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Cyclodextrin derivative of hyaluronan

L. Šoltés[a,*], R. Mendichi[b], E. Machová[c], B. Steiner[c], J. Alföldi[c], V. Sasinková[c], S. Bystrický[c], K. Balog[d]

[a]*Institute of Experimental Pharmacology, Slovak Academy of Sciences, SK-84216 Bratislava, Slovak Republic*
[b]*Instituto di Chimica delle Macromolecole, Consiglio Nazionale delle Ricerche, I-20133 Milano, Italy*
[c]*Institute of Chemistry, Slovak Academy of Sciences, SK-84238 Bratislava, Slovak Republic*
[d]*Faculty of Material Technology, Slovak Technical University, SK-91724 Trnava, Slovak Republic*

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Abstract

Conversion of hyaluronan (HA) to its β-cyclodextrin derivative (HA-β-CD) was accomplished by direct coupling of β-cyclodextrin (β-CD) molecules with carboxylic acid groups of the HA macromolecule. The intermolecular dehydration, yielding the HA-β-CD derivative, was performed by the action of diethyl azodicarboxylate and triphenylphosphine under mild, neutral conditions. The physico-chemical characteristics of the novel (bio)material, determined both in solution and solid state, were compared with those of native HA. The specific action of a hyaluronidase was exploited to advantage in studying the depolymerization kinetics of the two types (HA and HA-β-CD) of macrobiomolecules. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: Biopolymer(s); Cyclodextrin(s); Drug carrier; Hyaluronan; Hyaluronan derivative; Viscosurgery; Viscosupplementation

1. Introduction

The term ''Hyaluronan'' (HA) (Balazs et al., 1986) was assigned to a polysaccharide composed of regularly alternating units of D-glucouronic acid and *N*-acetyl-D-glucosamine linked by β-(1 → 3) and β-(1 → 4) linkages (Fig. 1A). This glycosaminoglycan, present in all tissues and body fluids of vertebrates, is a polyanion with the corresponding counter-cations Na^+, K^+, H^+, etc. The molecular weights of HA biopolymers, isolated from various sources, cover the range from hundreds of thousands to up to several millions of daltons. The aqueous solution of such high-molecular-weight HAs are pseudoplastic and exhibit shear-dependent viscosity and frequency-dependent elasticity (Larsen & Balazs, 1991; Fraser et al., 1997). As a result of these properties, ultrapure viscous HA (usually Na^+ salt) solutions were widely used as a ''viscosurgery'' aid for ophthalmic operations (Nimrod et al., 1992) and for ''viscosupplementation'' in the treatment of some joint diseases, especially osteoarthritis (Peyron, 1993; Kikuchi et al., 1996).

Cyclodextrins (CDs) are cyclic oligosaccharides composed of D-glucose units linked through α-(1 → 4) bonds (Fig. 1B). In (aqueous) solutions the CD molecules are characterized by their higher order structure (Fig. 1b) 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 (cavity diameter = 0.78 nm) forms inclusion complexes with a wide variety of amphiphilic or lipophilic drugs by taking up the whole drug molecule, or some part of it, into the cavity.

The idea to exploit CD-oligosaccharides or the polysaccharide of HA as a vehicle for pharmaceutics is not new, while our attempt to combine the favorable properties of both vehicles, i.e. CD and HA, seems to be original. Of the three types of reactive groups on the HA macromolecule – carboxyl, hydroxyl, and acetamido – carboxyl was selected to make it react with the (outer) hydroxyl groups of the β-CD oligosaccharide, yielding a (semisynthetic) biopolymer (Fig. 1C).

2. Experimental

2.1. Materials and chemicals

The sample of HA used was supplied by CONTIPRO (Ústí nad Orlicí, Czech Republic). The β-CD, triphenylphosphine, and diethyl azodicarboxylate applied were the

* Corresponding author. Tel.: +421-7-375-928; fax: +421-7-375-928.
E-mail address: exfasolt@savba.savba.sk (L. Šoltés)

