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Synthesis and antiherpetic activity of carboxymethylated and sulfated hyaluronan derivatives

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ABSTRACT

Native high molecular weight hyaluronan (HMW-HA, $M_W = 1 \times 10^6$ g/mol) and a thermally degraded low molecular weight hyaluronan (LMW-HA, $M_W = 1.3–1.4 \times 10^5$ g/mol) were carboxymethylated providing products with degrees of carboxymethylation (DS_{CM}) of up to 0.8. Sulfation of resulting carboxymethyl hyaluronan (CM-HA) and hyaluronan (HA) was performed by different sulfation procedures enabling the control of the degree of sulfation (DS_S) in the obtained new carboxymethyl hyaluronan sulfates (CM-HA-S) and hyaluronan sulfates (HA-S), respectively, in a range between 0.9 and 3.3. Both carboxymethylation and sulfation were found to take place preferentially at the primary hydroxyl groups of HA. The antiviral activity of these synthesized HA derivatives was tested against Herpes simplex virus type 1. Both HA-S and CM-HA-S derivatives with high DS_S values of about 3.0 exhibit a strong antiherpetic activity. The CM-HA derivatives were found to be not active and an additional effect of introduced carboxymethyl groups on the antiherpetic activity of CM-HA-S derivatives was not observed. In the case of HA-S, the antiviral efficacy can be correlated with the DS_S and becomes stronger with increasing DS_S values.

1. Introduction

Sulfated glycosaminoglycans like heparan, dermatan or chondroitin sulfate exhibit various biological properties including anticoagulant and antithrombogenic effects, antiviral activity, and the ability to stabilize and present growth factors in their active conformation (Hintze, Möller, Schnabelrauch, Bierbaum, Viola, Worch, & Scharnweber, 2009; Kindness, Long, & Williamson, 1980; Kreuger, Spillmann, Li, & Lindahl, 2006; Lever & Page, 2002; Turnbull, Powell, & Guimond, 2001). Unfortunately, lacking effective preparation procedures make the reproducible availability of these complex sulfated polysaccharides in larger quantities difficult. By contrast, the non-sulfated glycosaminoglycan hyaluronan (HA) can be produced in an industrial scale by fermentation using, e.g. *Streptococcus* bacteria. The chemical sulfation of HA using common sulfating agents like SO_3-DMF or SO_3 complexes in combination with tertiary amines offers an interesting approach to structural analogs of natural sulfated glycosaminoglycans (Barbucci, Magnani, Lamponi, Rappuoli, & Consumi, 2000; Casu, Naggi, & Torri, 2002; Nagira, Nagahata-Ishiguroa, & Tsuchiya, 2007; Schiller, Becher, Möller, Nimptsch, Riemer, & Schnabelrauch, 2010).

It is known that the biological activity of sulfated glycans like heparan or heparan sulfate is strongly dependent not only on the degree of sulfation but also on the position of sulfate groups and the presence of further anionic or even hydrophobic substituents in the anhydrosugar unit (ASU) (Kreuger et al., 2006; Shukla, Liu, Blaiklock, Shworak, Bai, Esko, Cohen, Eisenberg, Rosenberg, & Spear, 1999; Schmidtke, Wutzler, & Makarov, 2004; Zautner, Hammerschmidt, Wutzler, & Schmidtke, 2006). Recently it was reported that dextran derivatives bearing both, sulfate and carboxymethyl groups, show a significant anticoagulant activity. This efficacy is further enhanced by the introduction of additional sulfonated benzylamide groups whereas after reduction of the carboxymethyl groups in the carboxymethylated dextran sulfates most of the activity is lost (Huynh, Chaubet, & Jozefonvicz, 2001). Based on these findings one should expect that both the variation of the content of sulfate groups and the introduction of additional functional groups into hyaluronan sulfate (HA-S) may represent an efficient tool to modulate their biological properties. Due to the poly-functional nature of HA and its poor solubility in common non-aqueous solvents, controlled chemical modification of HA resulting in uniform products with

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regioselective distribution of introduced functional groups represents a challenging task for polymer chemists. At present, only a few examples of multi-substituted hyaluronan derivatives are described (Chen, Ito, Imanishi, Magnani, Lamponi, & Barbucci, 1997; Wada, Chirachanchai, Izawa, Inaki, & Takemoto, 1994) and no systematic studies on structure – antithrombogenic or antiviral activity – relationships have been reported.

It is well known, that heparan sulfates interact with herpes simplex virus glycoproteins during virus adsorption to host cells (Shukla & Spear, 2001; Spear, Shieh, Herold, WuDunn, & Koshy, 1992). This first step of the viral life cycle can be competitively blocked by polyanions (Herold, Gerber, Belval, Siston, & Shulman, 1996; Ramos-Kuri, Barron Romero, & Aguilar-Setien, 1996; Schmidtke, Karger, Meerbach, Egerer, Stelzner, & Makarov, 2003). Polyanions prevent virus infection of cells up to 90%. It is known that HA itself does not exhibit relevant antiviral activity. For sulfate half-esters of HA (commonly named as hyaluronan sulfates) an antiviral activity may be postulated, but it was not proved until now. Moreover there are no studies available dealing with the influence of the content of sulfate groups and their distribution within the ASU or the introduction of further anionic groups on antiviral activity.

