Solid-State NMR of *N*-Acylureas Derived from the Reaction of Hyaluronic Acid with Isotopically-Labeled Carbodiimides

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Abstract: Hyaluronic acid (HA) is a naturally-occurring linear polysaccharide consisting of alternating D-glucuronic acid and *N*-acetyl-D-glucosamine residues. Reaction of the carboxyl group of the glucuronate residues with 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide (EDC) in the presence of primary amines yielded only the *N*-acylurea adducts rather than the expected amide coupling products. To determine the nature of this linkage unambiguously and to deduce the primary structure of the *N*-acylurea products, ^{13}C- and ^{15}N-labeled 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide were synthesized. The isotopically-labeled carbodiimides were coupled to the carboxyl group of HA (molecular size ca. 2 000 000 Da) in water at pH = 4.75. The modified polysaccharides were then isolated, purified, and examined by cross polarization and magic angle spinning (CP-MAS) solid-state ^{13}C and ^{15}N NMR. The chemical shifts and states of protonation of the nitrogens confirmed the presence of two isomeric *N*-acylureas in unequal amounts and ruled out the presence of any unrearranged *O*-acylurea product.

Introduction

Hyaluronic acid (HA, Figure 1) is a viscoelastic biomaterial that, when present in concentrations as low as 0.1%, can be responsible for 80% of the total viscosity of certain biological fluids. HA is abundant in connective tissue, in the extracellular matrix, and in the vitreous body of the eye.[1] Hyaluronate is also believed to play an important role in cell proliferation and in the control of morphogenesis.[2] The use of HA esters as a drug delivery system has been reported recently.[3] Hyaluronic acid is also an important product in wound-healing[4] and in cosmetic[5] preparations.

Chemical modifications of HA have targeted both the hydroxyl and carboxyl functionalities.[6] However, in most cases, an unambiguous characterization of the final products was not provided.[7,8] A versatile chemical modification of HA would employ a bifunctional linker, e.g., one in which a variety of therapeutic agents could be attached to a sterically-accessible primary amine after modification of the HA. However, reaction of HA with 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide (EDC)[9] in the presence of an excess of either an aliphatic amine or 1,6-diaminohexane failed to provide polymeric materials in which the amine had been incorporated. Instead, the modified HA was tentatively characterized by ^1H NMR as the *N*-acylurea adduct of HA and EDC. On the basis of this observation, we hypothesized that functionalized carbodiimides could be designed, synthesized, and coupled to HA under similar conditions to provide polymeric adducts bearing appropriate functional groups. These functionalized biopolymers could then serve as potentially nonimmunogenic, biodegradable drug delivery agents.

In order to extend the carbodiimide methodology to further modifications of HA, it was necessary (a) to determine the primary structure of the EDC-modified HA unambiguously, (b) to differentiate between the possible isomeric *N*-acylurea or *O*-acylurea products, and (c) to quantify the degree of coupling. Thus, ^{13}C- and ^{15}N-labeled EDC were synthesized, and the HA adducts of these EDC isotopomers were prepared. Herein we report the details of the synthesis of the labeled carbodiimides, the synthesis and purification of the HA-EDC adducts, and the results of the CP-MAS solid-state ^{13}C NMR and ^{15}N NMR studies performed on the isolated, fibrous polymeric adducts.

Solid-state NMR has become a valuable analytical tool for the characterization of insoluble or high molecular weight biomaterials, such as fibrous[10] and membrane[11] proteins, native DNA,[12] and insect exoskeletons.[13] Cross polarization combined with magic angle spinning (CP-MAS)[14] gives well-resolved ^{13}C or ^{15}N NMR spectra, with signal intensities that are in general quantitative or nearly so, and without the extreme solubilization procedures that would be necessary to obtain solution NMR spectra. While the extensive panoply of techniques available to the solution NMR spectroscopist cannot in general be applied to the solid state, interpretation of chemical shift changes[11,12] and rotational resonance methods[15] nonetheless can give accurate chemical and structural information. The present work demonstrates the usefulness of these techniques for characterizing the chemical modification of a biopolymer at levels as low as 1% of all monomer units.

Experimental Section

General Procedures. High-resolution ^1H and ^{13}C NMR solution spectra were recorded on a General Electric QE-300 300-MHz spectrometer. Chemical shifts are given in ppm using residual $CHCl_3$ (7.26 ppm) as an internal standard, unless otherwise indicated. IR spectra were determined with a Perkin-Elmer 1600 FTIR instrument. [^{13}C]Carbon disulfide and [^{15}N]ethylamine hydrochloride were purchased from Cambridge Isotope Laboratories. Hyaluronic acid (Amvisc), obtained as its sodium salt (sodium hyaluronate, batch no. 5905) was provided by MedChem Products, Inc. 3-(Dimethylamino)propylamine, thiophosgene, and methyl chloroformate were purchased from Aldrich Chemical Co.

