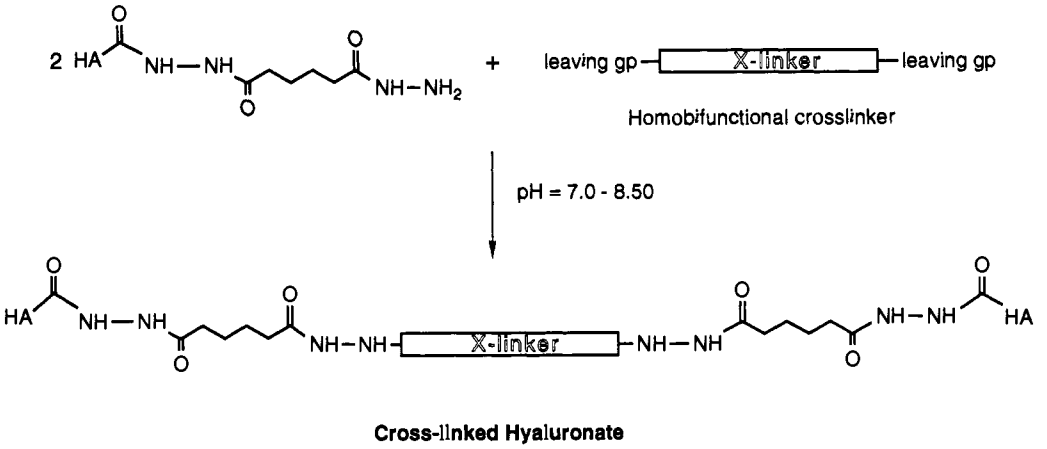
Table 1. Calculated and Measured Chemical Shifts in the HA Derivatives[a]

modified HA derivative	carbon position	% cross-linked	chemical shift (ppm) expected	chemical shift (ppm) measured
hydrazido-HA (**1**)	C-2, C-3		179	
	C-α	19	34.1	34.1
	C-β		25.9	25.4
HA-BS^3 (**2**)	C-2, C-3		179	
	C-α		34.1	33.8
	C-β		25.9	26.1
	C-4	22	179	
	C-α'		34.8	33.8
	C-β'		25.8	26.1
	C-γ'		29.9	28.1
HA-DMS (**3**)	C-2, C-3		179	
	C-α		34.1	35.1
	C-β		25.9	25.2
	C-4	12	172	
	C-α'		32.7	32.1
	C-β'		25.8	25.2
	C-γ'		29.9	28.6
HA-DTSSP (**4**)	C-2, C-3		179	
	C-α		34.1	33.3
	C-β	24	25.9	
	C-4		175	
	C-α'		35.5	33.3
	C-β'		35.8	33.3
HA-EGS (**5**)	C-2, C-3, C-4		179	
	C-α		34.1	32.4
	C-β		25.9	25.2
	C-5	10	173	
	C-α'		30.5	32.4
	C-β'		31.5	32.4
	C-α''		62.9	63.6

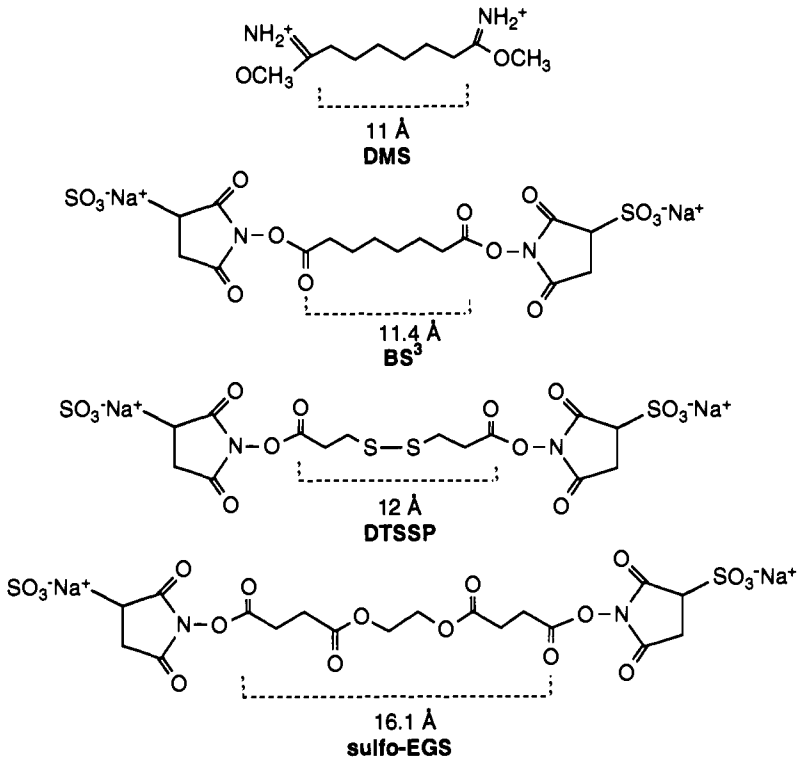
[a] Chemical shifts were calculated using substituent parameters.[20] Measured chemical shifts are given relative to external tetramethylsilane. Where this column is left blank, spectral overlap with HA resonances prevented accurate measurement of the cross-linker chemical shifts; where several identical shifts for different positions in the molecule are given, the individual peaks are not resolved.