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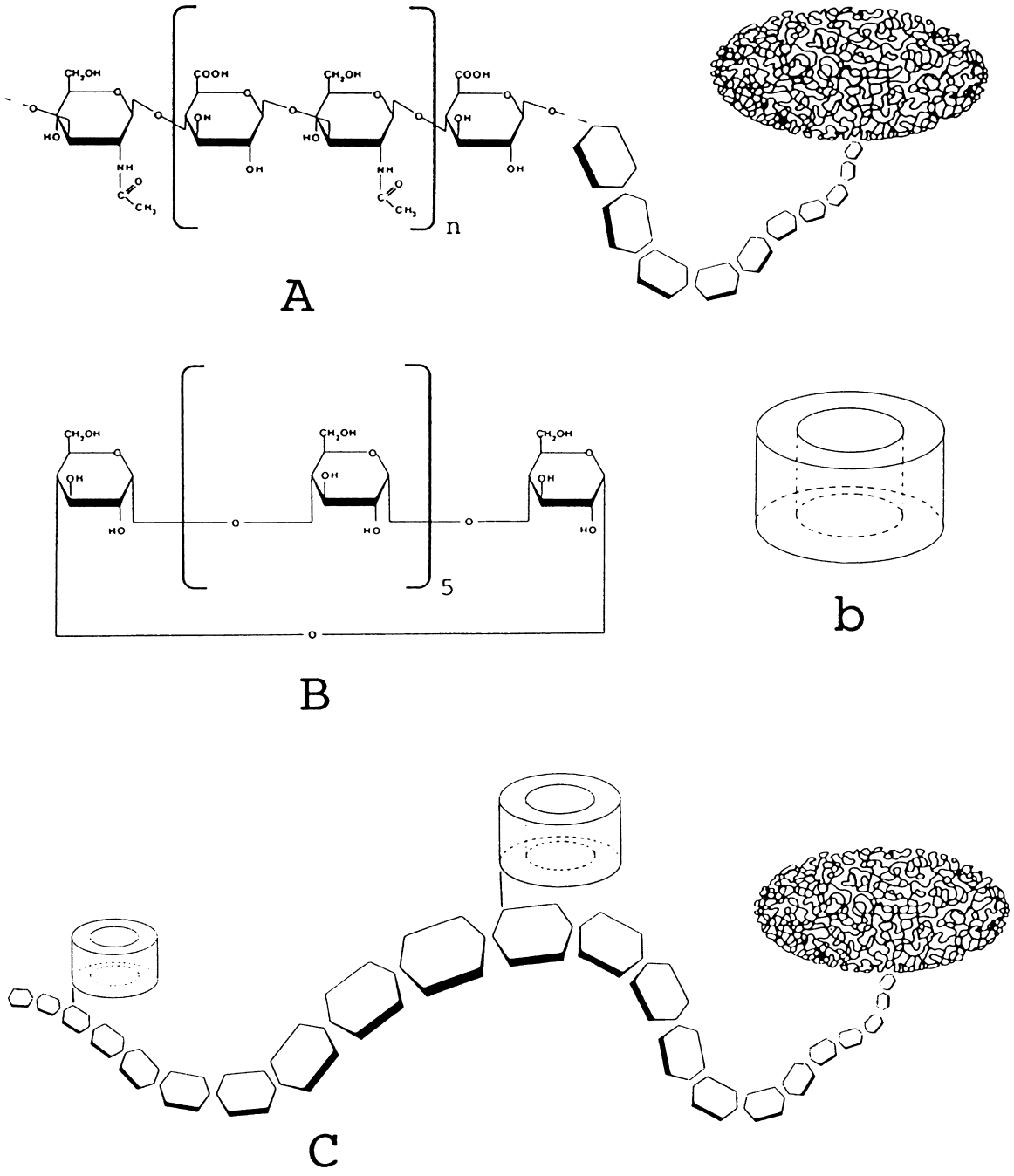

Fig. 1. Chemical formula of HA and sketch of its (bio)polymeric chain, A. Chemical formula of β-CD, B; and sketch of its higher order structure, b. Sketch of the CD derivative of HA, C.

products of FLUKA Chemie AG (Steinheim, Germany). Anhydrous dioxane, anhydrous ethanol, KOH, NaOH, NaCl, and $NaNO_3$ were purchased from MERCK (Darmstadt, Germany). The 8-anilino-1-naphthalene-sulfonic acid (ANS) probe applied was the product of SIGMA (St. Louis, MO, USA). Water used was of MILLI-Q_{RG} quality (Water Purification System; MILLIPORE Corporation, Bedford, MA, USA). The bovine testicular hyaluronidase (E.C.3.2.1.35) was purchased from the Institute of Sera and Vaccines (SEVAC, Prague, Czech Republic).