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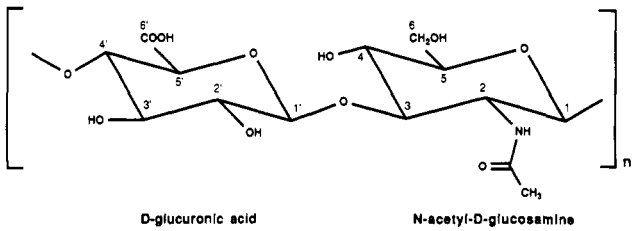

Figure 1. The repeating disaccharide unit in hyaluronic acid.

All solvents used were Optima (HPLC grade), obtained from Fisher Chemical Co.

[^{13}C]Ethyl Isothiocyanate (1). In an ice-cooled 2-mL conical vial equipped with a condenser and a stir bar were placed, via syringe, [^{13}C]carbon disulfide (500 mg, 6.0 mmol, 99% ^{13}C enriched) and 0.59 mL of 11.25 M NaOH (264 mg, 6.0 mmol). To this mixture was added ethylamine (297 mg, 6.0 mmol) as a 33% solution in water. The mixture was heated at 80 °C for 2 h. The resulting red solution was cooled down to 30–40 °C, and methyl chloroformate (620 mg, 6.0 mmol) was added to the mixture via syringe. The ethyl isothiocyanate separated as a brown oil on top of the reaction mixture and was carefully removed by pipet to a small test tube, dried with magnesium sulfate, and subjected to bulb-to-bulb distillation using a Kugelrohr oven with collection in a U-tube at −78 °C to give the [^{13}C]ethyl isothiocyanate **1** (180 mg, 31% yield), bp 130–140 °C: ^{1}H NMR ($CDCl_3$) δ 1.36 (t, 3 H, J = 7.0 Hz, CH_3), 3.53–3.57 (m, 2 H, CH_3CH_2); ^{13}C NMR ($CDCl_3$) δ 129.4 (N=^{13}C=S), 40.1 (CH_3CH_2), 15.5 (CH_3); IR (neat) 2073 cm^{-1} (N=^{13}C=S).

[2-^{13}C]-1-Ethyl-3-(3-(dimethylamino)propyl)thiourea (3). [^{13}C]Ethyl isothiocyanate (**1**) (150 mg, 1.70 mmol) was transferred with 1 mL of chloroform to a 2-mL conical vial. To this mixture was added via syringe an equimolar amount of 3-(dimethylamino)propylamine (**2**) (173 mg, 1.70 mmol). The mixture was stirred overnight at room temperature under nitrogen. The chloforom was removed by rotary evaporation, and the crude thiourea (300 mg, 96% yield) was used in the next reaction without further purification: ^{1}H NMR ($CDCl_3$) δ 1.18 (t, 3 H, J = 7.3 Hz, $CH_3CH_2NH^{13}C(S)NH$), 1.60–1.69 (m, 2 H, $CH_2CH_2CH_2N(CH_3)_2$), 2.21 (s, 6 H, $N(CH_3)_2$), 2.32–2.41 (m, 2 H, $CH_2CH_2CH_2N(CH_3)_2$), 3.30–3.50 (m, 4 H, $CH_3CH_2NH^{13}CSNHCH_2$); ^{13}C NMR ($CDCl_3$) δ 180.5 ($NH^{13}CSNH$).

[2-^{13}C]-1-Ethyl-3-(3-(dimethylamino)propyl)carbodiimide (4). The labeled thiourea **3** (280 mg, 1.50 mmol) was dissolved in 2 mL of acetone. To this mixture was added HgO (352 mg, 1.60 mmol), and the mixture was heated at 80 °C for 3 h, gradually turning black upon the formation of HgS. The reaction mixture was filtered through Celite, concentrated by rotary evaporation, redissolved in chloroform, dried ($MgSO_4$), and transferred to the microdistillation apparatus. The chloroform was evaporated with a gentle stream of nitrogen, and the residue was subjected to bulb-to-bulb distillation under reduced pressure. [2-^{13}C]EDC (132 mg) was isolated as a colorless oil in 57% yield after distillation: ^{1}H NMR ($CDCl_3$) δ 1.22 (t, 3 H, J = 7.2 Hz, $CH_3CH_2N=^{13}C=N$), 1.68–1.73 (m, 2 H, $CH_2CH_2CH_2N(CH_3)_2$), 2.22 (s, 6 H, $N(CH_3)_2$), 2.34 (t, 2 H, J = 7.1 Hz, $CH_2CH_2CH_2N(CH_3)_2$), 3.21–3.27 (m, 4 H, $CH_3CH_2N=^{13}C=NCH_2$); ^{13}C NMR ($CDCl_3$) δ 16.6 ($CH_3CH_2N=^{13}C=N$), 29.3 ($=NCH_2CH_2CH_2N(CH_3)_2$), 41.4 ($CH_3CH_2N=^{13}C=N$), 44.8 ($=NCH_2CH_2CH_2N(CH_3)_2$), 45.4 ($N(CH_3)_2$), 56.8 ($=NCH_2CH_2CH_2N(CH_3)_2$), 140.5 (N=^{13}C=N); IR (neat) 2073 cm^{-1} (N=^{13}C=N).

