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ORIGINAL RESEARCH

Molecular characterization of two host–guest associating hyaluronan derivatives†

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ABSTRACT: Molecular characteristics were determined of two high-molecular-weight water-soluble hyaluronan derivatives, namely β-cyclodextrin (HA-β-CD) and *N*-acylurea (EDC-HA). The weight-average molecular weight (M_w) of HA-β-CD and of EDC-HA, determined with a multi-angle light scattering detector connected on-line to a size exclusion chromatographic system, was respectively 185.3 and 86.8 kDa. However the M_w value determined for the equimolar mixture of the two HA derivatives equaled 556.0 kDa. Similarly, the gyration radius of the above equimolar mixture, Rg = 80.6 nm, was significantly greater than the values found for the single HA derivative, i.e. 40.2 nm for HA-β-CD and 23.8 nm for EDC-HA. These data indicate that the two kinds of substituents, bound to the polymeric chains, form host–guest/inclusion complexes resulting in polymacromolecular associates/aggregates. Copyright © 2003 John Wiley & Sons, Ltd.

KEYWORDS: hyaluronan derivatives; host–guest/inclusion complexation; light scattering; polymacromolecular aggregation/association; size exclusion chromatography; supramolecular biosystems

INTRODUCTION

The term 'hyaluronan' was assigned by Balázs *et al.* (1986) to a polysaccharide composed of regularly alternating units of D-glucuronic acid and *N*-acetyl-D-glucosamine linked by β-(1→3) and β-(1→4) linkages. This (endogenic) glycosaminoglycan, discovered first by Meyer and Palmer (1934) in the vitreous humor of cattle eyes, is present in all tissues and body fluids of vertebrates. Under physiological conditions, i.e. *in vivo*, hyaluronan is a polyanion where the corresponding counter-cations are Na^+, K^+, H^+, etc. The pK_a value of the polymer, estimated to be 2.9, indicates that the sample recovered from an acidic solution (pH < 2.9) should yield hyaluronic acid (the H^+ form), while at neutral as well as at alkaline pH conditions the samples isolated are salts, i.e. hyaluronates. In the present article the term hyaluronan (HA) is, however, preferably used, accepting thus the suggestion of Balázs *et al.* (1986).

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†Dedicated to Dr Endre A. Balázs, the architect of the 'Hyaluron-a-Polis'.

Abbreviations used: CD, cyclodextrins; DEA, diethylazodicarboxylate; DS, degree of substitution; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; HA, hyaluronan; LS, light scattering; MALS, multi-angle light scattering; MES, 2-(*N*-morphodino)ethanesulfonic acid; SEC, size exclusion chromatography; SCV, single capillary viscometer.

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At present, on producing the (commercially available) HA samples, originating from rooster combs, *Streptococcus zooepidemicus*, *Streptococcus equi*, etc., a whole range of physico-chemical procedures have to be applied, such as protein enzymatic digestion, HA ion-pair (e.g. cetylpyridiniumchloride) precipitation, membrane/molecular ultrafiltration, HA non-solvent precipitation and/or lyophilization. The mean molecular weight of the endogenic, native biopolymer ranges from hundreds of thousands up to several millions of Daltons. Independently of the polymer molecular weight, the (sodium, potassium) hyaluronate samples are very soluble in water as well as in aqueous salt solutions. However the polymer H^+ form or, e.g., Cu^{2+}-, Zn^{2+}- (macro)chelate complexes demonstrate significantly different solution properties (Mangani *et al.*, 1999; Burger *et al.*, 2001) when the solvent is H_2O or, e.g., the physiological (NaCl) solution. In the case of cetylpyridinium^{1+} as counter-cation, formed during the HA ion-pair precipitation from the aqueous solution, the cetylpyridinium hyaluronate can be extracted/dissolved in organic solvents, e.g. in an aliphatic alcohol.

The hydrodynamic/rheological behavior of the aqueous HA solutions is so exceptional that most other polymer solutions fail to duplicate its viscoelastic properties (Oviatt and Brant, 1993). In solution, HA chains assume an expanded 'somewhat stiff' random coil conformation. The size of the HA 'globule' varies with pH and salt concentration, as would be expected for a flexible polyelectrolyte (Cleland, 1968). Gibbs *et al.* (1968) found that at pH 2.5 the HA solutions were more elastic and

exhibited a 'paste-like' nature on gentle shaking or stirring, while at pH 1.5 and at pH 7 these solutions showed entirely different dynamic rheological behavior. The authors have suggested that the paste-like behavior may be attributed to a pronounced stiffening of the HA chains. They assumed the existence of a critical balance between the repulsive forces (provided by the ionized carboxyl groups) and the attractive interactions (electrostatic or hydrogen bonds mediated) operating between the molecular chain elements. At alkaline pH, hydrogen bonds taking part in the structural organization of HA chains are destroyed and this results in a large loss of the intrinsic stiffness and the formation of a more compact, flexible random coil (Ghosh *et al*., 1993).