2.2. Synthesis

Reactant A consisted of 1.14 g of β-CD, dispersed in 100 ml of dioxane, and of 0.87 g triphenylphosphine. Reactant B contained 0.3 g HA, suspended by 20 ml dioxane, and 0.44 g diethyl azodicarboxylate. To accomplish the synthesis, both reactants, A and B, were mixed together and stirred overnight (for $\approx$ 20 h) at ambient temperature. After the reaction, most of the dioxane was evaporated (without heating) under reduced pressure. Ethanol

(150 ml) was admixed to the product, which was of pasty consistency. The insoluble part was recovered by filtration using a glass-fiber filter. The sample obtained was then dissolved in water (300 ml), and the aqueous solution was concentrated by ultrafiltration through an Amicon PM-10 membrane (mol. wt. cut-off $\geq$ 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 0.53 g. On titrating the sample, carboxyl groups with an aqueous KOH solution (0.1 mol/l) (Rinaudo and Hudry-Clergeon, 1967), their content in the HA-β-CD derivative was lower by $\approx$ 9.2% than that present in the native HA biopolymer.

2.3. Light-scattering

Static light scattering (LS) measurements of the native HA biopolymer were carried out at 35°C using a multi-angle laser light-scattering (MALLS) device DAWN DSP-F (Wyatt Technology, Santa Barbara, CA, USA), the vertically polarized laser wavelength being 632.8 nm. The angular distribution of the intensities of scattered light was monitored at 18 fixed angles ranging from 14.5° to 158.3° with respect to the incident beam. The investigated HA biopolymer was dissolved in aqueous NaCl (0.15 mol/l). The LS 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 HA solution was determined at 35°C and at a wavelength of 632.8 nm by a KMX-16 differential refractometer (LDC Milton Roy, Rochester, NY, USA). The dn/dc value was found to be 0.150 ml/g.

The dn/dc value found for HA-β-CD derivative was 0.151 ml/g. As the sample solution was opalescent, static LS measurements of this derivative were not performed.

2.4. Size-exclusion chromatography

Analysis of the distribution of molecular weights of HA and HA-β-CD samples was performed using a slightly modified (Mendichi and Giacometti Schieroni, 1997) 150-CV plus type size-exclusion chromatography (SEC) instrument (Water, Milford, MA, USA) equipped with three on-line detectors connected in series, namely with a single capillary viscometer (SCV), a UV-VIS spectrophotometer, and the MALLS detector (Mendichi et al., 1995). At the applied eluent flow-rate of 0.2 ml/min, the stainless-steel capillary (ϕ = 0.020 in.; length = 20 in.) of the SCV detector demonstrated a shear-rate value $\leq$ 250 s^{-1}; the Reynold's number was $\approx$ 11. The SCV data acquisition and analysis software used was MILLENNIUM 2.15 (Waters). The UV-VIS spectrophotometer, set at 206 nm, served as the biopolymer concentration detector. The setup of the MALLS detector was similar to that operable at static LS measurements (cf. Section 2.3).

The SEC column set consisted of a guard pre-column and two commercial PL aquagel-OH columns (particle size = 15 μm; Polymer Laboratories, Church Stretton, UK) in which aqueous NaCl (0.15 mol/l) was run as the mobile phase; the separation temperature was set to 35°C and the sample injection volume equaled 200 μl.

2.5. Nuclear magnetic resonance spectroscopy

Proton decoupled ^{13}C NMR spectra of HA and HA-β-CD samples dissolved in D_2O were measured at 40°C using the BRUKER AVANCE DPX FT spectrometer at 75.46 MHz field frequency. The ^{13}C chemical shifts were referenced to internal standard of acetone (δ = 29.83 ppm).

2.6. UV spectroscopy

The UV absorption spectra of both HA and HA-β-CD biopolymers (0.1% w/v solution in a 0.15 mol/l aqueous NaCl) were obtained with the UV-VIS spectrophotometer (Specord M 40; Zeiss, Jena, Germany).

