Nonbiodegradable Hyaluronan Derivative Prepared by Reaction with a Water-Soluble Carbodiimide

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Reaction of hyaluronan (HA) with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) yielded a water-soluble polysaccharide with 40 % of modified HA carboxyl groups. ^{1}H and ^{13}C NMR measurements revealed that hyaluronan contained EDC bound by a nonspecific acylurea-type linkage. A test with testicular hyaluronidase showed that the modified hyaluronan was resistant to enzymic degradation.

Hyaluronan (hyaluronic acid, HA), discovered 65 years ago, belongs to the most attractive biomaterials. HA is composed of disaccharide repeating units of D-glucuronic acid and *N*-acetyl D-glucosamine. The unique viscoelastic properties of its aqueous solution are exploited widely in *e.g.* medical, pharmacological, and cosmetic applications. In the biological environment, HA can be depolymerized by action of enzymes as well as oxygen radicals. Many hyaluronan derivatives and conjugates with chemotherapeutics and physiologically active substances have been prepared and commercialized [1]. Hydroxyl and carboxyl groups in HA are the sites for linking other molecules. Activation of the HA hydroxyl groups is usually performed by the cyanylation agents such as cyanogen bromide [2]. More efficient and less hazardous chemistry employs the HA functionalization through the carboxyl groups.

Water-soluble carbodiimide is a widely used activator of carboxyl groups in the glycoconjugate chemistry. Recently, we have provided evidence of high reactivity of polysaccharide carboxyls with hydrazide groups of adipic acid dihydrazide in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) [3]. Attempts of other authors to couple amino groups to HA in the presence of EDC failed or led to very low yields [4—6]. Instead, coupling reaction of carbodiimides to HA resulted in acylurea-type adducts. To differentiate between the two types of possible acylurea products, namely *N*-acylurea and *O*-acylisourea derivatives of HA, the solid-state NMR spectroscopy was employed [7]. The exclusive presence of *N*-acylurea derivatives of high-molecular-mass HA was deduced.

Here we report the characterization of water-soluble derivative of HA prepared by reaction with EDC at mild conditions. Good water solubility of the product enabled its analyses by conventional NMR as well as testing of its resistance to the enzymic (testicular hyaluronidase) degradation.

EXPERIMENTAL

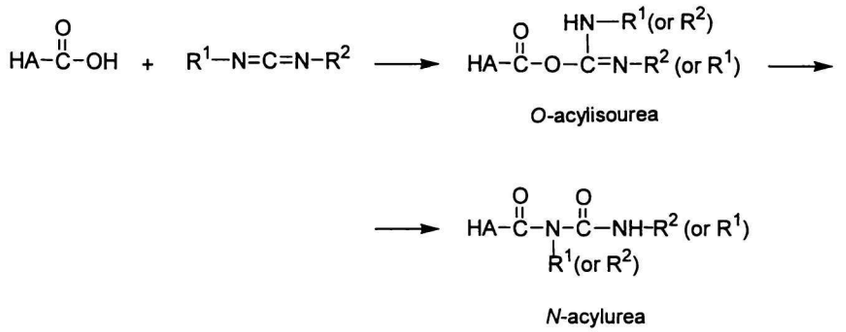
The sample of the high-molecular-mass hyaluronan (M_r = 250 000, polydispersity D = 1.15) was from CONTIPRO Chemical Co. (Czech Republic). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and 2-(*N*-morpholino)ethanesulfonic acid (MES) and its sodium salt were from Sigma (U.S.A.), other chemicals were from Lachema (Czech Republic). The bovine testicular hyaluronidase (EC 3.2.1.35) was purchased from the SEVAC a.s., Prague (Czech Republic).

The content of the free carboxyl groups in the original HA and the final product of EDC-HA was determined by the method of potentiometrical titration. The solution of the polysaccharide, after passing through the cation exchanger Dowex 50X × 2 (H^+-form), was titrated with aqueous solution of KOH using a combined electrode to the point of equivalence. The degree of substitution was calculated as the difference in the contents of carboxyl groups determined for EDC-HA and HA.

For NMR measurements the samples were dissolved in 0.5 cm^3 of D_2O (99.99 mole %) in 5-mm tubes. Spectra were recorded at 25 °C, on a Bruker DPX AVANCE 300 spectrometer, operating at 300 MHz for ^{1}H and 75.46 MHz for ^{13}C. The acetone was used as internal standard (δ = 2.225 for ^{1}H and δ = 31.07 for ^{13}C). The ^{1}H spectra were measured immediately after dissolving HA-EDC adduct.