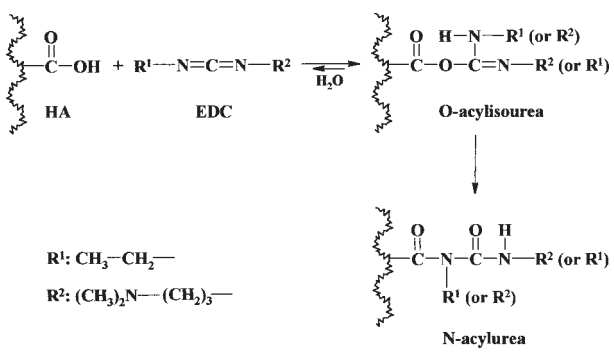
The three types of reactive groups on the HA macromolecule, i.e. *N*-acetyl, carboxyl and hydroxyl, are involved in the chemical modification/derivatization of this biopolymer:

- Deacetylation of the -NH-$COCH_3$ groups present in the native HA was performed by heating (80–100°C) of the aqueous HA solution alkalinized with NaOH. The amino derivative formed was then re-acetylated with [^{3}H]- or [^{14}C]-acetanhydride (Orviský *et al*., 1990). Although such deacetylation–acetylation treatment has been successfully applied to label the HA macromolecules, the recovered biopolymer has a significantly lower mean molecular weight. Alkaline or (strong) acidic pH conditions alone or combined with sample heating are accompanied with degradation of the HA macromolecules and 'denaturation' of their higher order structure (Šoltés *et al*., 1996).
- Esterification, namely the reaction of the -COOH (-COO^-) groups, present in the HA macromolecule, has been carried out with various aliphatic/aromatic alcohols. While the HA ester (HYAFF®; Fidia Farmaceutici S.p.A., Abano Terme, Italy) with a low degree of substitution (DS) maintains the water solubility of the sample, the HA derivatives with a higher DS value are insoluble both in H_2O and in aqueous (salt) solutions.
- Eterification reactions with participating -OH groups of the HA have most frequently been involved at crosslinking of HA macromolecules and subsequent hydrogel (HYLAN®; Biomatrix Inc., Ridgefield, NJ, USA) formation. HYLAN® A, for example, is an ~6 MDa (mean molecular weight) hyaluronan derived at rooster comb tissue treatment with formaldehyde. It is believed that the HYLAN® A polymer might be 'crosslinked' via a small amount of the link-protein-type molecule present in the preparation (Dr Endre A. Balázs, Matrix Biology Institute, Ridgefield, NJ, USA; personal communication). The mean molecular weight of HYLANs®, prepared by this method, varies between 5 and 25 MDa. The protein content of the hydrogel formed ranges from 0.4 to 0.8%. HYLAN® B, a solid gel, is prepared even at room temperature within minutes by a readily occuring reaction of HA hydroxyls with divinyl sulfone in aqueous alkaline solution.

Hyaluronan itself, discovered almost 70 years ago (Meyer and Palmer, 1934), is a biopolymer with many applications. The unique viscoelastic properties of the aqueous HA solutions are exploited widely in ophthalmology (for 'viscosurgery'), rheumatology (for 'viscosupplementation'), in cosmetic preparations, etc. Many hyaluronan water-soluble/insoluble derivatives, conjugates, (hydro)gels bearing, e.g., physiologically active substances/chemotherapeutics have been prepared and commercialized (Chabreček *et al*., 1990; Šimkovic *et al*., 2000). Novel polymer analog reactions are currently being searched for, with a great interest in finding efficient, non-destructive methods for the modification/derivatization of HA macromolecules. The new attractive HA biomaterials are applied as drug carriers, scaffolds for reconstruction of missing tissue, etc.

Carbodiimides belong to widely used (water-soluble) activators/reactants in glycoconjugate chemistry. Water-soluble hydrochloride salts of carbodiimides are exploited, e.g., for the modification/derivatization of proteins, for reacting with the substrate carboxyl group(s) resulting in efficient/easy formation of amides. Besides its high reactivity, the hydrochloride salt of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC; cf. Scheme 1), CAS no. 25952-53-8, is characterized by good solubility properties: solubility in water (100 mg/mL) and solubility in methylene chloride (20 mg/mL).

Cyclodextrins (CDs) are cyclic oligosaccharides composed of D-glucose units linked through α-(1→4) bonds. In (aqueous) solutions the CD molecules are characterized by their higher order structure with a void cavity in the center and hydroxyl groups occupying the outer surface (Szejtli, 1982). As the cavity exhibits a hydrophobic character, CDs (in aqueous solutions) can host non-polar molecules of appropriate dimensions. The β-CD, CAS no. 7585-39-9, forms inclusion complexes with a wide variety of amphiphilic or lipophilic substances/drugs



Scheme 1.

by taking up the whole drug molecule or some part of it into the cavity (cavity diameter = 0.78 nm).

The aim of the paper presented here is to provide broader knowledge on the findings observed at molecular characterization of two original (semisynthetic) water-soluble high-molecular-weight HA substances, namely the EDC-HA and HA-β-CD derivatives, each one individually as well as in the form of a bi-component mixture. For this goal a multi-angle light scattering (MALS) photometer was applied both in off-line batch mode and connected on-line to a size exclusion chromatographic (SEC) system. The multidetector SEC system used was equipped with four on-line detectors: a MALS photometer, a single capillary viscometer, a diode-array UV–vis spectrophotometer, and a differential refractometer.