2.7. Fluorescence spectroscopy

Steady-state fluorescence (FL) spectra were recorded with an SLM-AMINCO Model 8100 instrument (BIORI-TECH, Chamarande, France). The so-called molecular-probe technique was exploited to investigate the changes in FL of the ANS molecules (Na^+ salt) when free or complexed with β-CD. The instrument operated at excitation/emission wavelengths = 350/400–720 nm, with a 399 nm bandpass filter.

2.8. Circular dichroism

The circular dichroism (CD) spectra of both HA and HA-β-CD biopolymers were measured using a J-720 spectropolarimeter (JASCO International Ltd, Tokyo, Japan). The samples were dissolved in water (0.4 mg/ml) and measured at ambient temperature.

2.9. Infrared spectroscopy

The infrared (IR) spectra were measured using a NICOLET MAGNA-IR 750 apparatus. The samples were pressed into KBr pellets with the sample to KBr ratio of 1 : 100. At a resolution of 4 cm^{-1}, 128 scans were averaged. In order to obtain more exact band positions, the Fourier self-deconvolution technique (FT) was applied using the OMNIC 3.2 software (bandwidth 50 cm^{-1}; enhancement factor 2.3).

2.10. Differential scanning calorimetry

The differential scanning calorimetry (DSC) measurements were performed by DSC-20 and TG-50 devices (Mettler–Toledo AG, Greifensee, Switzerland) on an aluminum pan under nitrogen or air atmosphere. The heating rate used was 10 deg/min, the gas-flow rate was 50 ml/min. The

Table 1
Molecular-weight parameters of HA and HA-β-CD biopolymers

Parameter	Unit	LS method	SEC method			
		MALLS (sample: HA)	MALLS (sample: HA)	SCV (sample: HA)	MALLS (sample: HA-β-CD)	SCV (sample: HA-β-CD)
M_n	(kDa)		350.7	326.5	106.2	107.4
M_w	(kDa)	647.1	659.4	666.0	185.3	186.2
M_z	(kDa)		1050.4	1066.1	302.9	301.7
$\langle Rg_z^2 \rangle^{1/2}$	(nm)	97.4	97.2		40.2	
$A_2 \times 10^3$	(mol ml/g^2)	1.94				

software for data analysis was Mettler GraphWare TA 72.2/5 (Mettler–Toledo AG).

2.11. Study of depolymerization kinetics

Hyaluronidase, 135 TRU (turbidity-reducing units), was added to 10 ml of a 0.2% (w/v) solution of biopolymer in aqueous $NaNO_3$ (0.1 mol/l). The enzyme reaction occurred at 37°C under gentle shaking. Sample aliquots (200 μl), withdrawn in the given time intervals, 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 gel-filtration analysis (Machová et al., 1998).