reaction of hydrazido-HA (**1**) with the homobifunctional cross-linkers depicted in Scheme 2 led to the production of four hyaluronate hydrogels (**2–5**), as depicted in Scheme 3. Upon addition of the cross-linker to a midly basic solution of hydrazido-HA (**1**) at pH = 8.50, polymerization (gelation) could be observed

Scheme 1. General Strategy for Generating Cross-Linked Derivatives of Hyaluronate



Scheme 2. Homobifunctional Cross-Linkers Used for Hydrogel Synthesis[a]



[a] The respective spacer arm lengths are indicated.

within approximately 30–90 s. The molar ratio of hydrazido-HA (**1**) to cross-linker was 1:1.4, except for the EGS derivative **5**, which was 1:0.36. The ratios were selected to give 10–25% cross-linking. The hydrogels thus obtained were purified by repeated washings with water and were allowed to swell in water at 8 °C. These clear and colorless gels swelled to approximately 10 times their original volume in a period of 10 days to 2 weeks, a property that is highly characteristic of hydrogels and some other covalently cross-linked macromolecular networks. The equilibrium water content[26] of hydrogel **2** was calculated to be 99.86%, and each of the hydrogels **2–5** showed very limited elasticity.

The variations in the chemical nature of the homobifunctional cross-linkers gave HA hydrogels with slightly different characteristics, although each one swelled in water to a gelatin-like (Jell-O) material. The swelling properties of each gel was dependent on the salt content of the buffer. Full swelling of these highly anionic gels was observed only at the lowest ionic strength. Placing each of the four hydrogels in 0.15 M NaCl (physiological saline) resulted in shrinkage and rigidification of the gel. A control experiment was run in parallel to the cross-linking reactions: native hyaluronate was dissolved in 0.1 M $NaHCO_3$ (pH = 8.50) at a concentration of 15 mg/mL, and DMS was added. Gelation was not observed in the control, and the components of the mixture remained entirely water-soluble, indicating that in the absence of hydrazido-HA (**1**) covalent cross-linking of the HA strands did not occur.

Figure 4 shows the CP–MAS spectrum of a lyophilized sample of the cross-linked derivative HA-DTSSP (**4**) (Figure 4A) and a difference spectrum in which the contributions of the backbone HA resonances have been subtracted out (Figure 4B). The hydrazido-HA (**1**) spectrum could not be used for the subtraction since the adipate carbons in this nonsymmetrical material lacked the chemical shift equivalencies found in the cross-linked materials.[12a] Using a combination of the DWD experiment and substituent parameters, the resonance frequencies of the cross-linker can be predicted and correlated with the residual peaks in the difference spectrum. Table 1 shows that a close correlation