3-(Dimethylamino)propyl Isothiocyanate (5). A mixture of $NaHCO_3$ (4.9 g, 58 mmol), 3-(*N*,*N*-dimethylamino)propylamine (1.0 g, 9.7 mmol), 40 mL of H_2O, and 50 mL of HPLC grade chloroform was stirred for 10 min. Thiophosgene (1.7 g, 14.5 mmol) was added to this mixture via syringe, and the reaction mixture was stirred for 1 h at room temperature, turning gradually from red to yellow. The chloroform layer was dried ($MgSO_4$), filtered, and concentrated by rotary evaporation. The desired product **5** was obtained as an orange oil in 35% yield. The crude product was used without further purification: ^{1}H NMR ($CDCl_3$) δ 1.83 (m, 2 H, $(CH_3)_2NCH_2CH_2CH_2$), 2.21 (s, 6 H, $N(CH_3)_2$), 2.37 (t, 2 H, J = 6.8 Hz, $(CH_3)_2NCH_2CH_2CH_2$), 3.58 (t, 2 H, J = 6.6 Hz, S=C=$NCH_2CH_2CH_2$); IR (neat) 2108 cm^{-1} (N=C=S).

[1-^{15}N]-1-Ethyl-3-(3-(dimethylamino)propyl)thiourea (6). Crude isothiocyanate **5** (450 mg, 3.10 mmol) was dissolved in 4 mL of chloroform. To this mixture were added [^{15}N]ethylamine hydrochloride (250 mg, 3.10 mmol, 99% ^{15}N enriched) and an equimolar amount of triethylamine (330 mg, 3.10 mmol). The ethylamine hydrochloride was initially only partially soluble in the system; however, after stirring overnight at room temperature, no traces of solid were observed, indicating that it had gradually dissolved. The reaction mixture was washed with 5% NaOH, extracted with chloroform, dried ($MgSO_4$), and concentrated by rotary evaporation, yielding 610 mg (quantitative) of crude thiourea **6**, which was used without further purification: ^{1}H NMR ($CDCl_3$) δ 1.17–1.20 (m, 3 H, $CH_3CH_2{}^{15}NHCSNH$), 1.69 (m, 2 H, $CH_2CH_2CH_2N(CH_3)_2$), 2.23 (s, 6 H, $CH_2CH_2CH_2N(CH_3)_2$), 2.41 (m, 2 H, $CH_2CH_2CH_2N(CH_3)_2$), 3.20–3.50 (m, 4 H, $CH_3CH_2{}^{15}NHCSNHCH_2$).

[1-^{15}N]-1-Ethyl-3-(3-(dimethylamino)propyl)carbodiimide (7). The same procedure described above for the synthesis of [^{13}C]EDC (**4**) was employed. Thus, thiourea **6** (600 mg, 3.20 mmol) was subjected to dehydrosulfurization with HgO (750 mg, 3.50 mmol) in refluxing acetone to afford 210 mg of [^{15}N]EDC (**7**) in 43% yield after distillation: ^{1}H NMR ($CDCl_3$) δ 1.20–1.24 (m, 3 H, $CH_3CH_2{}^{15}N=C=N$), 1.68–1.73 (m, 2 H, $CH_2CH_2CH_2N(CH_3)_2$), 2.20 (s, 6 H, $N(CH_3)_2$), 2.29–2.34 (t, 2 H, J = 7.5 Hz, $CH_2CH_2CH_2N(CH_3)_2$), 3.18–3.25 (m, 4 H, $CH_3CH_2{}^{15}N=C=NCH_2$); ^{13}C NMR ($CDCl_3$) δ 16.6, 29.3, 41.4, 44.8, 45.5, 56.9; IR (neat) 2108 cm^{-1} (^{15}N=C=N).