EXPERIMENTAL

Materials and chemicals. The sample of HA used was supplied by CPN Ltd, formerly CONTIPRO (Ústí nad Orlicí, Czech Republic). The molecular parameter, M_w, determined for this biopolymer was 647.1 kDa. Further data relating to this HA sample have been described in detail previously (Šoltés *et al*., 2002).

2-(*N*-morpholino)ethanesulfonic acid (MES), its sodium salt, as well as the hydrochloride of EDC were purchased from SIGMA (St Louis, MO, USA). Diethyl azodicarboxylate (DEA), β-CD, and triphenylphosphine used were the products of Fluka Chemie AG (Steinheim, Germany). Water used was of Milli-Q_{RG} quality (Water Purification System; MILLIPORE Corporation, Bedford, MA, USA). All other chemicals, of p.a. purity grade, were the products of Merck (Darmstadt, Germany) or of Lachema (Brno, Czech Republic).

Synthesis of EDC-HA derivative. Briefly, HA (130 mg) was swollen-dissolved in 20 mL of MES buffer (50 mM, pH 6.0) and 100 mg of EDC were added into the reaction vessel. The mixture of reactants was stirred for *ca* 10 h at room temperature. The reaction pH was maintained at 6.0–6.5 with addition of 0.1 M HCl. Then the reaction mixture was filtered through a paper filter (Whatman no. 3). The aqueous solution was concentrated by ultrafiltration through an Amicon PM-10 membrane (M_w cut-off ≥10 kDa). The resulting sample concentrate was repeatedly (total five times) treated by the dissolution and ultrafiltration steps mentioned earlier and dried by lyophilization. The sample yield was 105 mg; as found by the potentiometric titration, a 40% conversion of the HA carboxyl groups was achieved (Bystrický *et al*., 2001). On combining the sample FT-IR spectrum with ^{1}H and ^{13}C NMR spectroscopic analysis, the chemical structure of the derivative synthesized was deduced to be an *N*-acylurea derivative of HA (cf. Scheme 1).

Synthesis of HA-β-CD derivative. Briefly, a suspension containing 0.3 g of HA plus 0.44 g of DEA in 20 mL of dioxane was admixed to a dispersion of 1.14 g of β-CD plus 0.87 g of triphenylphosphine in 100 mL of dioxane. The reaction mixture was stirred overnight (for *ca* 20 h) at ambient temperature. Then most of the dioxane was evaporated (without heating) under reduced pressure. Ethanol (150 mL) was added to the reaction vessel. The product of pasty consistency was recovered by filtration using a glass-fiber filter. The 'solid' sample obtained was then dissolved in water (300 mL), and the aqueous solution was concentrated by ultrafiltration through an Amicon PM-10 membrane (M_w cut-off ≥10 kDa) and further treated as described above. The sample yield was 0.53 g; the content of the free carboxyl groups in the HA-β-CD derivative was lower by *ca* 9.2%, as compared with that found in the native HA sample. The FT-IR spectrum of the HA-β-CD sample was unequivocally indicative of esterification of HA macromolecules (Šoltés *et al*., 1999). The ^{13}C NMR spectrum of the derivative synthesized does not, however, allow to determination of which particular OH group(s) of β-CD was(were) involved in the reaction with HA carboxyl(s).

Light scattering. The measurements in the off-line batch mode were carried out at room temperature using a MALS photometer Dawn DSP-F (WYATT Technology; Santa Barbara, CA, USA); the vertically polarized He–Ne laser wavelength was 632.8 nm. The angular distribution of the intensities of the scattered light was monitored simultaneously, by means of an array of photodiodes. The calibration constant for transforming the photodiode output voltage into the Rayleigh factor, $R(\theta)$, was calculated using toluene as a standard [$R(\theta)_{toluene} = 1.406 \times 10^{-5}$ cm^{-1}]. The normalization of the photodiodes was carried out by measuring the intensity of the light scattered by the concentrated solution of bovine serum albumin (Sigma; A 7888)—a globular protein assumed to act as an isotropic scatterer. The sample investigated was swollen-dissolved (overnight) in aqueous NaCl (0.15 M). Prior the measurements each sample solution was filtered through an 0.2 μm cellulose acetate filter (Millipore Corporation) into the measuring cell, K5. The MALS data acquisition and analysis software used was Astra 4.50 (Wyatt Technology). (The angle fit order = 2; the concentration fit order = 1.)

The specific refractive index increment, dn/dc, of the sample solution was determined at 35°C, at a wavelength of 632.8 nm, by a KMX-16 differential refractometer (LDC Milton Roy; Rochester, NY, USA). The dn/dc value found (0.150–0.151 mL/g) was fairly identical for each sample solution investigated.