Here, we report on the synthesis of carboxymethyl hyaluronans (CM-HAs) using both high molecular weight native and hydrolytically degraded HA as starting materials. Carboxymethyl hyaluronan sulfates (CM-HA-S) and also HA-S with varying degree of sulfate groups have been prepared from the obtained CM-HA derivatives and from HA, respectively, and the impact of different anionic groups on antiherpetic activity was studied.

2. Materials and methods

2.1. Materials

Hyaluronan (HA, high molecular weight HA (HMW-HA, from *Streptococcus*, $M_W = 1 \times 10^6$ g/mol)) was obtained from Aqua Biochem Dessau, Germany. Sulfur trioxide/N,N-dimethylformamide complex (SO_3-DMF, purum, ≥97%, active SO_3 ≥48%), sulfur trioxide/pyridine complex (SO_3-pyridine, pract.; ≥45% SO_3) and monochloroacetic acid (MCA, puriss., ≥99% T) were purchased from Fluka Chemie, Switzerland. Phosphate buffered saline (PBS buffer, 0.14 M NaCl, 0.0027 M KCl, 0.010 M PO_4^{3-}, pH = 7.4) was obtained from Sigma–Aldrich Chemie, Taufkirchen. Phosphonoformic acid (PFA, foscarnet, trisodium salt hexahydrate, Sigma–Aldrich), a well-known anti-herpetic agent was applied as positive control compounds in cytopathic effect inhibitory assays (Schmidtke, Schnittler, Jahn, Dahse, & Stelzner, 2001). Stock solution of PFA was prepared in cell culture medium (10 mg/ml) and stored at 4 °C until dilution in test media.

2.2. Analytical methods

^{1}H and ^{13}C NMR spectra were recorded in D_2O (99.9%, Aldrich), with a Bruker Advance 400 MHz spectrometer. The signal of D_2O at 4.75 ppm was used as reference line. The KBr technique was employed for recording FT-IR spectra with a FT-IR-Spektrometer *FTS 175* (BIO RAD, Krefeld, Germany). Important parameters to characterize the chemical composition of the prepared HA derivatives are the degree of sulfation (DS_S) and the degree of carboxymethylation (DS_{CM}). DS_S and DS_{CM} values give the average number of sulfate groups and carboxymethyl groups, respectively, per disaccharide repeating unit of HA which is formed from β-D-*N*-acetylglucosamine and β-D-glucuronic acid. Based on this definition values for the total degree of substitution ($DS_S + DS_{CM}$) may range between 0 (unsubstituted HA) and 4.0 (complete substitution of all free hydroxyl groups within the disaccharide repeating unit). DS_S values of the hyaluronan derivatives were determined by estimation of the sulfur content using an automatic elemental analyzer (*CHNS-932*, Leco, Mönchengladbach, Germany). Conventional acid–base titration was used to determine DS_{CM} values. Molecular weight determination was performed by gel permeation chromatography (GPC) analysis using a Jasco PU 980 pump, and a combination of 3 Suprema-Gel columns with dimensions of 8 mm × 300 mm (diameter × length) and the specifications 10 μm to 100 Å, 10 μm to 1000 Å and 20 μm to 30,000 Å referring to grain size and pore size, respectively. A double detection system consisting of a Postnova Analytics PN 3000 (15°) laser light scattering (LLS) detector and a Jasco RID-1531 refraction index (RI) detector was employed. Absolute values of weight-average (M_W) molecular weights have been determined using the LLS detection system. Calculation of polydispersity PD ($PD = M_W/M_n$) was performed on the basis of number-average molecular weight (M_n) and M_W values obtained from conventional calibration with commercially available pullulan standards (PSS Polymer Standards Service, Mainz, Germany).

2.3. Low molecular weight hyaluronan (LMW-HA)

700 ml of a 1% (w/v) solution of HMW-HA in distilled water was heated in a steam autoclave (Hirayama Manufacturing Corp., Japan) at 130 °C for 90 min. After cooling down to room temperature, the solution was purified by dialysis against distilled water followed by lyophilization of the solution and drying of the resulting polymer in vacuum. Yield: 90%. FT-IR and ^{13}C NMR spectra of the prepared LMW-HA were in accordance with literature data (Magnani, Lamponi, Rappuoli, & Barbucci, 1998) and identical to corresponding spectra of native HMW-HA. The values for the molecular weight of the three samples, LMW-HA 1, 2 and 3, respectively (Table 1), results from different preparations using always the same HMW-HA starting material.

2.4. Carboxymethyl hyaluronan (CM-HA)

4.98 mmol HMW-HA (sodium salt) was suspended in isopropanol/water (200/100, v/v) at room temperature under nitrogen. After stirring over night, NaOH (40% (w/w) in water) and MCA were added according to the chosen molar educt ratio given in Table 1. The solution was heated to 60 °C and stirring was continued for the reaction time given (see Table 1). After cooling to room temperature, the lower phase was separated and diluted with methanol, the precipitated product was dissolved in water and the pH was adjusted to pH 7.5 with 2 N HCl. The solution was dialyzed against distilled water followed by lyophilization of the aqueous solutions and drying of the resulting polymers under vacuum. Yields and DS_{CM} values of CM-HA 1-CM-HA 8 are given in Table 1.