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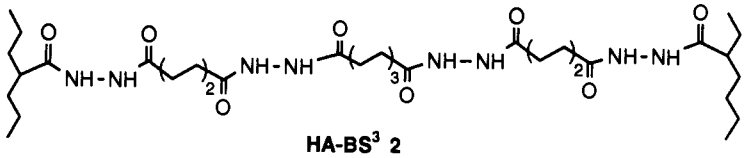
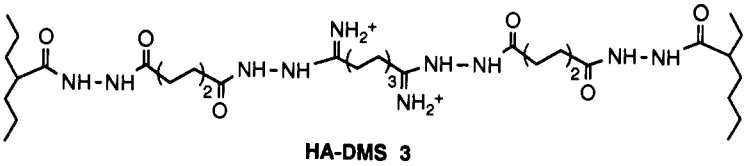
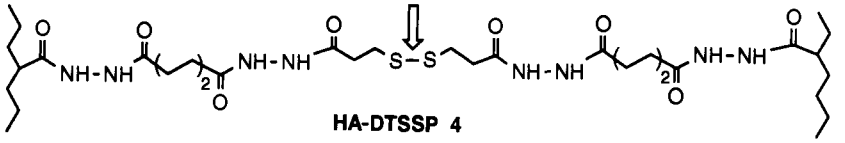
Scheme 3. Hyaluronate-Derived Hydrogels[a]



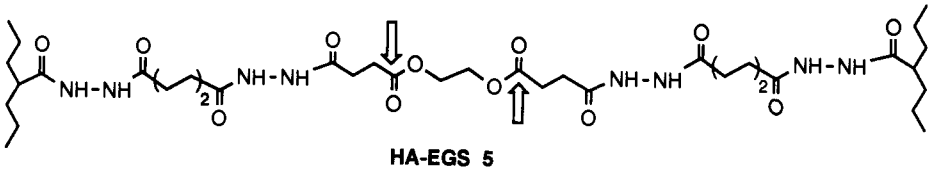
HA-BS^3 2



HA-DMS 3



HA-DTSSP 4



HA-EGS 5

[a] The open arrows indicate the potential sites for reductive or hydrolytic cleavage.

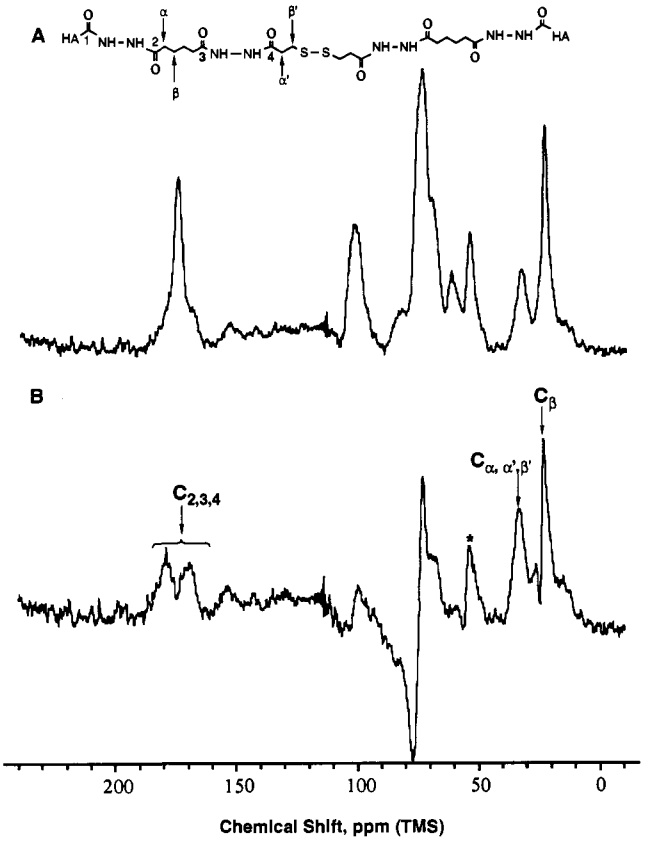


Figure 4. Solid-state CP–MAS ^{13}C NMR spectrum of HA-DTSSP (**4**) (A) and difference spectrum of HA-DTSSP (**4**) minus native HA (B).

exists between observed and calculated peaks. However, the three predicted peaks in the region between 34 and 36 ppm were not resolved; only a single peak at 33.3 ppm was observed. The cross-linker line widths were on the order of 2 ppm, probably due to conformational heterogeneity. The peak intensities could again be integrated in the parent (unsubtracted) spectra, indicating that 22–26% cross-linking of the HA had occurred.

Predicted and experimental peak positions for each derivative are given in Table 1. It is evident that substituent parameters predict the cross-linker chemical shifts with acceptable accuracy. Integration of nonoverlapped cross-linker resonance intensities in the unsubtracted spectra allowed estimation of the efficiency of cross-linking, which was in the range 10–25% of the available glucuronate moieties and varied among individual preparations. Although cross-linking sometimes appeared to exceed the extent of monofunctionalization, this was attributed to batch-to-batch variability in the original functionalization of HA.