Modified Hyaluronan

HA (130 mg) was dissolved in 20 cm^3 of MES buffer



R^1 = CH_3 - CH_2 -

R^2 = $(CH_3)_2N$ - CH_2 - CH_2 - CH_2 -

Scheme 1

(c = 0.05 mol dm^{-3}, pH 6.0) and 100 mg of EDC was added. The reaction mixture was stirred for 10 h at room temperature. The pH was maintained at 6.0—6.5 with addition of 0.1 mol dm^{-3} HCl. After completion of the reaction, the mixture was filtered through a paper filter (Whatman No. 3). The reagent excess and the low-relative-molecular-mass components were removed by repeated ultrafiltration (Amicon PM 10) of solution. The purification process effectiveness was monitored by using HPLC. The high-relative-molecular-mass product was freeze-dried. The yield of EDC-HA product was 105 mg. The infrared spectra were taken using a Nicolet Magna-IR 750 instrument.

Kinetics of Enzymatic Degradation

Commercial testicular hyaluronidase, 135 TRU (turbidity-reducing units), was added to 10 cm^3 of a solution (ρ = 0.002 g cm^{-3}) of the biopolymer in aqueous $NaNO_3$ (c = 0.1 mol dm^{-3}). The reaction was carried out under a gentle shaking at 37 °C. Sample aliquots (200 mm^3), withdrawn in the intervals 5, 10, 30, 60 min were heated for 5 min in a boiling water bath. After cooling, the precipitated enzyme was separated by centrifugation and the clear supernatant was submitted to HPLC analysis. Two columns in series packed with Biospher GM 300 and GM 1000 were used with HPLC instrument (Vývojové dílny, Czech Republic) equipped with differential refractometric detector. Pullulan standards were used as molecular-mass reference materials [8].

RESULTS AND DISCUSSION

Attempt of chemical binding of amines to HA by an EDC activation of HA carboxyl groups revealed that the only product detected was an acylurea adduct of EDC and HA. As the acylurea products of HA could be also commercially interesting [6], we have focused on the more detailed chemical characterization including their enzymic degradability.

The reaction of a carbodiimide with carboxyl groups of HA starts with the addition of diimide system of EDC to carboxylate to give *O*-acylisourea product (Scheme 1). In the absence of strong nucleophile (*e.g.* hydrazine) *O*-acylisourea can further undergo a structural rearrangement or less probably slowly hydrolyze back to carboxylic groups [9]. Structural rearrangement of *O*-acylisourea proceeds through an intramolecular acyl transfer to stable *N*-acylureas (Scheme 1). By keeping the set of experimental conditions (see Experimental) we have received 40 % conversion of carboxyl group as found by potentiometric titration. As HA is considered to be very sensitive to acid or basic degradation, the reaction was carried out at pH 6 for prolonged time. However, the small degradation was still unavoidable. The chemical bond formation of HA with EDC was observed on infrared spectra by appearing of new bands at 1701 cm^{-1} ν(C=O), 1645 cm^{-1} ν(amide I), and 1555 cm^{-1} ν(amide II).

In the ^{1}H NMR spectrum of product, the signals in the range 3.4—4.8 were assigned to HA [10], and singlet at 2.17 was attributed to CH_3 group of *N*-Ac. The remaining signals belong to acylurea parts of the structure: 1.35 (t) $\mathbf{CH_3}CH_2N$, 3.07, 3.06 (both s) $N(CH_3)_2$, 2.09 (overlapped multiplets) $CH_2\mathbf{CH_2}N$. The chemical shift of the CH_3 proton at 1.35 suggests the *O*-acylisourea structure [6].

In the ^{13}C NMR spectrum (Fig. 1) of HA-EDC adduct, except of signals of HA [10], there are also the chemical shifts of acylurea: 14.47 $\mathbf{CH_3}CH_2$, 25.11 $CH_3\mathbf{CH_2}$, 37.10, 56.00, 50.60 $CH_2CH_2CH_2$. The presence of CH_2 groups was confirmed using the DEPT pulse sequence. The signals for C=O group at 156.72, 156.20 indicate the presence of two *N*-acylurea isomers. The more intensive signal at 156.20 may belong to isomer with $NHCH_2CH_3$ arrangement [7].