IR (KBr): 3421 (s, OH), 2925 (CH_3, CH_2), 1616 cm^{-1} (C═O, CO—NH). ^{1}H NMR (D_2O, 343 K): $\delta = 2.51$ (s, CH_3), 3.85 (t, H-2, $^3J_1 = 8.47$, $^3J_2 = 8.16$), 3.98 (m, H-4′ and H-5′), 4.04 (m, H-3), 4.18–4.26 (m, H-5, H-6, H-3′ and H-4), 4.32 (m, H-2′), 4.37 (m, H-6′), 4.47 (s, new CH_2, large), 4.58–4.76 (m, new CH_2, small), 4.95 (d, H-1′, $^2J = 7.43$), 5.10 (d, H-1′, $^2J = 7.91$). ^{13}C NMR (D_2O, 343 K): $\delta = 23.21$ (CH_3), 55.01 (C-2′), 61.50 (C-6′, free), 69.44 (C-4′), 70.41 (C-6′, carboxymethylated), 71.26 (new CH_2, large, main product), 71.87–72.16 (new CH_2, small), 73.29, 73.76 (C-2), 74.43, 74.58, 75.03 (C-3), 76.20 (C-5′), 77.20 (C-5), 81.13, 80.68 (C-4), 82.91, 83.53 (C-3′), 101.01, 101.30 (C-1′), 103.18, 103.66 (C-1), 174.39, 174.79, 175.83, 178.26 (C-6, C═O, CO—NH).

Table 1
Characteristics of high and low molecular weight hyaluronans (HMW-HA, and LMW-HA, respectively), and reaction conditions and characteristics for the synthesized carboxymethyl hyaluronan (CM-HA) derivatives.

Sample	Molar ratio[a]	Reaction time[b] (h)	DS_{CM}	Yield (%)[c]	M_n[d] (g/mol)	M_W[e] (g/mol)	PD[f]
HMW-HA	–	–	–	–	1,019,625 *394,940*	1,174,865 *1,894,675*	*–4.80*
CM-HA 1	1:2.9:1	2	0.2	61	22,155 *62,750*	37,920 *135,145*	*–2.15*
CM-HA 2	1:2.9:1	4	0.3	38	11,795 *27,370*	14,910 *52,190*	*–1.91*
CM-HA 3a	1:5.8:2	2	0.4	65	16,820 *46,820*	26,200 *86,755*	*–1.85*
CM-Ha 3b	1:5.8:2	2	0.3	70	17,455 *50,465*	28,210 *92,685*	*–1.84*
CM-HA 4a	1:5.8:2	4	0.5	48	10,167 *34,635*	15,395 *58,210*	*–1.68*
CM-HA 4b	1:5.8:2	4	0.5	52	8685 *32,370*	16,670 *55,015*	*–1.70*
CM-HA 5[g]	1:5.8:2	4	0.8	51	3760 *22,185*	7075 *34,085*	*–1.54*
CM-HA 6[h]	1:5.8:2	4	0.6	68	6600 *19,480*	9940 *30,080*	*–1.54*
CM-HA 7	1:11.6:4	2	0.4	69	17,060 *39,040*	23,230 *74,565*	*–1.91*
LMW-HA 1	–	–	–	86	84,945 *219,975*	133,470 *552,475*	*–2.51*
LMW-HA 2	–	–	–	91	85,535 *215,390*	138,660 *543,985*	*–2.53*
LMW-HA 3	–	–	–	71	77,000 *209,470*	142,555 *576,460*	*–2.75*
CM-HA 8[i]	1:5.8:2	2	0.5	58	15,580 *43,005*	22,625 *78,335*	*–1.82*

[a] Molar ratio HA:NaOH:MCA.
[b] Reaction temperature for all syntheses: 60 °C.
[c] Related to used HA.
[d] Number-average molecular weight, determined by LLS (upper row) and conventional calibration with pullulan standards (lower row, in italics) detection.
[e] Weight-average molecular weight, determined by LLS (upper row) and conventional calibration with pullulan standards (lower row, in italics) detection.
[f] Polydispersity (determined by conventional calibration with pullulan standards and calculated from M_W/M_n).
[g] Second carboxymethylation, starting material: CM-HA 3a.
[h] Second carboxymethylation, starting material: CM-HA 4a.
[i] Starting material: LMW-HA 3.

2.5. Hyaluronan sulfate derivatives

2.5.1. Tetrabutylammonium salt of HA (TBA-HA) and carboxymethyl hyaluronan (TBA-CM-HA)

2.0 g (4.98 mmol) HA (sodium salt) and CM-HA, respectively, were dissolved in distilled water (400 ml) at room temperature over night. The solution was stirred with 20 g of Dowex WX 8 ion exchanger (tetrabutylammonium form) over night and after filtration, the polymer solution was lyophilized and dried under vacuum at 40 °C. Yield: 90%.

2.5.2. General remarks and work-up procedure for the sulfation reactions

The high- and low-sulfated HA-S were prepared according to a recently described procedure (Hintze et al., 2009).

The sulfated products were isolated from the reaction mixture by precipitation into acetone (2.8 l) and neutralized using ethanolic NaOH solution. The formed sodium salt of the HA-S and CM-HA-S, respectively, were washed several times with acetone and purified by dialysis against distilled water followed by lyophilization of the aqueous solutions and drying of the resulting polymers under vacuum.

2.5.3. High-sulfated HA-S and CM-HA-S

3.22 mmol TBA-HA and 2.55 mmol of TBA-CM-HA (DS_{CM} = 0.4–0.5), respectively, were suspended in DMF under argon at room temperature. The SO_3-DMF complex (64.4 mmol and 51.00 mmol, respectively, polymer:SO_3 ratio = 1:20), dissolved in 20–40 ml DMF, was added and the reaction solution was stirred for 1 h at room temperature. Work-up was performed as described above. Yields and DS_S values given in Table 1.