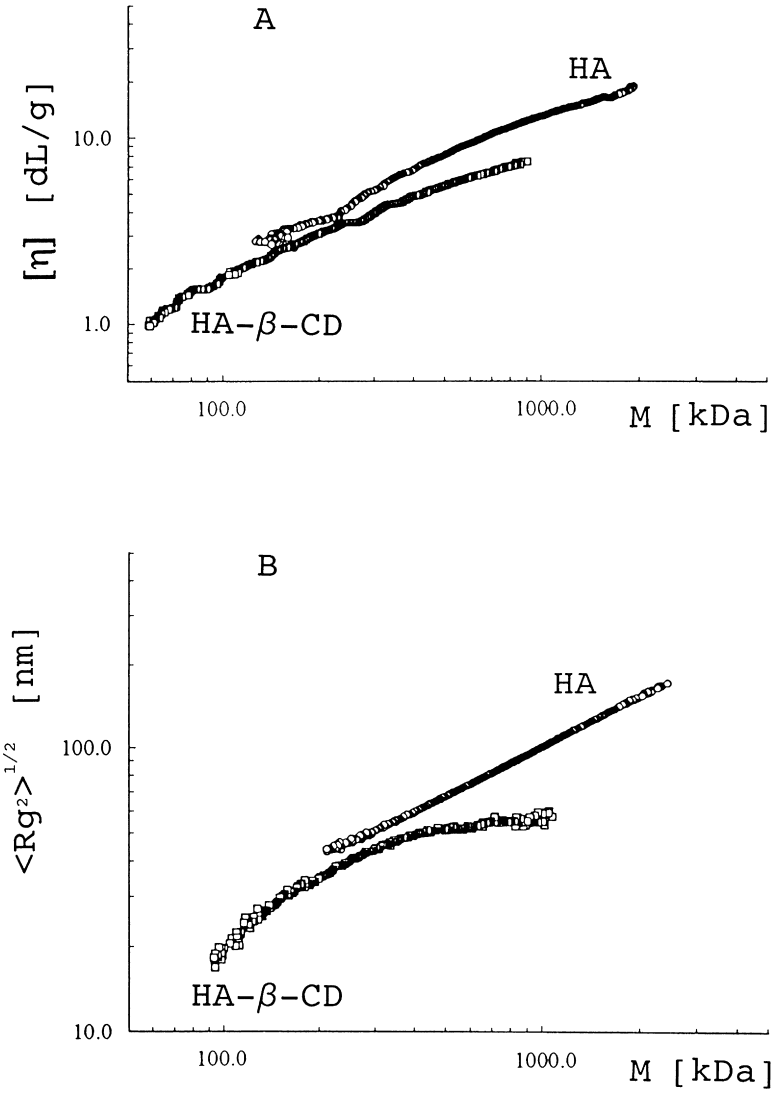

Fig. 2. Dependence [η] vs. M (panel A) and $\langle Rg^2 \rangle^{1/2}$ vs. M (panel B) observed on analyzing the HA and HA-β-CD samples by the SEC method

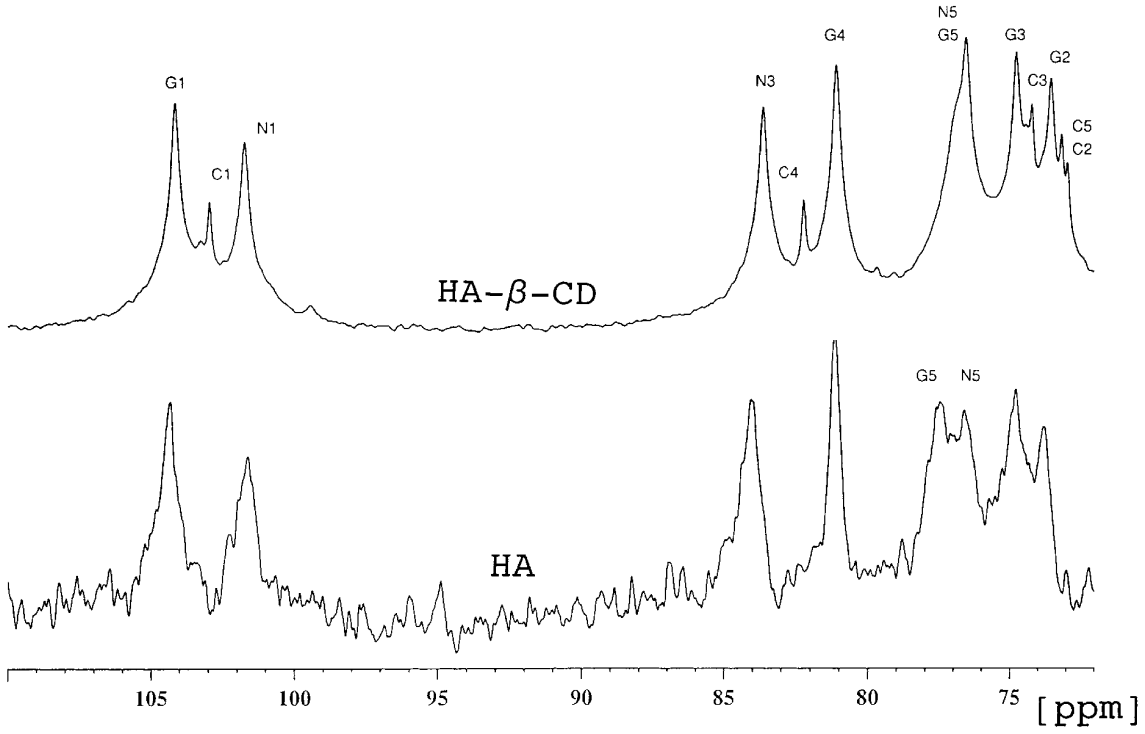

Fig. 3. Proton decoupled ^{13}C NMR spectra of HA and HA-β-CD biopolymers. (The N, G and C letters denote the *N*-acetyl-D-glucosamine, D-glucuronic acid and β-CD D-glucose units.)

