Chemical Modification of Hyaluronic Acid by Carbodiimides

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Hyaluronic acid (HA) is a linear polysaccharide with repeating disaccharide units of glucuronic acid and *N*-acetylglucosamine and is found in the extracellular matrix of connective tissues. Reaction of high molecular weight sodium hyaluronate (NaHA, MW $\sim 2 \times 10^6$) with EDC at pH 4.75, either in the presence or absence of a primary diamine, gave the *N*-acylurea and *O*-acylisourea as NaHA–carbodiimide adducts. None of the expected intermolecular coupling with the amine component was observed. On the basis of this new observation, this method for chemical modification of HA was used in conjunction with new synthetic carbodiimides to prepare HA derivatives bearing lipophilic, aromatic, cross-linked, and tethered functional groups. The degree of conversion to NaHA–acylurea products appears to depend upon both the characteristics of various carbodiimides and the conformational structure of NaHA.

INTRODUCTION

Hyaluronic acid (HA), a naturally occurring linear polysaccharide, is composed of repeating disaccharide units of glucuronic acid and *N*-acetylglucosamine (*1*, *2*). Both monosaccharides have the β-D-anomeric configuration at C-1. The linkage from glucuronic acid to *N*-acetylglucosamine is (1→3) and the linkage from *N*-acetylglucosamine to glucuronic acid is (1→4). The nomenclature for this repeating disaccharide unit is [→4)-*O*-(β-D-glucopyranuronosyl)-(1→3)-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→] (*2*) (Figure 1). HA is widely distributed in animal tissues, present in high concentrations in synovial fluid and the vitreous body of the eye, and in loose connective tissues of rooster comb, umbilical cord, and dermis (*3*). All HA molecules are thought to have the same primary structure, but differences occur in the degree of polymerization of the HA polysaccharide chains obtained from different tissues (*4*). Sodium hyaluronate (NaHA) is characterized by its large hydrodynamic volume (*5*). NaHA with a molecular weight of 1 million has an intrinsic viscosity of 3000 mL/g (*6*). It absorbs water, cushions cells, and lubricates the soft tissues of joints. The wide application of the purified, high molecular weight HA in eye surgery is an example of the utility of HA as a noninflammatory, viscoelastic biomaterial (*7*).

Studies on the chemical modification of hyaluronic acid have been mainly concerned with its *cross-linking* and *coupling*. Divinyl sulfone, bisepoxides, formaldehyde, and bishalides have been used to cross-link HA to produce highly swollen gels or virtually insoluble, plastic materials, depending upon the degrees of cross-linking (*8a*,*b*, *9*, *10*, *11a*). Coupling reactions, on the other hand, can also alter the properties of HA. For example, extensive esterification of HA with monofunctional organic halides can produce water-insoluble films (*11b*). These chemically modified HA are thought to have surgical and medical value as long-lasting biomaterials, and as potential drug-delivery vehicles.

The generation of a free amino group on HA for further coupling reaction has been a subject of much interest, both at the polymer and oligosaccharide level. Preparation of an alkylamine derivative of HA oligosaccharide modified at its reducing end was used for radioactive labeling (*12*). For high molecular weight HA, alkaline N-deacetylation of its glucosamine moiety produced a free amino group on HA polymer chain, but concomitant degradations of HA via β-elimination in the glucuronic acid moiety was observed (*13*). Hydrazinolysis of high molecular weight HA ($>2 \times 10^6$ Da) was performed at 100 °C, to give a partially N-deacetylated HA, but the molecular weight was decreased by 1 order of magnitude (*14*).

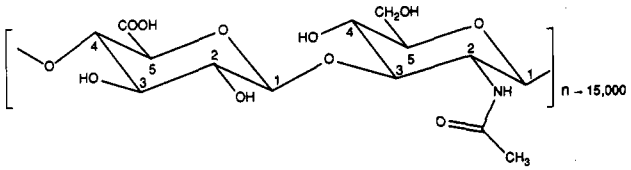

Figure 1. Hyaluronic acid is a linear polysaccharide, with repeating disaccharide units of glucuronosyl-β-1,3-*N*-acetylglucosamine linked by β-1,4-glycosidic bonds.

It was reported that the carbodiimide-catalyzed reaction of HA with monofunctional amines such as glycine methyl ester led to the formation of an amide linkage (*15*). Since carbodiimide–carboxylate reactions could be performed in aqueous solutions under mild conditions, we chose to explore carbodiimide-promoted coupling of HA carboxylic acid group with simple aliphatic diamines. When we began our studies using HA, difunctional amines, and carbodiimides, we expected to obtain an undegraded HA derivative with a free amino group. This was not realized, however, and only products from coupling of the carbodiimide to HA were observed. We now report the evidence for this reaction and the exploitation of this unexpected observation to prepare functionalized and cross-linked derivatives of HA.