Size exclusion chromatography. The multidetector SEC system consisted of an Alliance 2690 separation module (Waters, Milford, MA, USA) and of four on-line detectors connected in series: a home-made single capillary viscometer (SCV), a UV–vis spectrophotometer (model 996 PDA; Waters), the MALS Dawn DSP-F photometer (Wyatt Technology), and a differential refractometer (DRI; model 410; Waters). The experimental conditions consisted of: 0.15-M aqueous NaCl as the mobile phase; 35°C; 0.2 mL/min eluent flow-rate; 200 μL sample injection volume; 0.2 mg/mL injected sample concentration. Each sample, prior its SEC analysis, was clarified by filtration through an 0.2 μm cellulose acetate

filter (Millipore Corporation). The column set was composed of a guard pre-column and two stainless-steel columns (both 7.8 mm × 30 cm) packed with TSK gel PW (G6000 and G5000, 17 μm particle size) from TosoHaas (Montgomeryville, PA, USA).

SCV: the dimensions of the capillary tube were 0.02 inch of internal diameter and 20 inch of length. At the applied eluent flow-rate of 0.2 mL/min the SCV detector demonstrated an approximate shear-rate of 250 s^{-1}.

UV–vis: light absorbance was measured at 206 nm.

DRI: since the instantaneous polymer concentration was monitored by the UV–vis spectrophotometer the function of the DRI detector was to check the sample impurities not absorbing at 206 nm.

RESULTS

The molecular characteristics (M_w, Rg) of the EDC-HA derivative were preliminarily estimated by the off-line batch MALS measurements. Table 1 summarizes the data observed for the EDC-HA biopolymer by both the off-line batch MALS and on-line SEC-MALS methods. The SEC-MALS profile recorded on analyzing the EDC-HA sample is represented in Fig. 1. A small secondary peak was unambiguously revealed also by the UV–vis and DRI detectors. The UV–vis spectrum of this low-molecular-weight sample component/impurity indicated one absorbance maximum at far-UV light. Along with the EDC-HA chromatographic record, Fig. 1 shows the SEC calibration curve determined on combining the output signals of the MALS and UV–vis monitors. The (initial) steep part of the curve might indicate a tendency of the sample molecules to aggregate. However the $Rg = f(M)$ power law function observed (cf. Fig. 2) indicates relatively low dimensions of the EDC-HA macromolecules. Moreover, at a certain molecular weight the Rg value of the EDC-HA biopolymer is (significantly) lower than the value of the HA-β-CD derivative.

The SEC-multi-detector analysis of the HA-β-CD biopolymer revealed the presence of a low-molecular-weight impurity (cf. Fig. 3). Yet, contrary to the EDC-HA sample analysis, the calibration curve observed on investigating the HA-β-CD biopolymer did not indicate any tendency to self-aggregation of sample macromolecules. The $[\eta] = f(M)$ power law function determined

Table 1. Molecular characteristics of the two HA derivatives synthesized

SAMPLE		EDC-HA		HA-β-CD	
Parameter	Unit	MALS	SEC-MALS	SEC-MALS	SEC-SCV
M_n	kDa	—	51.0	106.2	—
M_w	kDa	91.2	86.8	185.3	—
M_z	kDa	—	156.1	302.9	—
Rg	nm	25.2	23.8	40.2	—
$[\eta]$	dL/g	—	—	—	3.60

M_n = number-average molecular weight; M_z = z-average molecular weight; $[\eta]$ = limiting viscosity number (intrinsic viscosity).

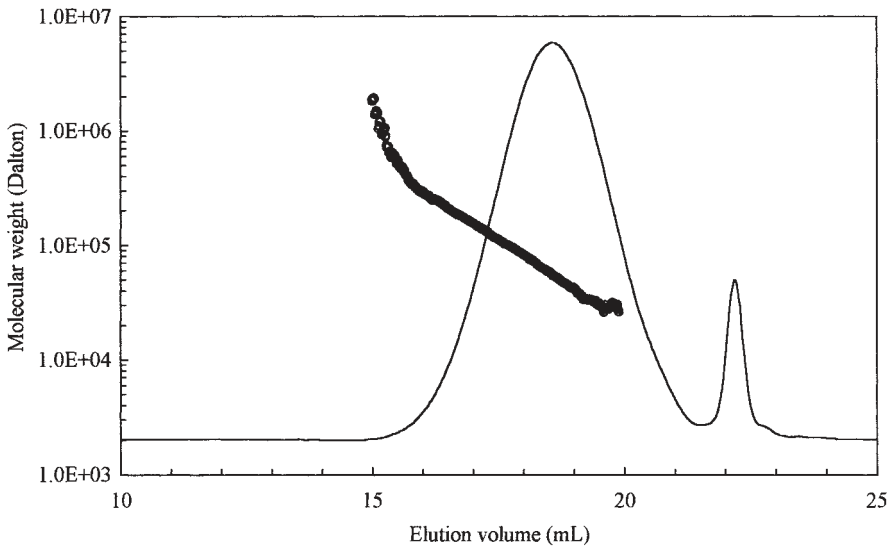


Figure 1. SEC-MALS record of the EDC-HA derivative and the SEC calibration curve.