3. Results

3.1. Characterization of the biopolymers in dissolved state

LS, SEC: The weight-average of the molecular weights, M_w, of native HA biopolymer determined by static LS measurements equaling 647.1 kDa was in excellent agreement with the corresponding values found by the SEC method, i.e. 659.4 and 666.0 kDa (Table 1). Both the *z*-average of the root-mean-square radius of gyration $\langle Rg_z^2 \rangle^{1/2} = 97.4$ (97.2) nm and the value of second virial coefficient $A_2 = 1.94 \times 10^{-3}$ mol ml/g^2, determined for the HA biopolymer, corresponded well with the values determined for HA samples in aqueous solutions of similar ionic strength (Ueno et al., 1988). Both the width and the remarkable symmetricity of the chromatogram observed on analyzing the native HA sample were indicative of a relatively narrow polydispersity of this biopolymer. This was well supported by the values $M_w/M_n = 1.88$ (2.04) and $M_z/M_w = 1.59$ (1.60), where M_n and M_z are the number- and z-average of the sample molecular weights.

As evident from the molecular-weight parameters listed in Table 1, the polymeric chain of the native HA partially degraded at synthesis of the HA-β-CD derivative. However, the values $M_w/M_n = 1.74$ (1.73) and $M_z/M_w = 1.63$ (1.62) of HA-β-CD biopolymer also indicated that the derivative synthesized was characterized by narrow polydispersity.

The double-logarithmic dependence of the intrinsic viscosity number $[\eta]$ on the molecular weight (M) of the HA and HA-β-CD biopolymers is represented in Fig. 2 (panel A). As evident, at a certain molecular weight the value $[\eta]$ of the HA-β-CD derivative is lower than that of the native HA sample. The high viscosities of the solutions of HA and HA-β-CD, whose macromolecules exceed the *M* values of hundreds of thousands of daltons, may result in a non-Newtonian sample-flow behavior. With this flow type, it is mandatory to minimize the shear-rate, which was achieved to a certain extent by proper selection of the capillary diameter and length in the SCV detector (cf. Section 2.4).

The most interesting observation, however, is the difference in conformation of the macromolecules of the two samples – the native HA and the HA-β-CD derivative – in the solution. As seen in Fig. 2 (panel B), at a certain molecular weight, the value of $\langle Rg^2 \rangle^{1/2}$ of the native HA biopolymer (significantly) exceeds that of the HA-β-CD derivative. While the slope of the double-logarithmic $\langle Rg^2 \rangle^{1/2}$ vs. *M* dependence for the native HA sample, equaling the value of 0.58, is in good agreement with the reported data (Fouissac et al., 1992), the curvi-linear shape of this dependence found in the case of the HA-β-CD derivative resembles dependences observed for branched polymers.

NMR: The ^{13}C nuclear magnetic resonance (NMR) spectrum of the HA-β-CD biopolymer (Fig. 3) shows the presence of CD. The position of its chemical shifts are practically identical with those of the CD itself (Bock et al., 1984). No differences were recorded between the chemical shifts of the carbon atoms of the native HA (Toffanin et al., 1993) and its β-CD derivative, apart from

Fig. 4. FT-IT spectra of HA and HA-β-CD samples.

the overlapping of signals G5 and N5 in the spectrum of HA-β-CD at 76.3 ppm, which were distinct in the spectrum of the native HA biopolymer at 77.2 ppm and 76.3 ppm. This finding may indicate a change in non-bonding interactions in HA-β-CD macromolecules. The NMR spectrum of the HA-β-CD sample does not allow to determine which particular OH group of β-CD was involved in the reaction with HA carboxyl.

UV: The UV absorption spectra of both the HA and HA-β-CD biopolymer were qualitatively identical. Each sample showed only a weak absorbance in the far UV-light region with a peak at 206 nm.