General Procedure for Coupling of Labeled EDC to Sodium Hyaluronate. Sodium hyaluronate was dissolved in water such that the concentration of the HA solution was approximately 4 mg/mL. The pH of the solution was adjusted to 4.75 using 0.01 N HCl. A 0.5 mg/mL solution of the labeled carbodiimide (either **4** or **7**) in H_2O was prepared, and the pH was adjusted to 5.00 by addition of 0.1 N HCl. The carbodiimide solution was then added dropwise to the HA solution over a period of 15 min. The pH of the reaction mixture was maintained at 4.75 throughout the entire course of the reaction by titration with 0.01 N HCl. The proton consumption was measured in order to quantitate the degree of coupling. For workup, sodium chloride (5% w/v) was added to salt out the modified HA. The mixture was then added to 3 volumes of 95% ethanol, and the modified HA was obtained as a stringy white precipitate. The precipitate was squeezed dry and redissolved in the original volume of water for a second precipitation. The precipitation and redissolution sequence was repeated three times to ensure the removal of all low molecular weight organic impurities (unreacted starting material). The resulting solution of HA was clear and viscous and was placed on a lyophilizer for 48 h.

The ^{1}H NMR samples were prepared by dissolving 10 mg of modified HA in 1 mL of D_2O and adding 10 μL of 1 N NaOD to decrease the viscosity of the solution. The standard used was 3-(trimethylsilyl)-propanesulfonic acid, sodium salt hydrate, set at δ 0.00 ppm. The reactions were carried out at room temperature with carbodiimides **4** and **7** and also at 4 °C with compound **7**. The maximum theoretical coupling was 20% based on molar equivalents used. However, 3.0 and 4.8% coupling were observed for the reactions of **4** and **7** with HA, respectively, based on H^+ consumption data. Proton consumption data was not available for the reaction of **7** with HA at 4 °C. The reaction times were 2 h at room temperature and 16 h at 4 °C; longer reaction times did not give increased coupling: ^{1}H NMR (D_2O) δ 1.09 (t, 3 H, CH_3CH_2N), 1.65 (m, 2 H, $NCH_2CH_2CH_2N(CH_3)_2$), 2.19 (s, 6 H, $N(CH_3)_2$), 2.34 (t, 2 H, $NCH_2CH_2CH_2N(CH_3)_2$), 3.12 (m, 4 H, $CH_3CH_2NCONHCH_2$).

Solid-State NMR Spectroscopy. Solid-state NMR spectra were obtained using a home-built spectrometer operating at a field of 7.1 T (301.42-MHz ^{1}H frequency). Both ^{13}C and ^{15}N spectra were acquired with cross polarization and magic angle spinning[14] using a home-built probe incorporating Doty Scientific stator and rotors. Typical sample quantities were 250 mg. Spinning speeds were 3–4.5 kHz. Proton, ^{13}C, and ^{15}N rotating frame frequencies were 55–60 kHz during both cross polarization and decoupling. Recycle delays were usually 3 s, which was quite adequate to allow full relaxation of the proton spin system, and were limited by sample heating rather than by relaxation. Usually, 10 000–30 000 transients were averaged/spectrum. The cross polarization contact time was 1 ms for ^{13}C; a contact time of 3 ms was used for ^{15}N to ensure full magnetization transfer even to unprotonated nitrogen species. Under these experimental conditions, the sample temperature was approximately 27 °C. Protonated and unprotonated resonances were distinguished using the delayed decoupling experiment;[16] delays for dipolar dephasing were 40 μs for ^{13}C and 80 μs for ^{15}N; in both cases these timings were found empirically to create a null in the signal of immobile singly-protonated species. Other assignments were made by comparison of solid- and solution-state chemical shifts, or by comparing the spectra of labeled modified hyaluronate polymers with unlabeled modified samples on the one hand, and with unmodified hyaluronate on the other.

^{13}C chemical shifts were referenced to the downfield line of adamantane and converted to the tetramethylsilane scale by adding 38.56 ppm.[17] ^{15}N chemical shifts were likewise measured with respect to solid NH_4Cl, and 14.8 ppm was added to express these shifts with respect to 5.6 M NH_4Cl in H_2O.[18]