EXPERIMENTAL PROCEDURES

General Experimental. Infrared spectra were taken on a Perkin-Elmer 1430 spectrophotometer. Only important, diagnostic peaks are reported. UV-vis spectra were taken on a Perkin-Elmer Lambda 4B spectrophotometer. Gas chromatography (GC) was performed on a Varian 3700 gas chromatograph (programmable temperature control) equipped with a flame ionization detector (FID), connected to helium carrier gas and interfaced to a HP 3380A integrator. The column used was DB-5 megabore (15 m × 0.5 mm) fused silica, at 30 psi He pressure. Thin-layer chromatography (TLC) was performed with

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Macherey-Nagel Polygram Sil G/UV$_{254}$ (40 × 80 mm, 0.25-mm silica gel) plastic TLC plates. Spots were visualized under UV light and/or under visible light by spraying the TLC plate with a ninhydrin spray solution (0.5% w/v of ninhydrin in water-saturated 1-butanol) or by dipping the TLC plate into a phosphomolybdic acid (10% w/w solution in EtOH) followed by heating with a heat gun. Flash column chromatography was performed under nitrogen pressure on Merck silica gel G (400–230 mesh). Low-resolution electron-impact mass spectra were obtained by using a Hewlett-Packard Model 5980A mass spectrometer interfaced to a HP 5710A GC. Only molecular ions (M^+) are listed. NMR spectra were obtained on a GE QE-300 spectrometer operating at 75 MHz for ^{13}C and 300 MHz for ^1H. Unless otherwise specified the chemical shifts for compounds dissolved in organic solvents are reported as ppm (δ) with $CDCl_3$ as standard. Chemical shifts of modified HA are reported as ppm (δ) with 3-(trimethylsilyl)-1-propanesulfonic acid sodium salt as the standard. The pH of the HA or modified HA samples was raised to about 14 by adding NaOD. The following abbreviations are used for peak multiplicities: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet. Concentration of HA was measured by the carbazole assay using glucuronolactone as standard (*16*). Intrinsic viscosity (IV) of HA and modified HA was measured with Cannon-Ubbelohde semimicro dilution type viscometer (size 75) at 37 °C. Hyaluronic acid was provided by MedChem Products, Inc., as its sodium salt, NaHA, which was purified from rooster comb by modifications of procedures described elsewhere (*17*). Unless otherwise stated, the molecular weight of HA used was from 1.5 to 2 million. Denatured ethanol containing 5% of methanol and 5% of 2-propanol was used in the purification of HA and its derivatives. The ninhydrin test was used to quantify the free primary amino groups of the amine-functionalized HA and D-glucosamine was used as the standard. The ratios of the reagents were the ratios of the molar equivalents based on the reacting functional groups. In the case of NaHA, it was the molar equivalents of its dissacharide units.

Procedures for Chemical Reactions *N*-(Benzyloxycarbonyl)-1,6-hexanediamine (8). To a solution of 1,6-hexamethanediamine (11.6 g, 100 mmol) in 50 mL of $CHCl_3$ was added dropwise at room temperature a solution of benzyl chloroformate (1.83 g, 10 mmol) in 50 mL of $CHCl_3$. The addition proceeded for 1 h, during which a white precipitate of the amine chloride was formed. The agitation was continued at room temperature for 5 h, the reaction mixture filtered, and the filtrate washed with water. The removal of the excess unreacted diamine was monitored by silica gel thin-layer chromatography ($CHCl_3$/MeOH/Et_3N = 90/10/5). The ninhydrin spray showed the disappearance of the purple spot at the bottom line on TLC plate, indicative of the complete removal of the unreacted 1,6-hexanediamine. The dried residue was weighed as 2.0 g. The crude product (1.2 g) was purified by column chromatography (silica gel, 0.8% MeOH, 5% Et_3N in $CHCl_3$) to provide 486 mg of the pure product (40.5%). ^1H NMR ($CDCl_3$): δ 1.26–1.43 (8 H), 2.60 (t, J = 6 Hz, 2 H, CH_2NH_2), 3.11 (m, 2 H, $-CH_2NHCOO-$), 5.08 (s, 2 H, CH_2Ph), 7.35 (m, 5 H, *Ph*). ^{13}C NMR ($CDCl_3$): δ 26.48 and 29.93 ($-CH_2-$) 33.60 (CH_2NH_2), 41.03 ($-CH_2NHCOO-$), 66.58 (CH_2Ph), 128.03, 128.47, and 136.70 (*Ph*), 156.38 (C=O).

***N*-[3-(Dimethylamino)propyl]-*N'*-[6-[(benzyloxycarbonyl)amino]hexyl]thiourea (9).** To a solution of *N*-(benzyloxycarbonyl)-1,6-hexamethylenediamine (8) (50 mg, 0.20 mmol) in 5 mL of $CHCl_3$ was added dropwise at room temperature a solution of 3-(dimethylamino)propyl isothiocyanate 7 (40 mg, 0.28 mmol) predissolved in 5 mL of $CHCl_3$. After stirring for 6 h at room temperature, the solvent was evaporated to give 77 mg of the crude product (91%, GC). ^1H NMR ($CDCl_3$): δ 1.27–1.68 (10 H, $-CH_2-$), 2.19 (6 H, R-N*Me*$_2$), 2.30–2.39 (2 H, CH_2NMe_2), 3.11 (2 H, $-CH_2NHCOO-$), 3.13–3.52 (4 H, $-CH_2NH-$), 5.00 (s, 2 H, CH_2Ph), 7.27 (m, 5 H, *Ph*). ^{13}C NMR ($CDCl_3$): δ 26.02, 28.64, and 29.54 ($-CH_2-$), 40.59, 44.31 (N*Me*$_2$), 44.41, 44.47, 66.20 (CH_2Ph), 127.70, 127.75, 127.84, 128.22, and 136.45 (*Ph*), 156.24 (C=O), 181.80 (C=S).