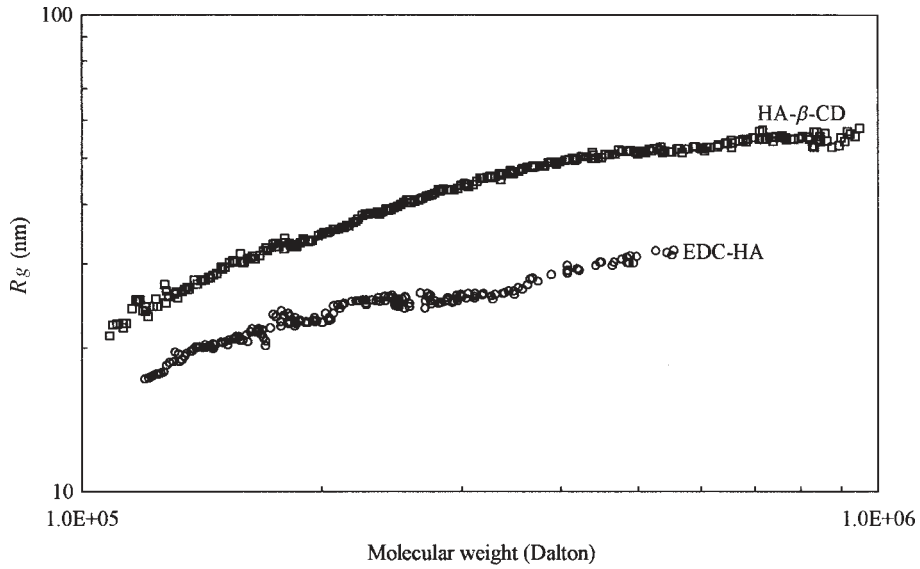


Figure 2. $Rg = f(M)$ power law functions for the EDC-HA and HA-β-CD derivatives analyzed.

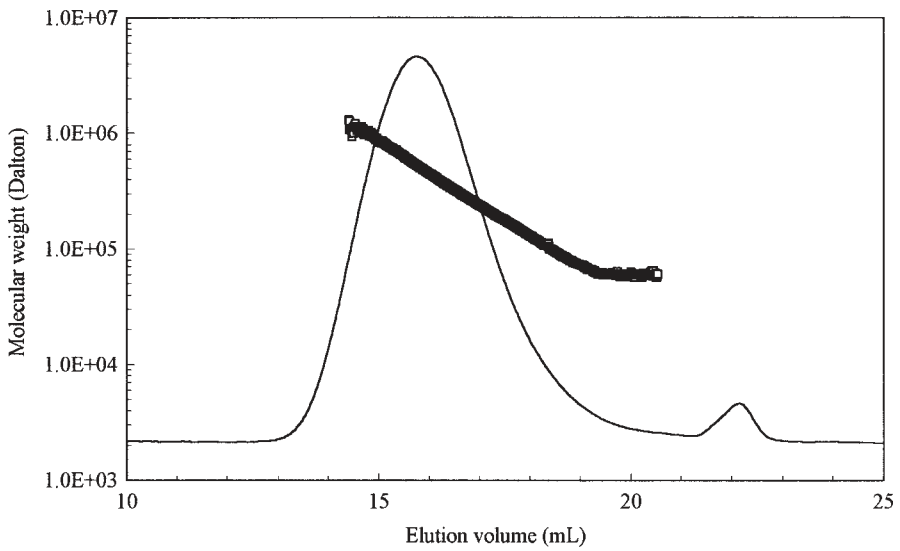


Figure 3. SEC-MALS record of the HA-β-CD derivative and the SEC calibration curve.

for the HA-β-CD sample on combining the output signals of three detectors, namely SCV, UV–vis, and MALS, is represented in Fig. 4.

The molecular-weight parameters of the mixtures of the EDC-HA and HA-β-CD biopolymers, determined with the off-line batch MALS method, are listed in Table 2. From a quantitative point of view, the values of M_w and Rg determined for the given mixtures significantly exceeded those observed on investigating each macromolecular component individually (cf. Table 1). [Since the nominal values, assuming a simple ([1] + [1]) additivity, would be: M_w = 276.5 kDa and Rg = 65.4 nm, the tendency of forming large(r) associates/aggregates of the macromolecules of the HA derivatives is meaningful.] The intensity of the scattered light determined for the EDC-HA or HA-β-CD biopolymer sample individually as well as for the mixture at equimolar concentration is represented in Fig. 5. As is evident, there is a great difference between the MALS signal determined experimentally and the one calculated on

Table 2. Molecular characteristics of EDC-HA and HA-β-CD mixtures

SAMPLE		[EDC-HA]/[HA-β-CD] ≈ [4]:[1]	[EDC-HA]/[HA-β-CD] ≈ [1]:[1]
Parameter	Unit	MALS	MALS
M_w	kDa	293.0	556.0
Rg	nm	69.6	80.6