2.5.3.1. HA-S 1 and 3. IR (KBr): 3486 (s, OH), 2964 (CH_3, CH_2), 1631 (C=O, CO—NH), 1245 cm^{-1} (SO_2). ^{13}C NMR (D_2O, 343 K): δ = 23.60 (NH—CO<u>C</u>H$_3$), 56.04 (C-2′), 68.34 (C-6′, sulfated), 69.57 (C-4′, unsulfated, small), 73.75, 74.25, 76.95, 77.17, 77.66, 78.71, 79.38, 80.71 (C-4′(sulfated), C-2, C-3, C-5′, C-5, C-4 and C-3′), 100.63 (C-1′), 102.01 (C-1), 175.04, 175.35 (C-6, C=O).

2.5.3.2. CM-HA-S 1 and 4. IR (KBr): 3470 (s, OH), 2961 (CH_3, CH_2), 1633 (C=O, CO—NH), 1243 cm^{-1} (SO_2). ^{13}C NMR (D_2O, 343 K): δ = 23.55 (NH—CO<u>C</u>H$_3$), 56.01 (C-2′), 67.91, 69.34, (C-6′, sulfated), 70.07, 71.11, (CH_2), 73.75, 74.44, 77.10, 77.62, 78.67 (C-4′, C-2, C-3, C-5′, C-5, C-4 and C-3′), 100.64 (C-1′), 101.95 (C-1), 175.07, 178.30 (C-6, C=O).

2.5.4. Low-sulfated HA-S and CM-HA-S

3.22 mmol TBA-HA and 2.86 mmol TBA-CM-HA (DS_{CM} = 0.3–0.4), respectively, were suspended in DMF under argon at room temperature. The SO_3-pyridine complex (22.54 mmol, and 20.02 mmol, respectively, polymer:SO_3 ratio = 1:7), dissolved in 20–40 ml DMF, was added and the reaction solution was stirred for 20 min at room temperature. Work-up was performed as described above. Yields and DS_S values given in Table 2.

2.5.4.1. HA-S 2 and 4. IR (KBr): 3435 (s, OH), 2917 (CH_3, CH_2), 1623 (C=O, CO—NH), 1236 cm^{-1} (SO_2). ^{13}C NMR (D_2O, 343 K): δ = 23.17 (NH—CO<u>C</u>H$_3$), 54.80 (C-2′), 67.67 (C-6′, sulfated), 68.86

Table 2
Reaction conditions and characteristics of synthesized hyaluronan sulfates (HA-S) and carboxymethyl hyaluronan sulfates (CM-HA-S).

Sample	Educt	DS_{CM}	Molar ratio SR[a]:HA	DS_S	Yield (%)	M_n[b] ($gmol^{-1}$)	M_W[c] ($gmol^{-1}$)	PD[d]
HA-S 1	HMW-HA	–	20:1[f]	3.0	83	32,255*51,590*	47,835 *88,505*	*–1.72*
HA-S 2	HMW-HA	–	7:1[g]	1.5	69	6265 *22,100*	12,880 *39,240*	*–1.78*
HA-S 3	LMW-HA	–	20:1[f]	3.2	66	26,925*41,390*	38,485 *69,950*	*–1.69*
HA-S 4	LMW-HA	–	7:1[g]	1.9	66	8020 *22,910*	14,135 *40,575*	*–1.77*
CM-HA-S 1	HMW-HA	0.4	20:1[f]	3.2	55	15,530*35,025*	24,970 *54,005*	*–1.54*
CM-HA-S 2[e]	HMW-HA	0.5	10:1[f]	2.8	55	70,950*97,970*	95,810*132,260*	*–1.35*
CM-HA-S 3	HMW-HA	0.3	7:1[g]	0.9	63	1820 *22,805*	10,475 *39,345*	*–1.73*
CM-HA-S 4	LMW-HA	0.5	20:1[f]	3.3	55	14,090*30,010*	22,090 *45,405*	*–1.51*
CM-HA-S 5	LMW-HA	0.4	7:1[g]	1.1	54	9165 *28,190*	12,350 *46,900*	*–1.66*

[a] Sulfating reagent.
[b] Number-average molecular weight, determined by LLS (upper row) and conventional calibration with pullulan standards (lower row, in italics) detection.
[c] Weight-average molecular weight, determined by LLS (upper row) and conventional calibration with pullulan standards (lower row, in italics) detection.
[d] Polydispersity (determined by conventional calibration with pullulan standards and calculated from M_W/M_n).
[e] In the presence of *p*-toluene sulfonic acid.
[f] SR:SO_3-DMF.
[g] SR:SO_3-pyridine.

(C-4′), 73.03–74.36 (C-2, C-3, C-5′), 77.45 (C-5), 81.93 (C-4), 82.97 (C-3′), 101.36 (C-1′), 103.68 (C-1), 175.39 (C-6, C=O).

2.5.4.2. CM-HA-S 3 and 5. IR (KBr): 3452 (s, OH), 2917 (CH_3, CH_2), 1612 (C=O, CO—NH), 1242 cm^{-1} (SO_2). ^{13}C NMR (D_2O, 343 K): 23.24 (NH—CO$\underline{C}H_3$), 54.95 (C-2′), 67.82 (C-6′, sulfated), 69.22 (C-4′), 70.42, 71.28, (CH_2), 73.25 (C-2), 74.12 (C-3), 74.59, 75.02, 77.19 (C-5 and C-5′), 81.62 (C-4), 82.97 (C-3′), 101.46 (C-1′), 103.65 (C-1), 174.30, 175.39 (C-6, C=O).