Fig. 2 presents dependence of the M_r values on the time of enzymic reaction. As expected, the native HA showed depolymerization with the highest rate at first 10 min. (The degree of polydispersity increased

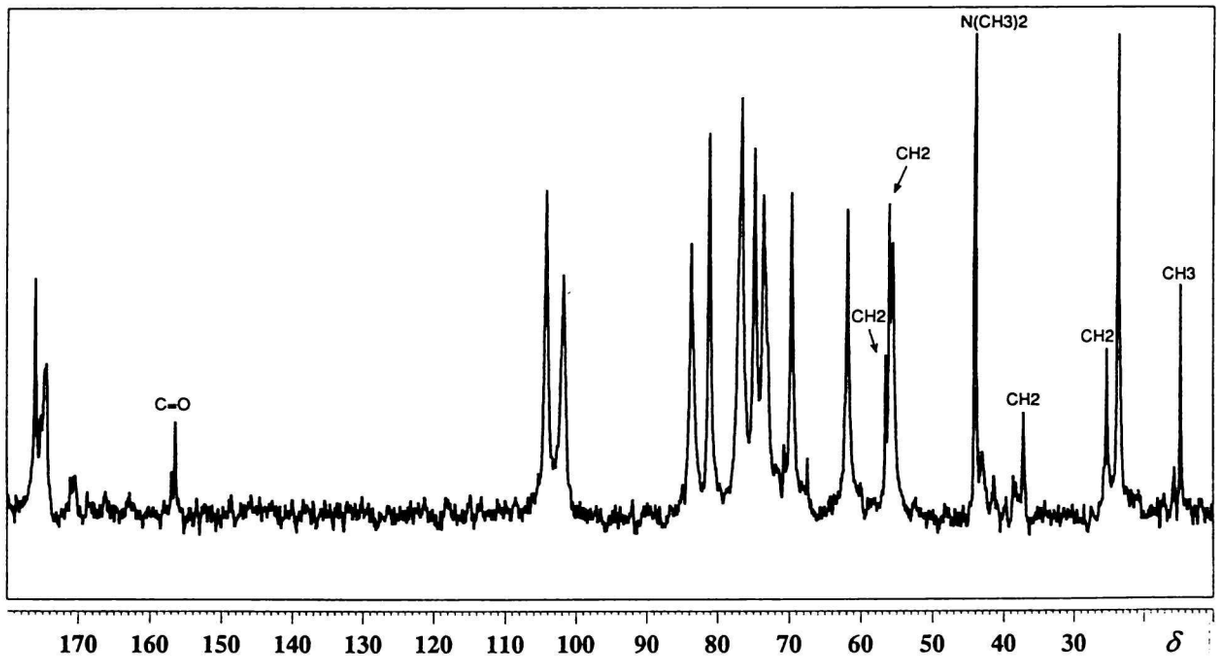


Fig. 1. Proton-decoupled ^{13}C NMR spectrum of EDC-HA in D_2O at 25 °C.

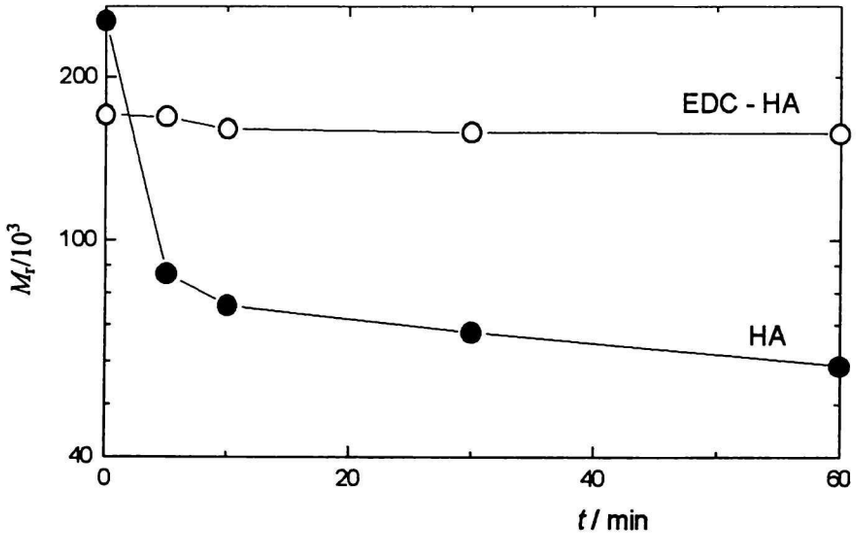


Fig. 2. Depolymerization kinetics of HA and EDC-HA by action of hyaluronidase.

from $D = 1.15$ to $D = 1.33$ at the same time.) On the contrary, EDC-modified HA did not show any depolymerization at all. This observation unveiled that roughly half of carboxyl groups that were modified can be enough to prevent the effective enzymatic attack by hyaluronidase. The chemical modification of HA with EDC can reduce biodegradability of HA while its biocompatibility may be retained.

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