In contrast to the hydrazido-HA (**1**), the chemical shifts for the cross-linked HA hydrogels showed clear evidence of conformational changes in theHA backbone. In HA-DTSSP (**4**), the major ^{13}C resonance was shifted upfield by 3.5 ppm. This was evident as side-by-side negative and positive peaks in the difference spectrum (Figure 4B), suggesting that the natural abundance sugar backbone resonances have been shifted upfield. These changes were also seen in the other cross-linked derivatives, e.g., in HA-BS^3 (**2**) (Figure 5).

The surface morphologies of the lyophilized cross-linked hyaluronate hydrogels **2–5** were examined by SEM.[21,22] The introduction of covalent cross-links into the HA derivatives was expected to produce porous three-dimensional networks. The high water content of the hydrogels (>99%) also suggested that upon freeze-drying, highly macroporous sponge-like materials should be produced. The hydrogels were flash-frozen, selected samples were gold-coated, and the materials were examined by SEM. The electron micrographs indicated the presence of highly porous and sheetlike surface structure in the cross-linked HA derivatives with average pore diameters ranging from 20 to 50 μm (Figures 6 and 7.) In contrast, native HA exhibited predominantly fibrous and irregular structures (Figure 6A). It is interesting to note that minor variations in the chemical nature

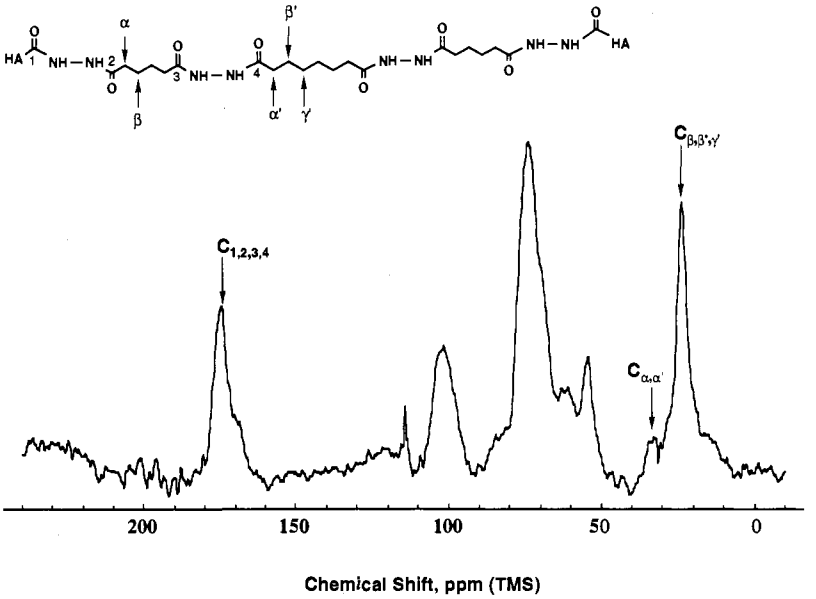


Figure 5. Solid-state CP–MAS ^{13}C NMR spectrum of HA-BS^3 (**2**).

of the homobifunctional cross-linkers could nonetheless lead to the production of biomaterials with significantly different surface characteristics. For example, HA-DTSSP (**4**) (Figure 6B,C) was observed to have continuous hexagonal-type pores, with an average diameter of 50 μm, that were not interconnected. The ester-containing HA-EGS (**5**) (Figure 6D,E) had discontinuous, interconnected pores with average pore diameters ranging from 20 to 50 μm. HA-DMS (3) (Figure 7A,B) showed relatively uniform continuous pore structure with pore diameters also ranging in size from 20 to 50 μm. Finally, electron micrographs of HA-BS^3 (**2**) (Figure 7C,D) showed elongated pores and a network with a predominantly "sheetlike" appearance. No correlation between the chemical structure of the cross-linkers and the surface morphologies of the hydrogels was apparent in these preliminary analyses.