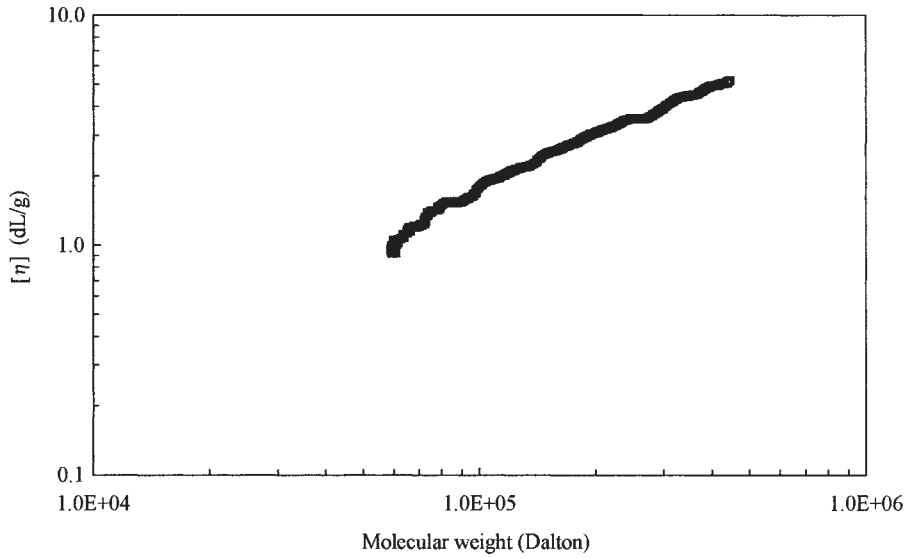


Figure 4. $[\eta] = f(M)$ power law function for the HA-β-CD derivative analyzed.

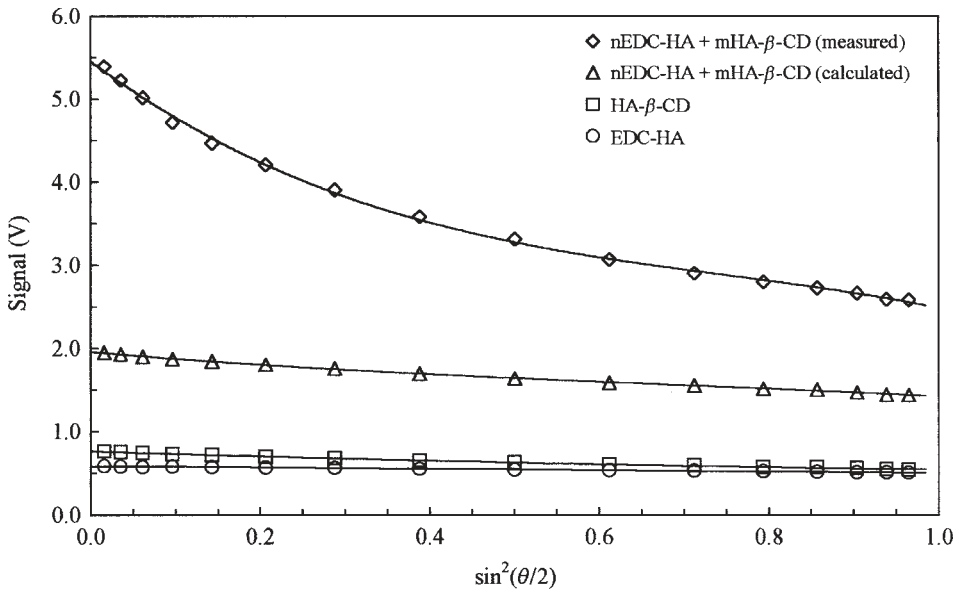


Figure 5. Intensities of the light scattered by the macromolecules of the two individual HA derivatives, i.e. EDC-HA or HA-β-CD, as well as by the nEDC-HA × mHA-β-CD associates/aggregates as measured or calculated on assuming a simple [1] + [1] additivity.

assuming the absence of association/aggregation of the macromolecules. The angular variation of the light scattering plotted in the form $P(\theta)^{-1}$ against $\sin^2(\theta/2)$ for the samples investigated is shown in Fig. 6. Since the $d[P(\theta)^{-1}]/d[\sin^2(\theta/2)]$ value is proportional to Rg^2, the dimension(s) of the 'globule' formed on association is significantly greater than the dimension(s) of each one single macromolecular component.

On injecting the EDC-HA and HA-β-CD ([1]:[1]) mixture into the SEC apparatus the results found were M_w = 156.1 kDa and Rg = 39.6 nm. This finding appears to suggest that during the SEC separation procedure the associates, formed unequivocally under static conditions, became dissociated due to the continually repeated disturbance of the thermodynamic equilibrium.