2.5.5. Sulfation of CM-HA in the presence of p-toluene sulfonic acid (CM-HA-S 2)

p-Toluene sulfonic acid (1.84 mmol) was added under argon to a suspension of the CM-HA (1.15 mmol) in DMF (50 ml) and the colloidal solution is stirred for 30 min at room temperature. After cooling to 4 °C in an ice bath, molecular sieve (A3) was added and subsequently a SO_3-DMF complex solution (11.50 mmol, molar polymer:SO_3 = 1:10) was dropped slowly into the solution. Stirring was continued for 1 h, the molecular sieve was removed by filtration and the polymer was precipitated into acetone (400 ml). After neutralization with ethanolic NaOH solution, the sulfated polymer was isolated by filtration and purified. Yields and DS_S values given in Table 2.

IR (KBr): 3470 (s, OH), 2961 (CH_3, CH_2), 1633 (C=O, CO—NH), 1243 cm^{-1} (SO_2). ^{13}C NMR (D_2O, 343 K): δ = 23.41 (NH—CO$\underline{C}H_3$), 55.78 (C-2′), 63.13 (C-6′, unsulfated), 68.11, (C-6′, sulfated), 70.73 (CH_2), 73.56, 75.00, 75.98, 76.74, 77.35, 78.39, 78.69 (C-4′, C-2, C-3, C-5′, C-5, C-4 and C-3′), 100.52 (C-1′), 101.97 (C-1), 175.07, 178.30 (C-6, C=O).

2.6. Determination of cytotoxicity and antiviral activity

Cytotoxicity and antiviral activity of polymers were examined on 2 days-old monolayers of Green monkey kidney cells (GMK) cells grown in 96-well plates using well-established assays (Schmidtke et al., 2001). To determine the 50% cytotoxic concentration (CC_{50}), cell monolayers were incubated with 9 serial dilutions (factor 2, each concentration in duplicate) of the respective compounds for 72 h (37 °C, 5% CO_2). Then, the cells were fixed and stained with a crystal violet formalin solution. Cytotoxicity was quantified spectrophotometrically with a plate reader as described previously (Schmidtke et al., 2001). In the cytopathic effect (CPE) inhibitory assay, 50 μl of drug solution and 50 μl of a constant amount of virus (0.1 multiplicity of infection for HSV-1 strain K1) were added to confluent GMK cell monolayers. The inhibition of the virus-induced CPE was scored spectrophotometrically 48 h post infection when untreated infected control cells showed maximum cytopathic effect. At least three independent assays were performed and used to calculate a mean dose–response curve with standard deviations. The polymer concentration required to reduce the CPE by 50% (IC_{50}) was calculated from this mean dose–response curve.

3. Results and discussion

3.1. Synthesis and characterization of hyaluronan derivavtives

3.1.1. Preparation of low molecular weight hyaluronan (LMW-HA)

LMW-HA was prepared by heating an aqueous solution of HMW-HA ($M_W = 1 \times 10^6$ g/mol) in a steam autoclave at 130 °C for 90 min. Under these conditions LMW-HA samples (LMW-HA 1–3) with a M_W of about 130,000–140,000 g/mol were obtained in a reproducible reaction. The PD was determined to range around 2.5 and shows a much more narrow molecular weight distribution as the native starting material (Table 1). The FT-IR and ^{13}C NMR spectra of the products obtained were identical with those of the starting high molecular weight HA and also congruent with spectral data published earlier (Magnani et al., 1998). Hence, no structural changes in the HA samples are detectable during thermal degradation.

3.1.2. Carboxymethylation of hyaluronan

In analogy to the preparation of other carboxymethylated polysaccharides (Liebert & Heinze, 1998; Wagner, Kautz, Röder, Schwalbe, Pachmann, Clement, & Schnabelrauch, 2004), carboxymethylation of HA was performed under alkaline conditions at 60 °C using monochloroacetic acid (MCA) as alkylating agent (Fig. 1). A ratio of 2.9–11.6 mol of NaOH per mol ASU of HA was used and the MCA amount was varied in a range between 1 and 4 mol per mol ASU of HA. The effect on the extent of carboxymethylation by variation of the NaOH and MCA ratio and the reaction time in the given ranges was only marginal and varied between 0.2 and 0.5 in the DS_{CM} value. The reaction conditions could be optimized by a 5.8 molar excess of NaOH and 2 molar excess of MCA and a reaction time of 4 h at 60 °C. In comparison to HMW-HA the