***N*-Ethyl-*N'*-octylthiourea (5).** The experimental procedure of the synthesis of 9 was adopted. Thus octylamine (402 mg, 3 mmol) and ethyl isothiocyanate (270 mg, 3 mmol) gave 660 mg of thiourea 5, which was 97.5% homogenous (GC). ^1H NMR ($CDCl_3$): δ 0.86 (t, J = 6.2 Hz, 3 H, $(CH_2)_7Me$), 1.24 (t, 3 H, CH_2Me_2), 1.35–1.47 (10 H), 1.58 (m, 2 H, $-CH_2CH_2NH-$), 3.38–3.45 (4 H, $-CH_2CH_2-NH-$), 5.77–5.80 (2 H, N*H*). ^{13}C NMR ($CDCl_3$): δ 13.97, 14.20, 22.51, 26.81, 28.91, 29.05, 29.11, 39.08, 44.34, 44.42, 181.12 (C=S).

***N*-Ethyl-*N'*-[6-[(benzyloxycarbonyl)amino]hexyl]thiourea (12).** The experimental procedure of the synthesis of 9 was adopted. Thus *N*-(benzyloxycarbonyl)-1,6-hexamethylenediamine (8) (100 mg, 0.4 mmol) and ethyl isothiocyanate (40 mg, 0.44 mmol) were combined in $CHCl_3$ to give 150 mg of the crude product with a purity of 86% (GC). ^1H NMR ($CDCl_3$) δ 1.18 (t, J = 7.2 Hz, 3 H, CH_3), 1.32 (4 H), 1.45–1.57 (4 H), 3.14 (t, J = 6.6 Hz, 2 H, $-CH_2NHCOO-$), 3.41 (4 H, $-CH_2NH-$), 4.96 (2 H, CH_2Ph), 7.26–7.33 (5 H, *Ph*). ^{13}C NMR ($CDCl_3$): δ 14.17, 25.81, 25.94, 28.64, 29.56, 38.94, 40.47, 43.91, 66.39 (CH_2Ph), 127.66, 127.90, 128.30, and 136.28 (*Ph*), 156.52 (C=O), 181.19 (C=S). MS: m/e 337.1 (M^+).

***N*-Ethyl-*N'*-(6-cyanohexyl)thiourea (15).** The experimental procedure of the synthesis of 9 was adopted. Thus 6-aminohexanenitrile (2.856 g, 22.8 mmol) and ethyl isothiocyanate (2.05 g) were combined in $CHCl_3$ to give 4.90 g of thiourea (15) (89.2% purity by GC). ^1H NMR ($CDCl_3$): δ 1.23 (t, J = 7.2 Hz, 3 H, CH_3), 1.52–1.73 (6 H), 2.37 (t, J = 7.0 Hz, 2 H, $CH_2C{\equiv}N$), 3.41–3.51 (4 H, $-CH_2NH-$), 5.77–5.80 (2 H, $-NH-$). ^{13}C NMR ($CDCl_3$): δ 13.68, 13.73, 16.39, 24.30, 25.19, 27.64, 38.45, 43.27, 119.32 (C≡N), 180.51 (C=S).

1,6-Hexamethylenebis(ethylthiourea) (23). To a solution of 1.351 g of ethyl isothiocyanate (15 mmol) in 10 mL of $CHCl_3$ was added dropwise 874 mg of 1,6-diaminohexane (7.5 mmol) predissolved in 10 mL of $CHCl_3$. The agitation continued for 2 h at room temperature as the reaction mixture gradually turned milky and viscous. The formed white solid was not soluble in hexane or Et_2O, poorly soluble in ethyl acetate and $CHCl_3$, and freely soluble in acetone and MeOH. Recrystallization of the crude product in MeOH improved its purity from 40% to over 75% (GC), and it was identified by ^1H NMR as bisthiourea 23. ^1H NMR ($CDCl_3$): δ 1.19 (6 H, CH_3), 1.22 (4 H), 1.56 (4 H), 3.46 (8 H, $-CH_2NH-$), 6.26–6.32 (4 H, N*H*). This material was used without further purification.

***p*-Phenylenebis(ethylthiourea) (26).** To a solution of 811 mg of *p*-phenylenediamine (7.5 mmol) in 15 mL of acetone was added 1.351 g of ethyl isothiocyanate (15 mmol). The reaction mixture was stirred 12 hr at room temperature and the formed precipitate was isolated by filtration, washed with cold methanol, and dried. The light brown precipitate was soluble in MeOH and DMSO, but not soluble in acetone, benzene, or $CHCl_3$. The solid product (510 mg) showed UV absorbance on silica gel TLC (R_f 0.6, CH_3Cl/MeOH/Et_3N = 90/10/5), and was homo-

Hz, 6 H, CH_3), 3.45–3.49 (q, J = 6.1 Hz, 4 H, $-CH_2NH-$), 7.31 (s, 4 H, *Ph*).