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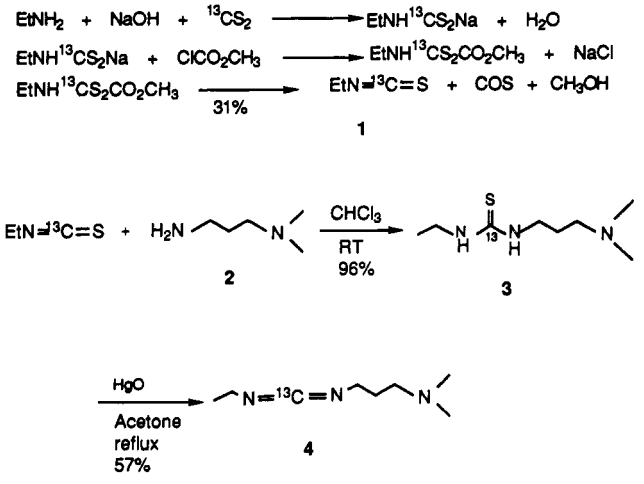
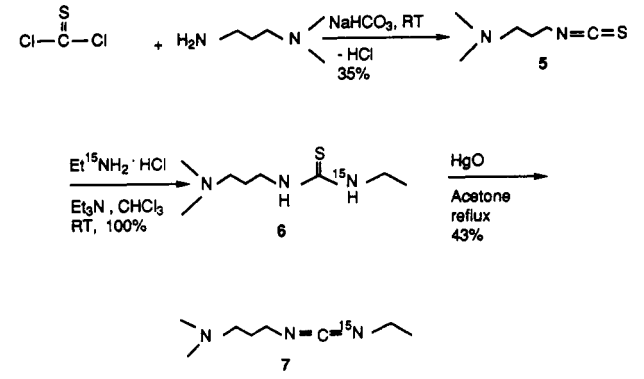
Scheme I. Synthesis of [2-^{13}C]EDC



Scheme II. Synthesis of [1-^{15}N]EDC



Results and Discussion

[2-^{13}C]EDC selectively labeled at the central sp-hybridized carbon was synthesized in three steps (Scheme I). The reaction of [^{13}C]CS_2 with ethylamine and sodium hydroxide, followed by treatment with methyl chloroformate, afforded [^{13}C]ethyl isothiocyanate (**1**) in 31% yield after bulb-to-bulb distillation.[19] Reaction of [^{13}C]ethyl isothiocyanate with 3-(dimethylamino)-propylamine (**2**) provided thiourea **3** in 96% yield. Thiourea **3** was subjected to dehydrosulfurization[20] to give the [2-^{13}C]-labeled carbodiimide **4** in 32% yield after bulb-to-bulb distillation.

The synthesis of [1-^{15}N]EDC labeled on the *N*-ethyl nitrogen of the carbodiimide was accomplished in three steps (Scheme II). Treatment of thiophosgene with 3-(dimethylamino)propylamine in the presence of sodium bicarbonate afforded 3-(dimethylamino)propyl isothiocyanate (**5**) in 35% yield.[21] Treatment of isothiocyanate **5** with [^{15}N]ethylamine hydrochloride in the presence of an equimolar amount of triethylamine afforded thiourea **6** in quantitative yield. Dehydrosulfurization of thiourea **6** with HgO in refluxing acetone afforded [1-^{15}N]EDC (**7**) in 43% yield after bulb-to-bulb distillation.

The isotopically-labeled carbodiimides were each coupled to HA at pH 4.75 at room temperature.[22] The pH of each reaction was maintained at 4.75 by addition of 0.01 N HCl. This pH was chosen, since under these conditions a sufficient number of free carboxylates are present, while at the same time the solution is sufficiently acidic to promote carbodiimide activation. At lower pH, HA solubility and integrity were compromised; at higher pH, insufficient reaction with EDC was observed. Subsequent studies with HA fragments of defined length (2–5 disaccharide units) have confirmed this pH as that which provides the opportunity for optimum coupling.[23] The maximum theoretical coupling was 20% based on the molar equivalents used. This value was selected to obtain sufficient coupling for observation of the ^{15}N and ^{13}C resonances for the EDC–HA adduct balanced against the declining efficiency of coupling at higher percentages of modification.

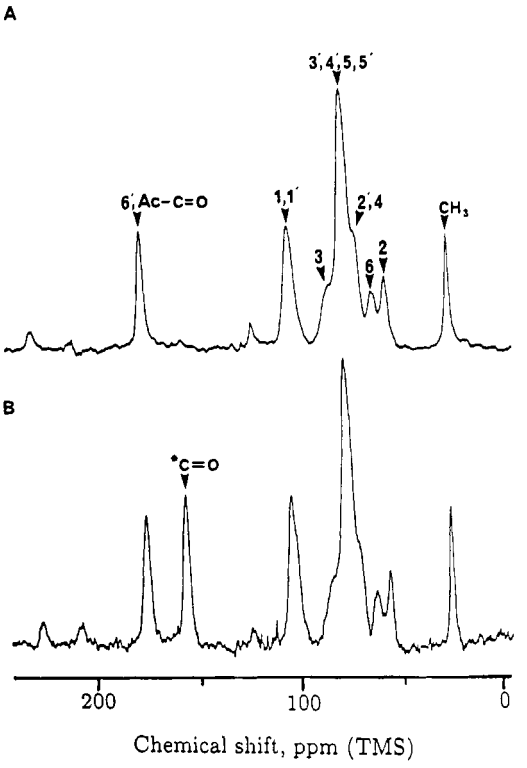


Figure 2. (A) ^{13}C CP-MAS NMR spectrum of native hyaluronate. (B) ^{13}C CP-MAS NMR spectrum of [^{13}C]EDC–HA adduct.