FL: On titrating the HA-β-CD biopolymer, at the initial addition of the ANS probe the emitted light maximum (at 565 nm) and its intensity were practically identical with the results found on working with the native HA biopolymer. On increasing the level of the ANS added, a relative enhancement of up to 31.4% of the emitted light intensity was observed for the HA-β-CD sample in comparison with the HA biopolymer. A slight shift in the emitted light maximum to 550 nm and 540 nm was recorded on investigating the HA biopolymer and the HA-β-CD sample, respectively. On overloading the ''β-CD binding sites'' in the HA-β-CD sample by a high ANS level, a quenching process must have taken place as both biopolymers emitted practically the same quantum of light of the same color (with a wavelength maximum of 550 nm). These observations correlate well with those found on investigating the ANS complexation with β-CD bound to a silica gel (Litwiler & Bright, 1992).

CD: Native HA has two chromophoric groups (*N*-acetyl and carboxyl) both of which exhibit n-π* electronic transitions in the wavelength region 200–225 nm. The CD spectra of both HA and HA-β-CD samples investigated showed one negative dichroic band centered at 208 nm. This band reflects the chiral environment of carboxyl and amide groups. The same values of ellipticities found for both samples support the proposition that the derivatization procedure (esterification reaction) did not change the orientation of *N*-acetyl and carboxyl groups and had no significant influence on the neighboring hydroxyl groups in the HA-β-CD derivative.

3.2. Analysis of the biopolymers in solid phase

IR: In the FT-IR spectrum of the HA-β-CD sample (Fig. 4) the carbonyl stretching band of the ester group can be clearly recognized at 1734 cm^{-1}. The intensity ratios of the overlapping bands in the native HA biopolymer at 1659 and 1612 $cm^{-1}$and at 1417 and 1377 $cm^{-1}$are also slightly changed (Gilli et al., 1994) as a result of sample esterification.

DSC: Table 2 lists the onset, middle point, and end point temperature of glass transition, as well as further parameters – heat effects – found for the two (bio)materials, HA and HA-β-CD.

Table 2
DSC parameters of HA and HA-β-CD (bio)materials

Sample	Environment	Glass transition			Heat effects		
		Onset (°C)	Middle point (°C)	End point (°C)	Temperature range (°C)	Heat (J/g)	Peak of exotherm (°C)
HA	Nitrogen	50	63	76	216–492	605	236
HA	Air	53	63	72	207–392	1302	239
HA-β-CD	Nitrogen	55	75	95	203–349	243	232
HA-β-CD	Air	68	85	102	209–418	468	391

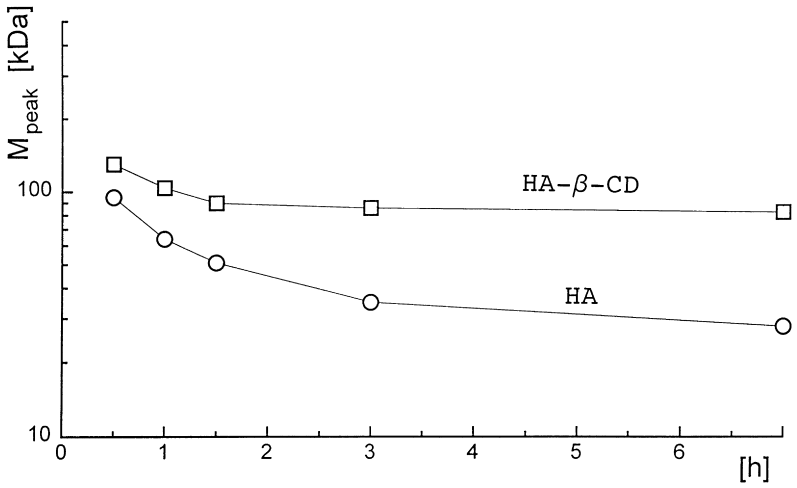

Fig. 5. Depolymerization kinetics of HA and HA-β-CD samples by action of hyaluronidase. (As pullulans were applied to calibrate the gel-filtration device, the M_{peak} values represent only apparent molecular weights of the HA and HA-β-CD biopolymers.)