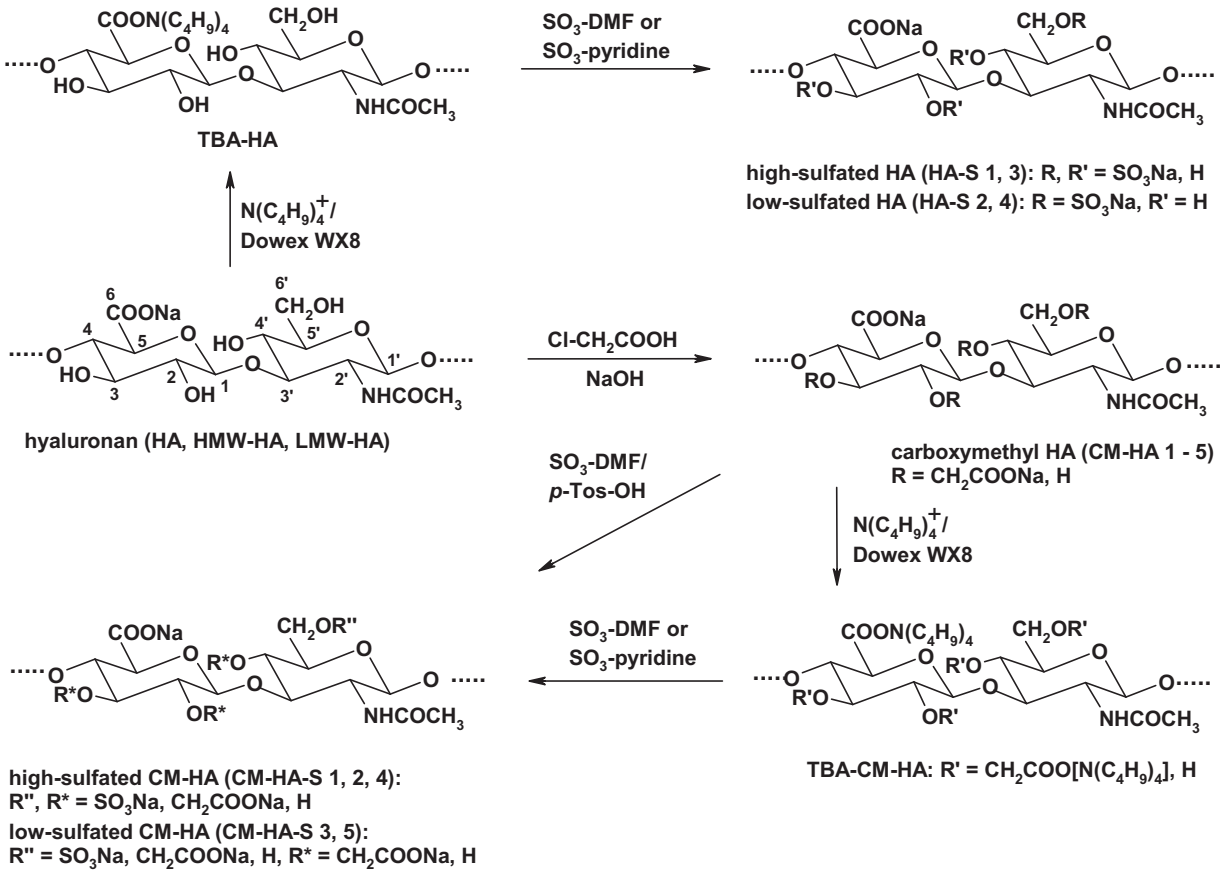

Fig. 1. Reaction scheme for the synthesis of hyaluronan sulfate (HA-S) and carboxymethyl hyaluronan sulfate (CM-HA-S).

carboxymethylation of LMW-HA 3 leads to products with a slightly higher DS_{CM} when using the same reaction conditions (Table 1).

The yield of the products was between 60 and 70% and decreased with increasing reaction time. Probably the drastic reaction conditions resulted in a dramatic decrease in the molecular weight and a leakage of substance during dialysis. Increasing the temperature of the carboxymethylation to 80 °C resulted in the formation of colored products.

Determination of the molecular weight by GPC measurements revealed that the carboxymethylation reaction causes a remarkable degradation of the polymer backbone. Starting with a HMW-HA of a M_W of 1×10^6 g/mol, CM-HA derivatives with a M_W ranging between 40,000 and 10,000 g/mol were obtained (see Table 1). Furthermore, an increasing quantity of MCA and NaOH and an extension of the reaction time resulted in products with a smaller molecular weight. Nevertheless the established procedure gave reproducible results with regard to DS_{CM} values and molecular weights as it is illustrated in Table 1 for samples treated under identical reaction conditions (see for example CM-HA 3a/CM-HA3b or CM-HA 4a/CM-HA 4b).

In order to increase the DS_{CM}, a second carboxymethylation run of CM-HA derivatives was performed (CM-HA 5 and 6, respectively). This strategy successfully used for various polysaccharides (e.g. for dextran, Wagner et al., 2004), resulted in the case of HA only in a slight increase in the DS_{CM} values of 0.2–0.5 presumably due to the lowered reactivity of the remaining free hydroxyl groups in the already carboxymethylated HA derivative. The molecular weight was drastically decreased by the repeated carboxymethylation procedure (Table 1).

The structure of the CM-HA was confirmed by NMR and 2D-NMR spectroscopy. All signals could be allocated to their corresponding H- and C-atoms, respectively. Compared to HA, a new signal at 71.26 ppm appeared in the ^{13}C NMR spectrum of CM-HA which could be assigned to the carbon atom of the CH_2 unit in the carboxymethyl group (Fig. 2). The analysis of 2D-NMR spectra in particular showed, that HA was carboxymethylated to about 60–70% on the primary hydroxyl group at C-6′. The residual 30% are statistically distributed among the secondary hydroxyl groups on C-2, C-3 and C-4′.