Table I. Chemical Shifts, Line Widths at Half Height, Relative Intensities (Calculated Intensities in Parentheses), and Assignment of the ^{13}C Spectrum of Native Hyaluronic Acid[a]

chemical shift (ppm)	line width (ppm)	relative intensity	assignment
24.7	1.9	0.87 (1)	acetyl-CH_3
55.7	3.8	1.00 (1)	2
62.3	4.9	0.96 (1)	6
70.1	4.5	1.59 (1–2)	2', 4
76.1	4.8	4.52 (4–5)	3', 4', 5, 5'
84.2	4.8	1.05 (1)	3
102.7	5.3	2.09 (2)	1, 1'
175.2	3.6	1.92 (2)	6', acetyl-CO

[a] Chemical shifts are referenced to external tetramethylsilane (TMS) and are uncorrected for bulk susceptibility.

The reaction of a carbodiimide with a carboxyl group generally proceeds through the addition of the free carboxylate to one of the double bonds of the diimide system to give an *O*-acylurea product.[24] In the presence of a nucleophile the acyl–nucleophile product will form, plus the urea of the carbodiimide. The second pathway, which accounts for hindered *O*-acylureas and occurs in the absence of added nucleophiles, is for the *O*-acylurea to rearrange to the more stable *N*-acylurea through an intramolecular acyl transfer. The scope of this chemical modification of high molecular weight HA by a variety of carbodiimides has been described recently.[25]

The ^{13}C CP-MAS spectrum of native hyaluronate is shown in Figure 2A. It was obtained at a spinning frequency of 3.63 kHz.

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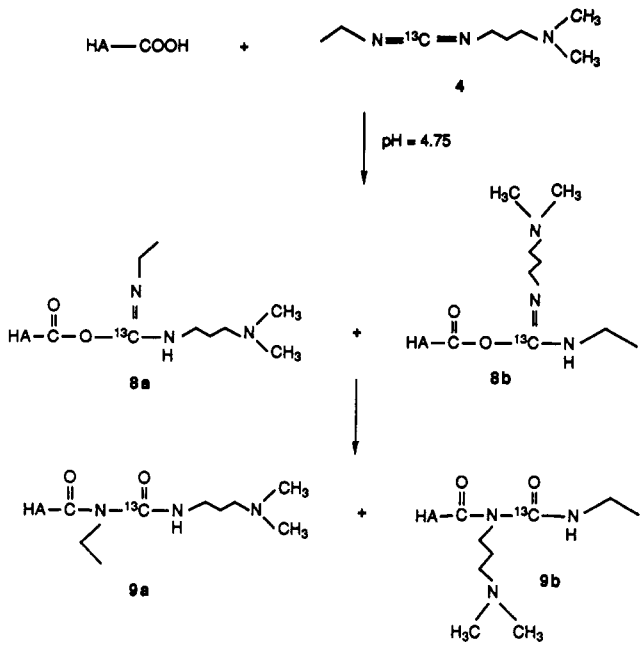
Scheme III. Possible Reaction Pathways for Coupling of [2-^{13}C]EDC with HA, Showing Regioisomeric *O*-Acylurea Adducts and *N*-Acylurea Rearrangement Products



Under these conditions at a field strength of 7.1 T, only the carbonyl groups of hyaluronate have significant rotational sideband intensity; these sidebands are labeled. The remaining lines were fit to Lorentzians, using a deconvolution program to disentangle the overlapped resonances in the main sugar carbon region. The chemical shifts, line widths, and relative intensities thus obtained are given in Table I. The summed intensity of all peaks, including the carbonyl sidebands, was normalized to 14 carbons. The unprotonated and methyl carbons were identified using a delay without decoupling sequence,[17] with a dipolar dephasing time of 40 μs. Methylene carbons can also be distinguished from methine carbons by this method if a somewhat shorter dephasing period is used. The remaining carbon signals were assigned by comparison with solution NMR.[26]

The data in Table I show that CP-MAS intensities obtained for this material are nearly quantitative; all peak intensities fall near integer numbers, with the exception of the strongly overlapped signals at 70.1 and 76.1 ppm. The sum of the latter pair of intensities corresponds nonetheless to that expected for six carbons. We can therefore anticipate that incorporation of isotope levels can be estimated from such spectra with an accuracy of around ±10%. The chemical shifts of this fibrous hyaluronate sample are uniformly nearly identical to those observed in solution NMR, with no more than 1 ppm deviation for the clearly resolved resonances or 3 ppm for the overlapped ones. Given that chemical shielding of sugar carbons is highly sensitive to sugar pucker changes or other conformational alterations, with shifts of 10 ppm or greater being noted in some cases,[27] these data strongly suggest that the conformations of hyaluronate in solution and in the fibrous state are very similar.