Ethyl[6-[(benzyloxycarbonyl)amino]hexyl]carbodiimide (13). To a suspension of mercuric oxide (100 mg) in dry acetone (15 mL) was added thiourea **12** (77 mg) predissolved in 5 mL of dry acetone and refluxed for 3 h. Black mercuric sulfide was formed and was removed by filtration through Celite, dried ($MgSO_4$), and concentrated to give 60 mg of the product with a purity of 93% (GC). ^{1}H NMR ($CDCl_3$): δ 1.11–1.45 (8 H), 3.06–3.16 (6 H, $-CH_2N{=}C{=}N-$ and CH_2NHCOO), 4.98 (2 H, CH_2Ph), 7.24 (5 H, *Ph*). ^{13}C NMR ($CDCl_3$): δ 16.58, 26.13, 26.29, 29.73, 31.00, 40.79, 41.34, 46.44, 66.38 (CH_2Ph), 127.91, 128.34, and 136.50 (*Ph*), 156.30 ($C{=}O$) IR: 2120 (s, $-N{=}C{=}N-$) cm^{-1}. MS: m/e 303.2 (M^+).

[3-(Dimethylamino)propyl][6-[(benzoyloxycarbonyl)amino]hexyl]carbodiimide (10) was prepared as described for carbodiimide **13**, except that the crude product was chromatographed with a column packed with silica gel ($CHCl_3/Et_3N$ = 95/5). Thus, 77 mg of the thiourea **3** gave 15 mg of pure carbodiimide **6**. ^{1}H NMR ($CDCl_3$): δ 1.24–1.74 (10 H), 2.22 (s, 6 H, $N(CH_3)_2$, 2.34 (t, J = 7.5 Hz, 2 H), 3.16–3.26 (6 H, $-CH_2N{=}C{=}N-$ and CH_2NHCOO), 5.08 (s, 2 H, benzyl methylene), 7.34 (5 H, benzene). ^{13}C NMR ($CDCl_3$): δ 26.23, 26.42, 29.28, 29.89, 31.11, 44.80, 45.47, 46.51, 66.55 (CH_2Ph), 128.04 and 128.46 (*Ph*), 156.38 ($C{=}O$). IR: 2126 (s, $-N{=}C{=}N-$) cm^{-1}. MS: m/e 360 (M^+).

Ethyltridecylcarbodiimide (3) was prepared as described for carbodiimide **13**. Thus 320 mg of *N*-ethyl-*N'*-tridecylthiourea (**2**) gave 180 mg of carbodiimide **3**, which was 97.4% homogeneous by GC. ^{1}H NMR ($CDCl_3$): δ 0.87 (t, 3 H, $CH_3(CH_2)_{12}$), 1.21–1.33 (20 H, 1.57 (2 H, $-CH_2CH_2N{=}C{=}N-$), 3.17–3.24 (4 H, $-CH_2N{=}C{=}N-$). IR: 2120.1 cm^{-1} (s, $-N{=}C{=}N-$).

Ethyl-3-octylcarbodiimide (6) was prepared as described for carbodiimide **13**. Thus 600 mg of *N*-ethyl-*N'*-octylthiourea (**5**) gave 434 mg of carbodiimide **6**, which was 97.2% homogeneous. ^{1}H NMR ($CDCl_3$): δ 0.87 (t, 3 H, $CH_3(CH_2)_7$), 1.20–1.26 (12 H), 1.56 (2 H, $-CH_2CH_2N{=}C{=}N-$), 3.19–3.24 (4 H, $-CH_2N{=}C{=}N-$). IR: 2128.5 cm^{-1} (s, $-N{=}C{=}N-$).

Ethyl[6-(trifluoroacetamido)hexyl]carbodiimide (18) was prepared as described for carbodiimide **13**. Thus 108 mg of *N*-ethyl-*N'*-[6-(trifluoroacetamido)hexyl]thiourea (**17**) gave 90 mg of carbodiimide (**18**). GC showed the compound decomposed at elevated temperature. ^{1}H NMR ($CDCl_3$): δ 1.23 (t, J = 7.2 Hz, 3 H, CH_3), 1.25–1.58 (8 H), 3.19–3.26 (m, 4 H, $-CH_2N{=}C{=}N-$), 3.35–3.77 (2 H, $CH_2NHCOCF_3$). IR: 2128.5 cm^{-1} (s, $-N{=}C{=}N-$).

1,6-Hexamethylenebis(ethylcarbodiimide) (24). The dehydrosulfurization of bisthiourea **23** with mercuric oxide in acetone was performed for 2 h, with the oil bath at 72 °C. Higher temperature and longer reaction time may cause polymerization. Thus, the crude product after the reflux of 76.8 mg of bisthiourea **23** and 192 mg of HgO (in acetone) was purified by extracting with cold hexane to give 22.5 mg of **24**. ^{1}H NMR ($CDCl_3$): δ 0.87 (6 H), 1.21–1.41 (4 H), 1.54–1.61 (4 H), 3.19–3.27 (m, 8 H, $-CH_2N{=}C{=}N-$). IR: 2124 cm^{-1} (s, $-N{=}C{=}N-$).

***p*-Phenylenebis(ethylcarbodiimide) (27)** was prepared following the method above for biscarbodiimide **24**, using 108 mg of the aromatic bisthiourea **26**. The crude product was filtered through a silica gel column to give 45 mg of aromatic biscarbodiimide **27**, which was 97.3% homogeneous (GC). The compound was stored in a refrigerator and used within 3 days of preparation. ^{1}H [illegible] $-CH_2N{=}C{=}N-$), 6.98 (s, 4 H, $-C_4H_4-$). IR: 2120.1 cm^{-1} (s, $-N{=}C{=}N-$). MS: m/z 214.1 (M^+), intensity 100.