Discussion

Hyaluronic acid hydrogels with different physical and chemical properties were prepared from a unique hydrazido-modified HA and were characterized by CP–MAS NMR and SEM. Methodology previously used to prepare hydrazide-functionalized oligosaccharides of hyaluronate[12] with spectroscopically well-established linear connectivity was extended to high molecular weight HA (1.5×10^6). This methodology had three key features. First, all of the reaction components were water-soluble, eliminating the need to use organic cosolvents. Second, all reactions were conducted between pH 4.75 and 8.75, thereby preventing degradation of HA. No gross changes in viscosity were observed upon introduction of the hydrazido moieties. Third, the pendant hydrazido group allowed the introduction of covalent cross-links, both cleavable and noncleavable. The HA hydrogels were obtained by reaction with a variety of amine-specific homobifunctional cross-linkers with spacer arm lengths in the range 11–17 Å. These spacer arm lengths were selected on the basis of their propensity to promote interstrand cross-link formation.[23] The chemical nature of the cross-linker was varied to introduce hydrolytically labile ester linkages, a chemically reversible disulfide linkage, as well as charged imido groups, and the more stable hydrazide linkage. These seemingly minor variations in the cross-linkers nonetheless imparted to the HA hydrogels a variety of different physical properties that could be potentially exploited in biomaterial design.[12b]

The level of 10–25% cross-linking was selected for two reasons. First, a hydrogel retaining the chemical properties and biocompatibility of native HA was desired. Second, attempts to achieve higher levels of cross-linking were expected to give incomplete reaction and monofunctionalization in a too rigid framework. A systematic study is in progress to correlate percentage of cross-linking with degree of swelling and with physical properties of the hydrogel.

CP–MAS ^{13}C NMR spectroscopy is a powerful tool for the characterization of insoluble high molecular weight biopolymers. The spectra of lyophilized HA hydrogel samples were found to be highly reproducible, and estimates of cross-linking obtained from the intensities of different peaks in the same derivative were generally self-consistent. Integrated signal intensities in unsubtracted spectra (even with the formally nonquantitative SELTICS sequence) were essentially quantitative for all resonances except the carbonyl carbons. This can be rationalized on the basis that the chemical shielding anisotropies of sp^3 hybridized carbons are comparable to (or smaller than) the spinning frequencies employed. Comparison of summed sideband intensities under CP–MAS and SELTICS pulse sequences indicated the loss of approximately 15% intensity in the SELTICS sequence under the employed experimental conditions. Calculations based on integrated intensities were adjusted to reflect this experimental artifact.

In this study as well as in earlier CP–MAS NMR study of chemically modified HA,[19] the chemical shielding anisotropies of HA were not motionally averaged. In ordinary CP spectra (not shown), the carbonyl sidebands have about the intensity expected for rigid carbonyls. In addition, dipolar dephasing experiments used in both studies to assign the HA resonances[19] are very sensitive to motional averaging of the dipolar interaction. Neither the HA lines themselves nor the cross-linker resonances showed any evidence of such averaging. Although low angle (5–10°) librational motion could not be excluded, any larger amplitude motion, on a time scale faster than 1 ms is ruled out by the data.

Most intriguing is the *ca.* 3 ppm upfield shift in the major HA resonance in the cross-linked samples. We propose that this shift results from a conformational change in the sugar ring, possibly involving rotation around the $1 \rightarrow 3$ glycosidic linkage. An upfield shift could reflect an increase in steric crowding in the ring, perhaps as a result of a change in the sugar pucker. Even larger pucker-induced changes have been observed in DNA.[18] Unfortunately, it is difficult to fully explain this observation, since the peak