DISCUSSION

HA derivatives synthesis

The reaction of EDC with a carboxyl group of HA led to the formation of an *O*-acylisourea type intermediate (cf. Scheme 1). The *O*-acylisourea further underwent either a structural rearrangement through intramolecular

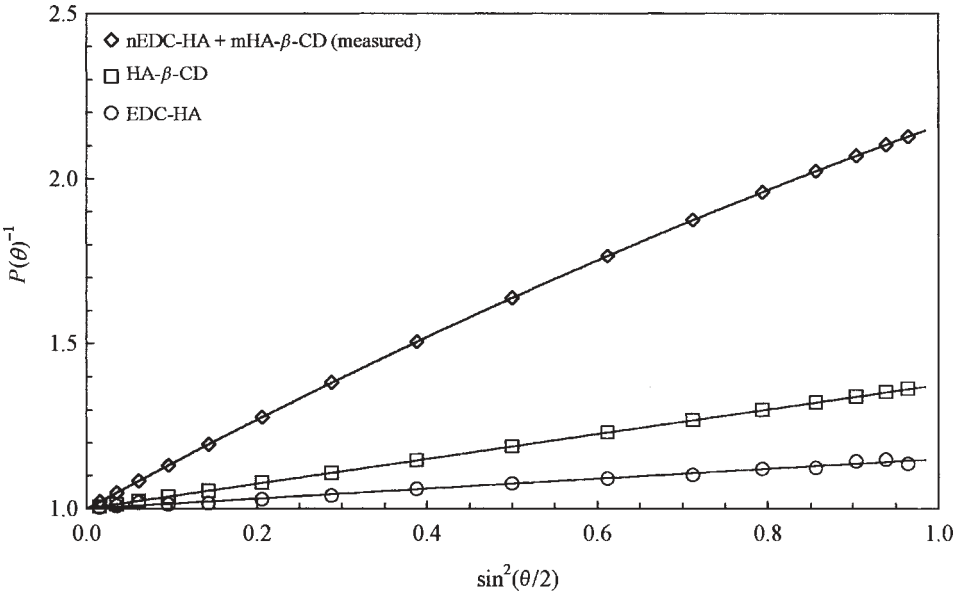


Figure 6. $P(\theta)^{-1}$ vs $\sin^2(\theta/2)$ plot of the two individual HA derivatives, i.e. EDC-HA or HA-β-CD, as well as of the nEDC-HA × mHA-β-CD associates/aggregates.

acyl transfer to a stable *N*-acylurea or the *O*-acylisourea intermediate may have hydrolyzed slowly back (Hoare and Koshland, 1967), yielding the HA carboxyls. Thus the low-molecular-weight component/impurity found in the EDC-HA sample could also have originated from the product decomposition/hydrolysis. Such a hypothesis is supported partly by the observation of a gradual increase of opalescence/turbidity of the EDC-HA sample solution when stored in the refrigerator. The most unfavorable finding from the EDC-HA sample molecular characterization was, however, a really 'massive' degradation of the native HA polymer chain during the reaction, represented by Scheme 1. The fact of the decrease of the M_w value from 647.1 kDa (valid for the native HA) to the value of 86.8 kDa did not classify the above derivatization reaction to be particularly proper from the point of view of the HA derivatization accompanied by sample molecular weight increase.

Preliminary assays for preparing a CD derivative of HA, e.g. on coupling β-CD to HA using epichlorohydrin or chloromethyloxirane, were completed successfully as concerns sample yield. They did, however, collapse from the point of view of maintaining the molecular weight of the resulting product. In order to process the reaction under mild conditions, the diethyl azodicarboxylate and triphenylphosphine system (Mitsunobu, 1981) was applied. On working with this system in an anhydrous aprotic solvent, alcohols are initially activated by the formation of alkoxyphosphonium salts, which subsequently alkylate acidic components. This reaction, which was successfully used in the synthesis and transformation of various kinds of natural products, exhibits stereospecificity, functional selectivity and regioselectivity. However, as evident from the molecular-weight parameter M_w = 185.3 kDa valid for the HA-β-CD biopolymer, its polymeric chain became partially degraded.

Light scattering/MALS

Static light scattering (LS), often called elastic or total intensity LS, belongs to the favorite methods in investigating colloids, such as solutions of macromolecules, micelles, associates/aggregates, as well as (semi)solid (dispersed) particles. The excess of the Rayleigh factor for a molecularly homogenic/uniform polymer solution is described by the following equation:

$$R(\theta) = K \cdot [M \cdot P(\theta) \cdot c - 2A_2 \cdot M^2 \cdot P^2(\theta) \cdot c^2 + \cdots] \quad (1)$$

or, more often, eqn (1) is rewritten to eqn (2), which converges more rapidly:

$$K \cdot c/R(\theta) = 1/[M \cdot P(\theta)] + 2A_2 \cdot c + \cdots, \quad (2)$$

where K is the optical constant, $K = [2\pi^2 \cdot n_0^2 \cdot (\mathrm{d}n/\mathrm{d}c)^2]/(\lambda_0^4 \cdot N_A)$, c is the concentration of the sample, M is the sample molecular weight, $P(\theta)$ is the intramolecular scattering function or the 'form factor', A_2 is the second virial coefficient, n_0 is the refractive index of the solvent, $\mathrm{d}n/\mathrm{d}c$ is the specific refractive index increment of the solute with respect to the solvent, λ_0 is the wavelength of the light in the vacuum, and N_A is the Avogadro's number.