3.1.3. *Sulfation of hyaluronan and carboxymethyl hyaluronan*

The introduction of sulfate ester groups into hyaluronan is well known (Magnani et al., 1998). Generally, first the sodium salt of HA is transformed into the stable tetrabutylammonium salt simply by stirring with a corresponding cation exchanger. The formed HA ammonium salt is not completely soluble in DMF but, in contrast to the sodium salt, it can be homogeneously suspended in DMF and sulfated with SO_3-DMF complex (molar ratio SO_3-DMF:HA = 20:1) at room temperature for 1 h. Nearly all of the four free OH-groups can be sulfated by this procedure and the values of DS_S for these high-sulfated products ranged between 2.9 and 3.3 (Table 2). Low-sulfated HA is obtained using SO_3-pyridine as sulfating agent and a molar ratio SO_3-pyridine:HA = 7:1. It is known that the SO_3-pyridine complex has a lower reactivity in polysaccharide sulfation than the SO_3-DMF one (Petit, Papy-Garcia, Muller, Courtois, Caruelle, & Courtois, 2004). This lower reactivity of SO_3-pyridine seems to has the advantage of a better control of the DS_S in the low-sulfated HA derivatives because a better homogenization of the reaction systems and hence a more uniform substitution along the polymer chains becomes possible. The reaction is carried out at room temperature for 20 min. Using SO_3-pyridine, the DS_S values range between 1.5 and 1.9 (Table 2).

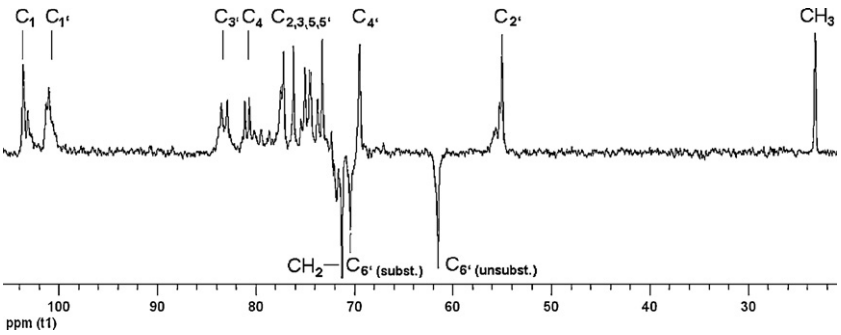

Fig. 2. ^{13}C NMR-DEPT 135 spectrum from carboxymethyl hyaluronan (CM-HA 6; DS_{CM} = 0.6).

The introduction of sulfate ester groups into CM-HA is performed with a SO_3-DMF and SO_3-pyridine complex, respectively, using the same procedures as described above for HA. Using SO_3-DMF, carboxymethyl hyaluronan sulfates with DS_S values ranging from about 2.8 to 3.3 can be obtained, whereas with SO_3-pyridine as sulfating agents DS_S only values of 0.9–1.1 are available.

From these data it can be concluded that sulfating HA and CM-HA with SO_3-DMF provides products with similarly high DS_S values of up to 3.3 whereas sulfation with SO_3-pyridine led to a remarkably higher DS_S in the case of HA (DS_S = 1.5–1.9) compared to CM-HA (DS_S = 0.9–1.1). This might be explained by the fact that in CM-HA primary hydroxyl groups have already been partly substituted by carboxymethyl groups and the remaining free secondary hydroxyls are less susceptible to the less reactive sulfation agent SO_3-pyridine.

Another route to sulfate CM-HA is to add *p*-toluene sulfonic acid to the reaction mixture which is known to promote the acylation of polysaccharides, probably by the formation of intermediary anhydride structures with the acylation reagents (Shimizu & Hayashi, 1989). Using this reaction system, CM-HA-S with high DS_S can be prepared (CM-HA-S 2, Table 2) and the extent of sulfation can be controlled by the used quantity of SO_3-DMF complex. A homogeneous sulfation in this reaction system is only possible for CM-HA derivatives with DS_{CM} values higher than 0.3, since educts with lower DS_{CM} values are not soluble in the *p*-toluene sulfonic acid/DMF system.

In Fig. 3, the ^{13}C NMR spectra of a CM-HA-S with high DS_S (C) in comparison to spectra of CM-HA (B) and HA (A) are depicted. As already seen in the CM-HA spectrum the signal at 71 ppm assigned to the CH_2 unit of the carboxymethyl group can also be observed in the CM-HA-S spectrum. Comparing the ^{13}C NMR spectra of non-sulfated derivatives (HA, CM-HA) and CM-HA-S, a low-field shift of the C-6′ signal in the *N*-acetyl-glucosamine unit from 61 to 68 ppm was observed after sulfation. This low-field shift is also found comparing the corresponding NMR data of HA and HA-S (e.g. δ = 68.34 ppm for HA-S 1 or 3) and is known to be typical for a sulfation at this position (Hintze et al., 2009). The disappearance of the signal for the unsubstituted C-6′ position at about 61 ppm in both high- and low-sulfated CM-HA-S indicates a complete substitution of the primary OH groups at this position by sulfate or carboxymethyl groups and a preferred sulfation of the C-6′ position which is also reported for HA and other polysaccharides (Barbucci, Magnani, Casolaro, Marchettini, Rossi, & Bosco, 1995; Philipp, Nehls, Wagenknecht, & Schnabelrauch, 1987). A defined assignment of further relevant signals in the ^{13}C NMR spectrum of CM-HA-S, especially those of C-2, C-3, and C-4′, is difficult because of the complexity of the obtained spectra in the range between 77 and 81 ppm where an overlay of several signals is observed. However comparing the signal pattern in this region with the corresponding one in the spectra of high-sulfated HA-S (e.g. HA-S 1 or 3, data given in the experimental section) and in accordance with recently published results on HA sulfation (Becher, Möller, Weiss, Schiller, & Schnabelrauch, 2010; Hintze et al., 2009), besides C-6′ sulfation, simultaneous sulfation of the secondary OH groups at C-2 and/or C-3 of the glucuronic acid unit and at C-4′ of the *N*-acetyl-glucosamine unit may be assumed.