General Procedure of Carbodiimide Reactions with NaHA. Sodium hyaluronate was dissolved in water to a concentration of ca. 4 mg/mL. For some reactions, as indicated below, amine was mixed with NaHA. The pH of the mixture was adjusted to 4.75 by 0.1 N HCl. The designed carbodiimide or commercially available carbodiimide was dissolved in water or 2-propanol, depending upon its solubility. After mixing HA and carbodiimide solutions, a pH increase was immediately observed. The reaction was monitored by pH meter and 0.1 N HCl was added dropwise to keep the pH at 4.75. The reaction was allowed to proceed for 2 h at room temperature. Then NaCl was added to make 5% w/v of the reaction mixture. Ethanol equal to 3 volumes of the reaction mixture was added and a stringy precipitate was obtained. The precipitate was redissolved for a second and third precipitation, thus removing all the unreacted or produced small organic compounds. The final precipitate was dissolved in deionized water to a concentration of no more than 6 mg/mL and was then lyophilized. The NMR sample solution of NaHA or modified NaHA was prepared by dissolving 5–10 mg of the lyophilized product into 1 mL of D_2O. The dissolution could be expedited by vortexing the sample and adding NaOD (sample pH ~14). The ratio of the reagents is defined as the molar equivalency ratio based upon the reaction of the carboxyl (HA) and carbodiimide functional groups.

***N*-Acylurea Products of HA and [3-(Dimethylamino)propyl]ethylcarbodiimide (EDC) (1a–e).** The above procedure was adopted in making these *N*-acylurea derivatives. The amount of reactants used and the analytical results are summarized as follows.

1a: The ratio of 1,6-diaminohexane/EDC was 3/1. The ratio of EDC/HA was 1/10. Thus NaHA (247.9 mg, 0.618 mequiv) and EDC (11.98 mg, 0.062 mmol) gave 221 mg of the lyophilized product (85% recovery). No amide linkage between HA and diaminohexane was formed. The purified product gave a negative ninhydrin test result. ^{1}H NMR (D_2O): δ 1.08 (t, J = 7.2 Hz, 3 H, CH_3CH_2N), 1.64 (m, 2 H), 2.18 (s, 6 H, $(CH)_3N$), 2.34 (t, J = 7.2 Hz, 2 H), 3.11 (m, 4 H, CH_2N).

1b: The ratio of 1,6-diaminohexane/EDC was 5/1. The ratio of EDC/HA was 1/10. Thus NaHA (246.4 mg, 0.615 mequiv) and EDC 11.78 mg, 0.062 mmol) gave 226 mg of the lyophilized product (88% recovery). The analytical results of ninhydrin and ^{1}H NMR are identical with those of **1a**.

1c: The ratio of 1,6-diaminohexane/EDC was 10/1. The ratio of EDC/HA was 1/10. Thus NaHA (247.7 mg, 0.618 mequiv) and EDC (11.84 mg, 0.062 mmol) gave 232 mg of the lyophilized product (89.5% recovery). The analytical results of ninhydrin and ^{1}H NMR are identical with use of **1a**.

1d: The ratio of 1,6-diaminohexane/EDC was 100/1. The ratio of EDC/HA was 1/10. Thus NaHA (122 mg, 0.304 mequiv) and EDC (11.78 mg, 0.062 mmol) gave 114 mg of the lyophilized product (85.4% recovery). The ninhydrin test result was negative.

1e: Thermally degraded NaHA with molecular weight of 60 000 was used in this reaction. The ratio of NaHA/1,6-diaminohexane/EDC was 1/1/1. Thus NaHA (264 mg, 0.66 mequiv) and EDC (126.5 mg, 0.66 mmol) gave 735 mg of the product which included **1e** and NaCl coprecipitate. No amide linkage between NaHA and diaminohexane was detected from NMR. The purified

during the ninhydrin test. ^{1}H NMR showed no amine α-methylene protons expected at δ 2.6. ^{1}H NMR (D_2O): δ 1.12 (t, J = 7.2 Hz, CH_3 of *N*-acylurea), 1.21 (t, CH_3 of *O*-acylisourea) (δ 1.12/δ 1.21 $\geq$ 4/1), 2.21 (s, $(CH_3)_2N$), 2.37, 3.15 (m, α-CH_2 of acylureas).

O-Acylurea Product of NaHA and EDC (1f). The above procedure was also followed. The reaction condition was the same as for **1e**, except that NaHA was not thermally degraded. Thus NaHA (250.8 mg, 0.627 mequiv) and EDC (120.2 mg, 0.627 mmol, in 20 mL of water) gave 873 mg of the product which included **1f** and NaCl coprecipitate. No amide linkage between NaHA and diaminohexane was detected from NMR. The purified product showed less than 1% of the free amino group during the ninhydrin test. ^{1}H NMR (D_2O): δ 1.12 (t, CH_3 of *N*-acylurea), 1.21 (t, J = 7.2 Hz, CH_3, of *O*-acylisourea) (δ 1.21/δ 1.12 $\geq$ 5/1), 2.21 (s, $(CH_3)_2N$), 2.38 (t, J = 7.2 Hz), 3.15 (m, α-CH_2 of acylureas).