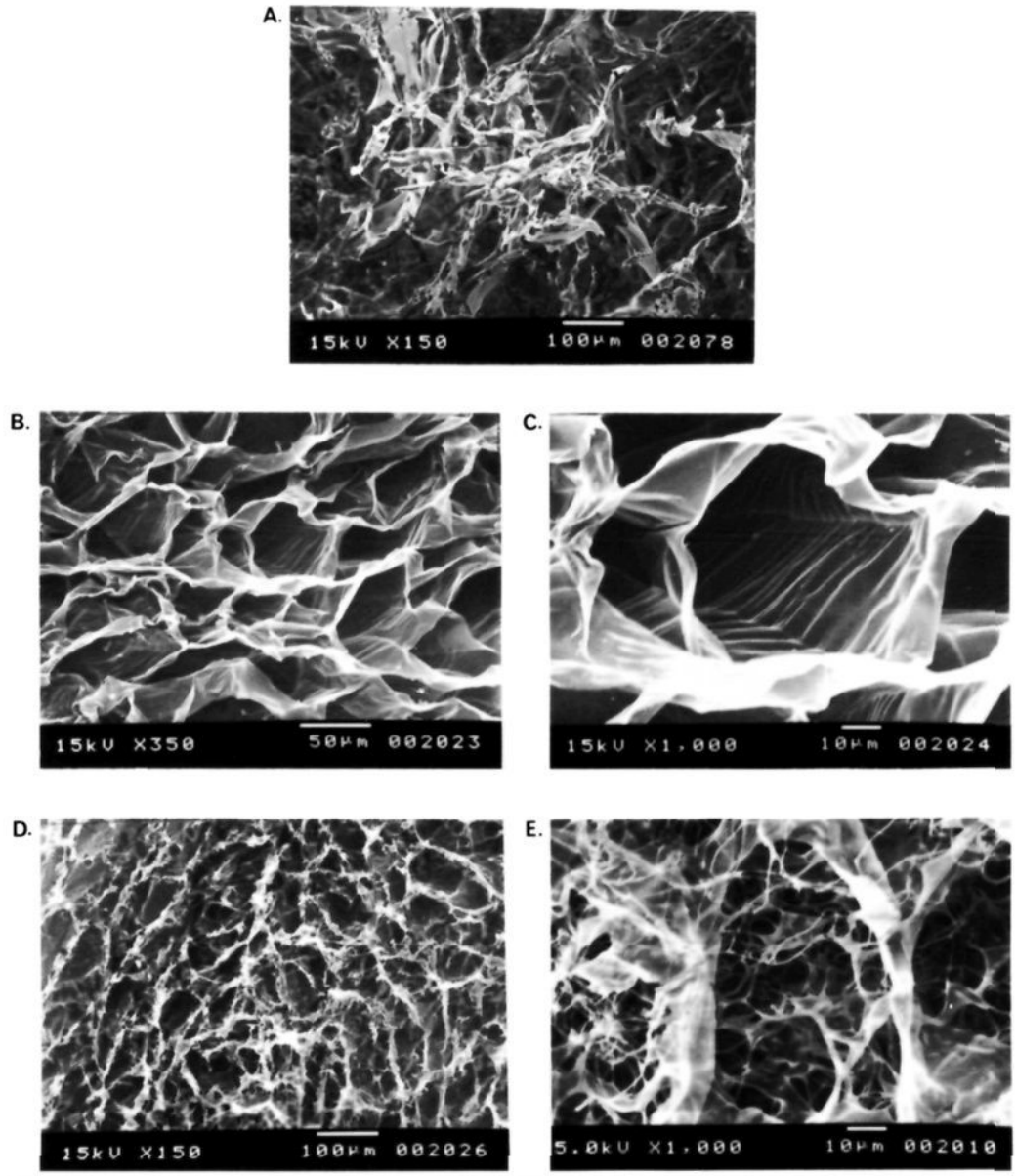


Figure 6. Scanning electron micrographs of a freeze-dried sample of native hyaluronate (A), a cross-section of HA-DTSSP (**4**) (B, C), and a cross-section of HA-EGS (**5**) (D, E).

affected contains signals from several carbons, most of which must experience the upfield shift. It was clear, however, that there were no significant shifts of either the anomeric C-1 or C-6 carbon resonances.

The broadened line widths of the cross-linker resonances suggest some disordering of these groups. Since the line widths of the HA itself did not increase significantly under cross-linking, it can be inferred that the polymer maintains a highly ordered and rigid conformation. Apparently, the flexibility necessary to accomplish the intramolecular cross-linking of the HA chains together was provided by the linkers, and the structural rigidity of the polymer itself was largely left intact by the modification.

These novel hydrogels may have a number of important biomedical applications.[12b] Interest is high in developing drug delivery systems for the delivery of peptides, proteins, and other biologically active molecules that are subject to rapid degradation under physiological conditions. As a result, several new polymeric systems that are capable of sustained release over a period of time have been developed.[24,25] The highly porous surface structure observed in the electron micrographs suggest that the HA hydrogels could have potential as carriers of biologically active molecules. These molecules could be covalently attached to the polymer backbone or could be physically dispersed within the matrix.