Reaction of hyaluronate with EDC selectively ^{13}C-labeled on the carbodiimide residue gave a polymeric adduct (Scheme III). This adduct was precipitated several times from ethanol to remove all traces of unreacted carbodiimide and small molecule byproducts. The purified polymer was studied by ^{13}C solid-state NMR, using CP-MAS. In addition to the native HA resonances, a new peak was identified at 156 ppm, consistent with a substituted urea (Figure 2B). The peak intensity corresponds to 0.025 ± 0.002 mol of linker/mol of monomer units. Unfortunately, this chemical shift does not unambiguously discriminate between the two *O*-acylurea isomers **8a** and **8b** or between them and the two *N*-acylurea isomers **9a** and **9b** which might be formed from them by rearrangement.

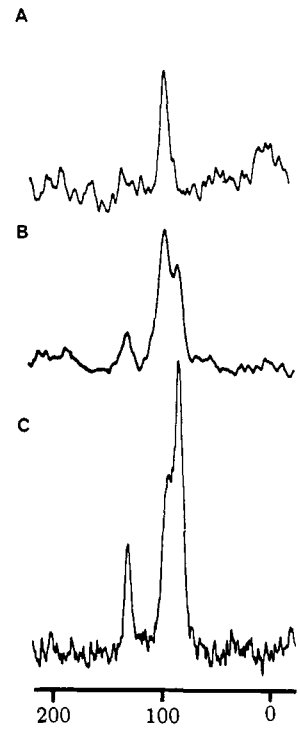
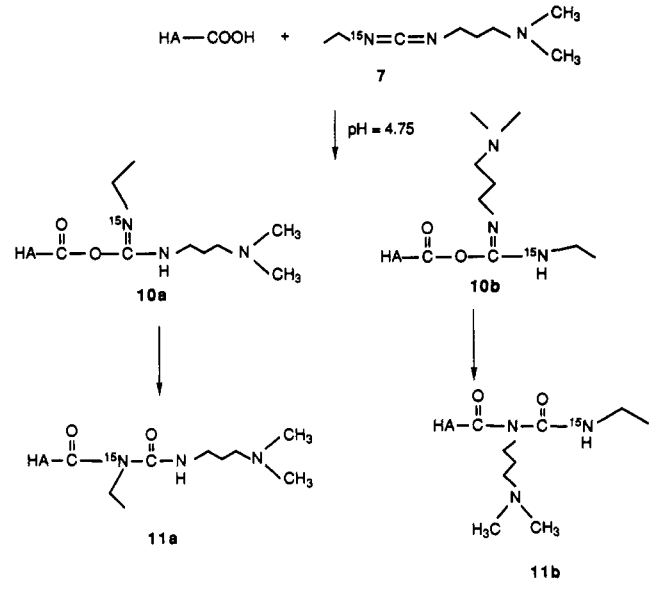


Figure 3. (A) ^{15}N CP-MAS NMR spectrum of native hyaluronate. (B) ^{15}N CP-MAS NMR spectrum of [^{15}N]EDC–HA adduct, 4 °C. (C) ^{15}N CP-MAS NMR spectrum of [^{15}N]EDC–HA adduct, 25 °C.

Scheme IV. Possible Reaction Pathways for Coupling of [1-^{15}N]EDC with HA, Showing Regioisomeric *O*-Acylurea Adducts and *N*-Acylurea Rearrangement Products



Next, EDC selectively ^{15}N-labeled on the ethylamine moiety was prepared and coupled with HA (Scheme IV). The ^{15}N solid-state CP-MAS NMR spectrum now showed three resonances (Figure 3C). The resonance at 96 ppm (relative to 5.6 M NH_4Cl in H_2O) was present also in the native polymer and was therefore assigned to natural abundance ^{15}N in the acetamido group of HA. Interrupted decoupling experiments showed the high-frequency peak at 133 ppm to be an unprotonated (tertiary) nitrogen. No significant MAS sidebands were observed even at slow spinning