Acylurea Product of NaHA and Carbodiimide 6 (7a–c). The above procedure was followed. 2-Propanol (3 mL) was used to dissolve the carbodiimide.

7a: The ratio of carbodiimide/NaHA was 1/20. Thus NaHA (454 mg, 1.13 mequiv) and carbodiimide **6** (10.82 mg, 0.056 mmol) gave 440 mg of **7a** (94.6% recovery). The proton uptake was 0.02 mmol. Intrinsic viscosity (IV): 2866 mL/g (starting NaHA, 2354 mL/g).

7b: The ratio of carbodiimide/NaHA was 1/10. Thus NaHA (381 mg, 0.95 mequiv) and carbodiimide **6** (18.2 mg, 0.095 mmol) gave 388 mg of **7b** (95.6% recovery). The proton uptake was 0.04 mmol. IV: 2730 mL/g (starting NaHA, 2354 mL/g).

7c: The ratio of carbodiimide/NaHA was 1/5. Thus NaHA (477 mg, 1.19 mequiv) and carbodiimide **6** (45.6 mg, 0.238 mmol) gave 465 mg of **7c** (89.0% recovery). The proton uptake was 0.1 mmol. IV: 2534 mL/g (starting NaHA, 2354 mL/g).

Acylurea Product of HA and Carbodiimide 3 (4a–c). The above procedure was followed. 2-Propanol (3 mL) was used to dissolved the carbodiimide. In all three reactions, the proton uptake was less than 0.005 mmol.

4a: The ratio of carbodiimide/HA was 1/20. Thus NaHA (444 mg, 1.1 mequiv) and carbodiimide **3** (15.4 mg, 0.055 mmol) gave 397 mg of **4a** (89.5% recovery). IV: 2401 mL/g (starting NaHA, 2354 mL/g).

4b: The ratio of carbodiimide/HA was 1/10. Thus NaHA (400 mg, 1.0 mequiv) and carbodiimide **3** (25.8 mg, 0.1 mmol) gave 386 mg of **4b** (90.8% recovery). IV: 2591 mL/g (starting NaHA, 2354 mL/g). Additional proton resonance to HA from **4b** was not observed, but acid hydrolysis of **4b** (pH = 1, boiling water bath, 4 h) produced ^{1}H NMR signals as follows. ^{1}H NMR (D_2O): δ 1.34, 1.37, and 2.14 (br band, aliphatic protons), 3.12 (α-CH_2 of acylureas).

4c: The ratio of carbodiimide/HA was 1/5. Thus NaHA (529 mg, 1.32 mequiv) and carbodiimide **3** (66 mg, 0.264 mmol) gave 573 mg of **4c** (96.3% recovery). IV: 2651 mL/g (starting NaHA, 2354 mL/g).

Acylurea Products of NaHA and Carbodiimide 10 (11a,b). The above procedure was followed.

11a: The ratio of carbodiimide/NaHA was 1/10. Thus NaHA (252 mg, 0.63 mequiv) and carbodiimide **10** (25.2 mg, 0.063 mmol) gave 232 mg lyophilized product **11a** (92% recovery). IV: 2760 mL/g (starting NaHA, 2354 mL/g). ^{1}H NMR (D_2O): δ 1.25, 1.58, 2.12 ($N(CH_3)_2$), 3.07 (α-CH_2 of acylureas), 7.39 (*Ph*).

11b: The ratio of carbodiimide/NaHA was 1/5. Thus NaHA (126 mg, 0.315 mequiv) and carbodiimide **10** (25.2 **11b** (94.5% recovery). The aqueous solution of **11b** was accidentally left stirring overnight without cooling. The temperature of the solution was measured as 42 °C. IV: 2323 mL/g (starting NaHA, 2354 mL/g). ^{1}H NMR (D_2O): δ 1.26, 1.42, 1.62, 2.21, 3.09 (α-CH_2 of acylureas), 7.41 (*Ph*).

Acylurea Product of NAHA and Carbodiimide 13 (14). The above procedure was followed. The ratio of carbodiimide/NaHA was 1/5. Thus NaHA (90.6 mg, 0.225 mequiv) and carbodiimide **13** (18 mg, 0.045 mmol) gave 105 mg of the lyophilized product **14**. The degree of coupling was measured as 16% by UV (240 nm). The proton uptake was 0.035 mmol. IV: 2734 mL/g (starting NaHA, 2354 mL/g). ^{1}H NMR (D_2O): δ 0.93 (CH_3 of *N*-acylurea), 1.33, 1.56, 3.15 (α-CH_2 of acylureas), 7.43 (*Ph*).

Acylurea Product of HA and Carbodiimide 18 (19). The above procedure was followed. The ratio of carbodiimide/NaHA was 1/5. Thus NaHA (400 mg, 1.0 mequiv) and carbodiimide **18** (60 mg, 0.2 mmol) gave 392 mg of **19** (85.2% recovery). The proton uptake was 0.06 mmol. IV: 3115 mL/g (starting NaHA, 2966 mL/g). ^{1}H NMR (D_2O): δ 1.11 (t, CH_3 of *N*-acylurea), 1.21 (CH_3 of *O*-acylisourea) (δ 1.11/δ 1.21 $\geq$ 5/1), 1.36, 1.49, 2.61 (CH_2NH_2 from decomposition of $CH_2NHCOCF_3$ in NaOD), 3.12 (α-CH_2 of acylureas).