The form factor $P(\theta)$, which relates to the angular variation of scattering, may be approximated by:

$$P(\theta) = 1 - [\mu^2 \cdot \langle s^2 \rangle]/3 + \cdots \quad (3)$$

where $\mu = (4\pi/\lambda) \cdot \sin(\theta/2)$ is a function of the angle (θ) between the detector and the primary incident light of

the wavelength (λ) of the light in the medium and $<s^2>$ denotes the mean square radius of the macromolecules. In the case of a polydisperse sample, the three molecular parameters, i.e. M, A_2 and $<s^2>^{1/2}$, correspond respectively to the weight-average molecular weight, M_w, the second virial coefficient, and the z-average of the root-mean-square radius of the macromolecules, $[<s^2>_z]^{1/2}$, in short referred to as the gyration radius, Rg.

On investigating the given mixtures of the two associating hyaluronan derivatives, determination of their two characteristics, M_w and especially Rg, by applying off-line batch MALS method is mandatory (cf. Table 2). On investigating any one single biopolymer, i.e. EDC-HA or HA-β-CD, the SEC-MALS method does not only provide M_w and Rg values but also further molecular characteristics, such as M_n and M_z parameters (cf. Table 1).

Multidetector size exclusion chromatography

The fractionation of the water-soluble HA derivatives can be virtually performed by different techniques, e.g. by SEC, hydrodynamic chromatography or field-flow fractionation. The multidetector SEC system applied was built up by assembling four on-line sample detecting devices. The set-up of the detectors is serial in the following order: SCV→UV–vis→MALS→DRI. The variables/settings, specified above, are an appropriate compromise warranting proper SEC separation/fractionation along with precise molecular characterization of the HA samples investigated (Mendichi and Giacometti Schieroni, 2002; Šoltés *et al*., 2002).

In the on-line SCV detector a differential transducer continuously monitors the pressure drop across a stainless-steel capillary tube. Slice by slice from the signals of the SCV and UV–vis detectors, the local $[\eta]$ value is monitored. The relationship of the $[\eta]$ values on the polymer molecular weight is usually represented in the form of the power law function $[\eta] = f(M)$. Considering the ultra low concentration of the sample flowing through the SCV detector, the $[\eta]_i$ values monitored (cf. Fig. 4) are real and the overall limiting viscosity value determined for the HA-β-CD biopolymer (cf. Table 1) is valid for this sample.

It is well known that the on-line MALS detector measures both the sample molecular weight, M, and the dimensions, the Rg values—of the macromolecules flowing through. Thus the SEC-MALS arrangement is of special advantage in determining the power law function, $Rg = f(M)$. The curvi-linear shapes of the Rg vs M dependence observed (cf. Fig. 2) provide indirect evidence on both sample polymolecularity and on differences of DS values for individual 'homopolymeric' fractions.

On balance, the whole experimental protocol of investigating the two synthesized HA derivatives either individually or in the given mixtures, particularly the data listed in Table 2 and the results represented in Figs 5 and 6, corroborate the following statement: The bi-component EDC-HA and HA-β-CD solution does not contain a simple mixture but rather associates/aggregates of the two HA derivatives; the angular variation of the scattered light is a direct indication of the large size of the polymacromolecular associates/aggregates formed.

Addendum

The mean molecular weight of HA in the synovial fluid of healthy human beings ranges from 1.6 to 10.9 MDa; the hyaluronan concentration equals 2–3 mg/mL (Balázs *et al*., 1967). In osteoarthritis the synovial fluid is more abundant and less viscous: the concentration of hyaluronan is decreased; the HA molecular weight is reduced (Peyron, 1993). These changes have been postulated to be co-responsible for the subsequent accelerated destruction of the cartilage (Kikuchi *et al*., 1996). Thus, logically, intra-articular HA injection therapy termed 'viscosupplementation' has been implemented in the treatment of traumatized arthritic joints.

Two hyaluronan preparations derived from rooster combs have been currently approved for intra-articular injection, viscosupplementation, in symptomatic patients with osteoarthritis of the knee. Hyalgan® (Fidia Farmaceutici S.p.A.) comprises HA of molecular weight of ~500 kDa. Synvisc® G-F 20 (Biomatrix Inc.) contains also the Hylan® A hydrogel.

'Host–guest associating hyaluronan derivatives' can be, however, classified as a novel potential/horizon in viscosupplementation of (osteo)arthritic joints: the intraarticular injection of a mixture of two associating high-molecular-weight HA derivatives along with an appropriate low-molecular-weight drug (competitor) would, to advantage, result in the formation of a macromolecular network directly *in situ* (Šoltés *et al*., 2000, 2001). The molecules of the drug (e.g. a nonsteroidal antiinflammatory drug—piroxicam, flurbiprofen) should initially completely block the process of association. (The very complexation of macromolecular components *in situ* will take place only after the drug has been cleared from the articular space.) In this way, one can obtain a viscosupplementing product (for osteoarthritis treatment) or a soft/pourable or solid gel, which can be used, e.g., in missing tissue reconstruction. The two macromolecular components would be the HA with the bond host (e.g. β-CD) and the HA with the bond guest (e.g. an adamantyl residue); the almost ball-shaped adamantyl group is one of the 'best' guest moieties, fitting very snugly into the host β-CD cavity (Cromwell *et al*., 1985; Amiel and Sébille, 1996; Rekharsky and Inoue, 1998).

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