Gels that degrade in response to a variety of biological stimuli should have widespread use in the biomedical sciences. Recently, HA gels that degrade in response to the presence of the hydroxy radical have been reported.[26] The hydroxy radicals were believed to be produced as a result of the onset of the inflammatory state. The combination of hydroxyl radicals and HA gels constituted a stimulus-responsive system with potential as a treatment for rheumatoid arthritis. The recently emerging field of tissue engineering[27] also suggests a potentially interesting application for these hydrogels. The highly porous three-dimensional structures of the HA hydrogels suggest that they may be appropriate biodegradable scaffolds for the adherence and growth of cells in three dimensions.[12b]

(27) Langer, R.; Vacanti, J. P. *Science* **1993**,*260*, 920–926.

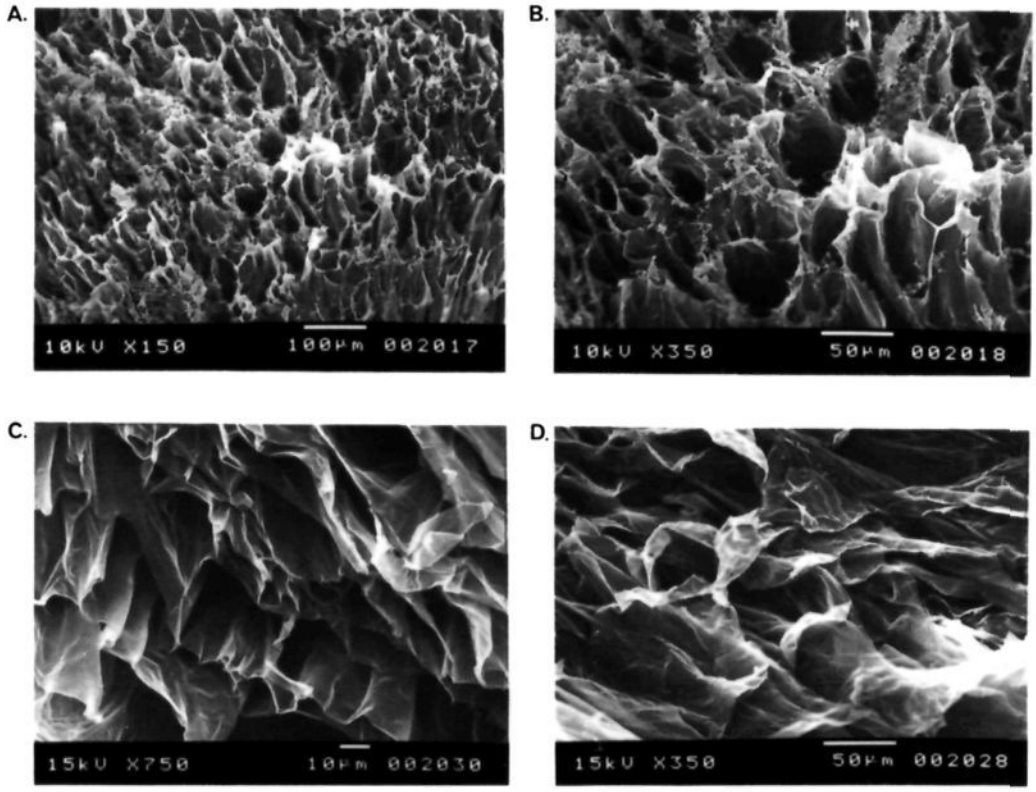


Figure 7. Scanning electron micrographs of a cross-section of HA-DMS (**3**) (A, B) and a cross-section of HA-BS[3] (**2**) (C, D).

In summary, we have presented a convenient methodology for the preparation of a functionalized high molecular weight HA derivative (**1**). Upon reaction with a number of commercially available homobifunctional cross-linkers, a series of novel hydrogels (**2**–**5**) with potentially varying physical and chemical properties were obtained. These cross-linked derivatives have been unambiguously characterized by solid-state ^{13}C NMR using CP–MAS. The surface morphology of the hydrogels has been studied by SEM. To our knowledge, these hydrogels represent the first examples of cross-linked derivatives of high molecular weight hyaluronate that have been unambiguously characterized. These hydrogels[12b] have potential for use as three-dimensional matrices for the incorporation and controlled release of biologically active compounds, as biodegradable scaffolds for cellular adhesion and proliferation in three dimensions, and as injectable substances for the potential treatment of osteoarthritis and rheumatoid arthritis.

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