NaHA Cross-Linked by Biscarbodiimide 24 (25a,b). The general procedure of the carbodiimide reaction with NaHA was adopted. The ratio of carbodiimide/NaHA was 1/10.

25a: The NaHA concentration was adjusted to 3.2 mg/mL and the carbodiimide concentration in 2-propanol was 2.75 mg/mL. Thus NaHA (423 mg, 1.05 mequiv) and biscarbodiimide **24** (11 mg, 0.05 mmol) gave 382 mg of **25a** (88% recovery). The proton uptake was 0.04 mmol. During the purification process, the precipitated NaHA derivative was only partially soluble in water. ^{1}H NMR (D_2O): δ 1.11 (t, CH_3 of *N*-acylurea), 1.36, 1.51, 3.12 (α-CH_2 of *N*-acylurea).

25b: The NaHA concentration was adjusted to 5.2 mg/mL and the carbodiimide concentration in 2-propanol was 0.92 mg/mL. Thus NaHA (416 mg, 1.04 mequiv) and biscarbodiimide **24** (11 mg, 0.05 mmol) gave 400 mg of **25b** (93.6% recovery). The proton uptake was 0.03 mmol. IV: 2554 mL/g (starting NaHA, 2354 mL/g).

NaHA Cross-Linked by Biscarbodiimide 27 (28a,b). The general procedure of the carbodiimide reaction with NaHA was adopted.

28a: The NaHA concentration was adjusted to 4.2 mg/mL and the carbodiimide concentration in 2-propanol was 1.5 mg/mL. The ratio of carbodiimide/HA was 0.18. Thus sodium hyaluronate (386 mg, 0.96 mequiv) and biscarbodiimide **27** (18 mg, 0.084 mmol) gave 389 mg of **28a** (96.4% recovery). The proton uptake was 0.12 mmol. The dried polymer from the first precipitation swelled in 200 volumes of water at 4 °C to form a cross-linked insoluble gel. The absorption of water appeared to reach an equilibrium after 5 days. Part of the gel was cut and stored in water at room temperature.

28b: The NaHA concentration was adjusted to 4.2 mg/mL and the carbodiimide concentration in 2-propanol was 0.89 mg/mL. The ratio of carbodiimide/HA was 0.10. Thus NaHA (404 mg, 1.01 mequiv) and biscarbodiimide **27** (10.7 mg, 0.05 mmol) gave 393 mg of **28b** (95% recovery). The proton uptake was 0.08 mmol. IV: 3073 mL/g (starting NaHA, 2354 mL/g). ^{1}H NMR (D_2O): δ 1.09 (t, CH_3CH_2 of *N*-acylurea), 1.19 (t, CH_3CH_2 of *O*-acylisourea) (δ 1.19/δ 1.09 $\geq$ 2/1), 1.62, 3.04 (α-CH_2 of acylureas).

***N*-Ethyl-*N'*-(6-aminohexyl)thiourea (16).** To a so-

THF was added 600 mg of borane–dimethyl sulfide (7.92 mmol). The reactor was heated to reflux, and dimethyl sulfide was distilled off. After 3 h, the mixture was cooled to room temperature and quenched with 3 mL of 1 N HCl. Then pH was adjusted to 9 with 1 N NaOH and saturated with K_2CO_3. Et_2O was added to separate the layers. The aqueous layer was extracted twice with $CHCl_3$, and the combined organics were concentrated to give crude thiourea **16** (52%). The ^1H NMR spectrum showed the ratio of the amine α-methylene protons over nitrile α-methylene protons as over 10/1. ^1H NMR ($CDCl_3$): δ 1.21 (t, J = 7.2 Hz, 3 H, CH_3CH_2NH), 1.24–1.64 (8 H, 2.66 (t, J = 6.6 Hz, 2 H, CH_2NH_2), 3.44 (4 H, $-CH_2NH-$), 5.92 (2 H, NH). ^{13}C NMR ($CDCl_3$): δ 14.29 (CH_3CH_2), 26.37, 26.58, 28.95, 33.34, 39.02, 41.87 ($-CH_2NH-$), 44.20 (CH_2NH_2), 181.25 (C=S).

***N*-Ethyl-*N'*-[6-(trifluoroacetamido)hexyl]thiourea (17).** To an aqueous solution of 350 mg of thiourea **16** (1.5 mmol) mixed with 5 mL of 0.2 M $NaHCO_3$ was added 480 mg of ethyl thiotrifluoroacetate (3 mmol). The reaction was continued at room temperature overnight and a white precipitate was formed. The product was isolated by ether extraction to give 317 mg of a white solid, which was 84.4% homogeneous by GC. ^1H NMR ($CDCl_3$): δ 1.24 (t, J = 7.2 Hz, 3 H, CH_3CH_2), 1.37–1.40 (m, 4 H), 1.58–1.64 (m, 4 H), 3.38 (t, J = 6.6 Hz, 2 H, $CH_2NHCOCF_3$), 3.47 (4 H, $-CH_2NH-$). ^{13}C NMR ($CDCl_3$): δ 14.18 (CH_3CH_2) 25.99, 28.62, 28.77, 39.06, 39.65, 44.08, 65.79, 117.74 (CF_3), 157.22 (C=O), 181.22 (C=S).