3.3. Depolymerization kinetics of the biopolymers

The kinetics of depolymerization of the HA and HA-β-CD samples, shown in Fig. 5, present the dependence of the sample M_{peak} on the time of enzymatic reaction. As evident, the depolymerization rate of the HA-β-CD derivative is markedly slower than that observed for the native HA biopolymer. This observation supports the assumption that the attack of the backbone of the HA-β-CD macromolecules by the hyaluronidase must were restricted to a considerable extent by the bulky substituent of the β-CD molecules.

4. Discussion

The preliminary assays for preparing a CD derivative of HA, e.g. by reacting HA with β-CD by using epichlorohydrin or chloromethyloxirane, were completed successfully as concerns the sample yield. They did, however, collapse from the point of view of maintaining the molecular weight of the resulting product. On analyzing possible sources of failure, HA itself was implicated. HA macromolecules are extremely sensitive to reaction conditions and they depolymerize and degrade under both alkaline and acid conditions and even more so on heating (Šoltés et al., 1996).

In order to process the reaction under mild, neutral conditions (at room temperature or below), the diethyl azodicarboxylate and triphenylphosphine system (Mitsunobu, 1981) was used. 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.

Evaluation of the whole experimental protocol clearly showed that on using the given conditions a β-CD derivative of HA ($M_w > 180$ kDa) was prepared. The novel HA-β-CD biopolymer or its analogs could be used to advantage as a substitute in all situations requiring the use of the (high-molecular-weight) HA biopolymer, e.g. as an aid for viscosurgery, viscosupplementation, or as a carrier for amphiphilic and lipophilic drugs.

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References

Balazs, E. A., Laurent, T. C., & Jeanloz, R. W. (1986). *Biochem. J., 235*, 903.
Bock, K., Pedersen, Ch., & Pedersen, H. (1984). *Adv. Carbohydr. Chem. Biochem., 42*, 199.
Fouissac, E., Milas, M., Rinaudo, M., & Borsali, R. (1992). *Macromolecules, 25*, 5613.
Fraser, J. R. E., Laurent, T. C., & Laurent, U. B. G. (1997). *J. Intern. Med., 242*, 27.
Gilli, R., Kačuráková, M., Mathlouthi, M., Navarini, L., & Paoletti, S. (1994). *Carbohydr. Res., 263*, 315.
Kikuchi, T., Yamada, H., & Shimmei, M. (1996). *Osteoarthritis and Cartilage, 4*, 99.
Larsen, N. E., & Balazs, E. A. (1991). *Adv. Drug Delivery Rev., 7*, 279.
Litwiler, K. S., & Bright, F. V. (1992). *Applied Spectroscopy, 46*, 169.
Machová, E., Kogan, G., Šoltés, L., Kvapilová, K., Šandula, J. (1998). *Reactive and Functional Polymers*, in press.

Mendichi, R., Maffei Facino, R., Audisio, G., Carini, M., Giacometti Schieroni, A., & Saibene, L. (1995). *Int. J. Polym. Anal. Charact.*, *1*, 365.

Mendichi, R., & Giacometti Schieroni, A. (1997). *Proc. ACS Fall Meeting, PMSE Division* (Vol. 77) Las Vegas, NV, USA (pp. 18).

Mitsunobu, O. (1981). *Synthesis*, *1*, 1.

Nimrod, A., Ezra, E., Ezov, N., Nachum, G., & Parisada, B. (1992). *J. Ocular Pharmacol.*, *8*, 161.

Peyron, J. G. (1993). *J. Rheumatology*, *20* (39), 10.

Rinaudo, M., & Hudry-Clergeon, G. (1967). *J. Chim. Phys.*, *64*, 1746.

Šoltés, L., Mislovičová, D., & Sébille, B. (1996). *Biomed. Chromatogr.*, *10*, 53.

Szejtli, J. (1982). *Cyclodextrins and their inclusion complexes*. Akadémiai Kiadó: Budapest, Hungary.

Toffanin, R., Kvam, B. J., Flaibani, A., Atzori, M., Biviano, F., & Paoletti, S. (1993). *Carbohydr. Res.*, *245*, 113.

Ueno, Y., Tanaka, Y., Horie, K., & Tokuyasu, K. (1988). *Chem. Pharm. Bull.*, *36*, 4971.