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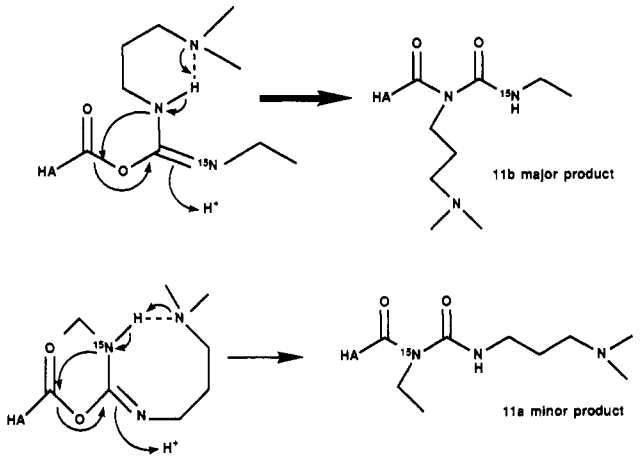
Scheme V. Proposed Route Leading to the Observed Major *N*-Acylurea Product (Heavy Arrow) and to the Minor Product (Lighter Arrow)



speeds, suggesting a small chemical shielding anisotropy (CSA). This tends to rule out formally double-bonded species, such as *O*-acylureas, which usually have large CSAs;[11] 1a additionally, the limited reference data available for *O*-acylureas suggest that they should appear at even higher frequencies. On the other hand, the chemical shift and CSA are entirely consistent with an *N*-acyl-*N*-alkylurea nitrogen such as that in compound **11a**, which would be formed by transfer of the acyl group to the ethylamine nitrogen.

The low-frequency peak, at 83 ppm, is a protonated nitrogen species with a chemical shift intermediate between that of a secondary amide and that expected for an alkylurea. Nonetheless, this resonance can be assigned to the latter species, **11b**. Its uncharacteristically high chemical shift can be attributed to the transfer of the acyl group to the other available nitrogen, resulting in increased double-bond character of the C–N bond and thus a paramagnetic shift. Deconvolution of the three peaks suggests a total isotope incorporation of 0.020 ± 0.004 mol of isotope/mol of monomer unit, close to that observed with the carbon label.

The effects of a lower reaction temperature (4 °C) on product distribution and on the rate of the rearrangement of *O*-acylurea were examined. Thus, [^{15}N]EDC was coupled to HA at 4 °C as previously described, and the isolated polymeric adducts were subjected to ^{15}N solid-state CP-MAS NMR spectroscopy. A longer reaction time (16 h) was allowed to compensate for the reduced reaction rate due to increased viscosity of the HA mixture at low temperature. The relative intensity of the two labeled species was unaffected by this reduction in reaction temperature; furthermore, no additional signals were observed (Figure 3B).

A rationale for the production of unequal amounts of isomeric *N*-acylureas in the coupling reactions of isotopically-labeled EDC is provided in Scheme V. It is likely that the rearrangement of the *O*-acylurea to an *N*-acylurea is catalyzed intramolecularly by one of the basic amine nitrogens. On the basis of this proposition, the rearrangement that involves a six-membered ring for proton abstraction by the terminal amine should be favored over the alternative eight-membered ring. This model accounts for the observation of **11b** as the major N-acylated product.

Formation of *N*-acylurea products from the reaction of a carboxylate with a carbodiimide in the absence of a nucleophile is well-documented.[28] Modifications of proteins with EDC have been reported to yield *N*-acylurea products that alter the DNA-binding properties of the modified proteins.[29] The reactions of *N*-acetylchondrosine, *N*-acetylchondrosine 6-sulfate, and heparin with EDC have been reported,[30] in which putative *O*-acylurea products were isolated and which rearranged to the corresponding *N*-acylureas upon alkaline treatment with $NaHCO_3$.

On the basis of the NMR results, therefore, we conclude that the initial O-acylated product of the coupling reaction between EDC and HA rearranges to give a mixture of two N-acylated isomers. These two isomers can readily be identified and characterized by solid-state NMR spectroscopy. The present results demonstrate the success of applying CP-MAS toward the detection of low-level chemical modifications in a biopolymer and in assigning unambiguous structures to complex macromolecules. The described approach could potentially be applied to the structural analysis of a variety of soluble and insoluble modified polymeric species. In addition, we have presented the first example of an unambiguous structural characterization of modified hyaluronate using solid-state NMR techniques. We are currently in the process of exploiting the carbodiimide methodology[31] for further functionalizations of hyaluronic acid, for potential use as a drug delivery vehicle, and for the production of novel biomaterials.

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Registry No. **1**, 141727-00-6; **2**, 109-55-7; **3**, 141727-01-7; **4**, 141727-02-8; **5**, 27421-70-1; **6**, 141727-03-9; **7**, 141727-04-0; $Et^{15}NH_2$·HCl, 84051-02-5; $^{13}CS_2$, 30860-31-2; $ClCO_2CH_3$, 79-22-1; thiophosgene, 463-71-8; hyaluronic acid, 9004-61-9.

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