Detrifluoroacetylation of HA Derivative 19 to Amine-Functionalized HA (20a). HA–trifluoroacetamide (**19**) (12.6 mg) was treated for 14 h at room temperature and pH 11. Then 0.1 N HCl was added to adjust the pH to 7.0–7.5. NaHA reaction products were precipitated with ethanol as described above. The precipitate was collected after centrifugation. The ethanol precipitation was repeated three times and the final aqueous solution was lyophilized to give 11.9 mg of a white, fibrous material. To test the amount of free amino group attached to the NaHA polymer chain, an aqueous solution of the NaHA–trifluoroacetamide (**19**) (1 mg/mL) was prepared and a quantitative ninhydrin test was performed. The percentage ratio of the detected free amine over NaHA was 3.1%. IV: 2865 mL/g (starting material (**19**), 3115 mL/g).

Detrifluoroacetylation of HA Derivative 19 to Amine 20b. The experimental procedure for **20a** was adopted except that the pH of the reaction mixture was adjusted to 11.5–12.0. Fourteen milligrams of the starting material (**19**) was used. The precipitate of **20b** was less stringy than that of **20a**. The lyophilized solid weighed 19.3 mg. Apparently, NaCl coprecipitated with and adhered to the NaHA derivative and was therefore also collected. The percentage ratio of the detected free amine over NaHA was 4.2%. IV: 2286 mL/g (starting material (**19**), 3115 mL/g).

RESULTS AND DISCUSSION

Commercially available carbodiimides have been widely used for carboxyl activation, such as in protein modification (*18*, *20*). EDC, [3-(dimethylamino)propyl]ethylcarbodiimide, for example, is a water-soluble carbodiimide. The following mechanism of the carbodiimide reaction in protein modifications is generally accepted: carbodiimide reacts with carboxyl groups to form an unstable, intermediate *O*-acylisourea, which, in the absence of nucleophiles, rearranges to a stable *N*-acylurea "by way of

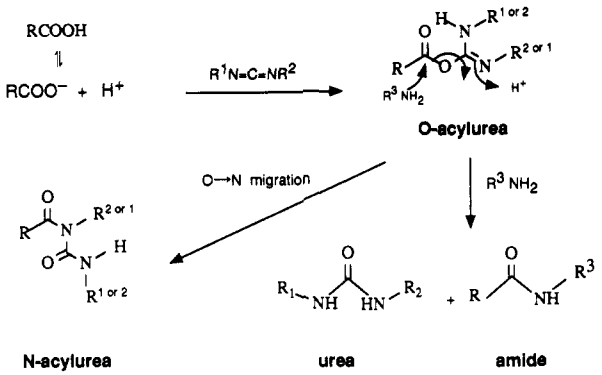

Figure 2. Mechanism of reaction between carboxylic acid and carbodiimide with and without primary amines.

a primary amine as nucleophile, the *O*-acylisourea formation is followed by a nucleophilic attack, forming an amide linkage between the amine and the acid (*18*) (Figure 2). An acidic environment is needed to catalyze the reaction, presumably through the protonation of the carbodiimide nitrogen. The reduced electron density at the carbodiimide central carbon is favorable to the nucleophilic attack of the carboxylate anion. At pH 4.75, carbodiimide nitrogens appear to be sufficiently protonated, while HA mainly exists as the carboxylate. The proton is not only a catalyst, but also a reagent. The stoichiometry of this process shows that one proton is consumed to form the urea compound. Thus the reaction can be monitored by the observed increase of pH and the consumption of hydrochloric acid during the reaction necessary to keep the reaction pH at 4.75.

Amidation reactions of glycosaminoglycans and primary amines catalyzed by carbodiimides have been reported. One example by Danishefsky and Siskovic is the reaction between glycine methyl ester (the amine component) and hyaluronic acid. The mechanism of the reaction was reported to be the same as mentioned above, i.e., the "condensation of the uronic acid moiety with EDC to form the *O*-acylurea followed by the displacement of the substituted urea by the amino group" (*15*). Similarly, we attempted to use diaminohexane and EDC to build an amide linkage between HA and the diamine.

The concentration of NaHA aqueous solution was adjusted to ca. 4 mg/mL, so that the solution would not be too viscous for thorough mixing with the added chemicals. A preliminary study without primary amine showed that carbodiimide was very reactive toward HA. The reaction started instantly at pH 4.75, as demonstrated by the immediate pH rise upon the addition of EDC. The attempted EDC-activated amidation using 1,6-diaminohexane was performed at the same pH. An initial pH surge was also observed. The equivalency ratios of the diamine to EDC in the reactions varied from 3/1 to 100/1, with the notion that the increased amine would favor the replacement reactions. The percentage equivalency ratio of EDC to NaHA was maintained at 10% as the base for comparison.

The reactions were allowed to proceed at room temperature for 2 h and in each case a pH increase was observed during the first hour at a progressively decreasing rate. This suggested that the formation of *O*-acylisourea had occurred, but it was not clear whether the replacement of amine or O → N migration of the *O*-acylisourea also proceeded during the reaction. Neither the displacement nor migration involve net proton gain or loss, according to the suggested mechanism (Figure 2). A ninhydrin test

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