The molecular weight of the sulfated HA derivatives was determined to range between 10,000 and 40,000 g/mol. An effect of the different sulfation agents on the resulting molecular weight could be detected. Less degradation was found to occur when SO_3-DMF was used as sulfating agent. Similar results were reported using SO_3 complexes of DMF and pyridine to sulfate anionic bacterial

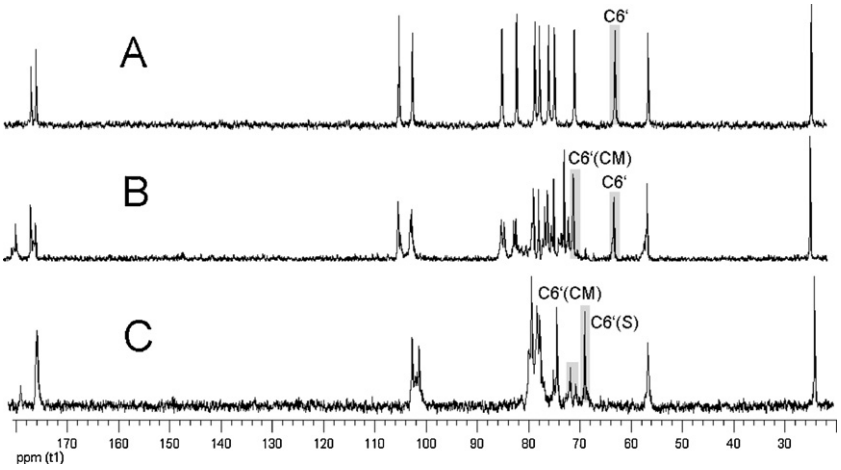

Fig. 3. ^{13}C NMR spectra from hyaluronan (A), carboxymethyl hyaluronan (B, CM-HA 4; DS_{CM} = 0.5) and carboxymethyl hyaluronan sulfate (C, CM-HA-S 4; DS_{CM} = 0.5, DS_S = 3.3).

Table 3
Antiherpetic activity of test compounds in Green monkey kidney (GMK) cells.

Sample	Educt	DS_{CM}	DS_S	IC_{50}[a] (μg/ml)
PFA[b]	–	–	–	12.5
HMW-HA	–	–	–	Not active
CM-HA 5	HMW-HA	0.8	–	Not active
HA-S 1	HMW-HA	–	3.0	0.5
HA-S 2	HMW-HA	–	1.5	37.5
HA-S 3	LMW-HA	–	3.2	2.4
HA-S 4	LMW-HA	–	1.9	29.6
CM-HA-S 1	HMW-HA	0.4	3.2	4.7
CM-HA-S 3	HMW-HA	0.3	0.9	Not active
CM-HA-S 4	LMW-HA	0.5	3.3	2.9
CM-HA-S 5	LMW-HA	0.4	1.1	Not active

[a] 50% inhibitory concentration (polymer concentration necessary to inhibit the HSV-1 induced cytopathic effect by 50%).
[b] Phosphonoformic acid.

polysaccharides like glucuronan (Petit et al., 2004). It is noticeable that all sulfated products have molecular weights in the same range, no matter if the starting material was low or high molecular weight HA. The considerable degradation might be due to the known susceptibility of the glycosidic bond in HA to hydrolytic cleavage caused by intermediately formed highly acidic —OSO_3H groups in the molecule (Nagahata, Tsuchiya, Ishiguro, Matsuda, Nakatsuchi, Teramoto, Hachimoro, Abe, 2004; Stern, Kogan, Jedrzejas, & Šoltés, 2007). Further studies are necessary to fully understand this phenomenon.

3.2. Cytotoxicity and antiviral activity of hyaluronan derivatives

None of the tested HA compounds prepared in this study exhibited cytotoxic effects up to a tested maximal concentration of 200 μg/ml.

The antiviral activity of the synthesized CM-HA-S derivatives was tested against Herpes simplex virus type 1 (HSV 1) in Green monkey kidney (GMK) cells and compared with those of the HA-S polymers. In the assay, biopolymer concentrations (IC_{50}) were determined able to inhibit the HSV-1 induced cytopathic effect by 50%. Therefore, the lower the measured IC_{50} value the higher is the antiviral activity of the polymer. PFA, a known antiviral medication used under the trade name Foscavir to treat herpes viruses including herpes simplex viruses types 1 and 2 (HSV-1, HSV-2), and drug-resistant cytomegalovirus (CMV) served as control. PFA acts as a structural analog of pyrophosphate that selectively inhibits the pyrophosphate binding site on virus-specific DNA polymerases at concentrations that do not affect cellular DNA polymerases (Crisp & Clissold, 1991; De Clercq, 2004; Villarreal, 2003). The antiviral activities of the prepared HA derivatives are summarized in Table 3. As expected neither HA nor CM-HA exhibit any antiherpetic activity in contrast to the sulfated derivatives.

The results clearly demonstrate the impact of the degree of sulfation on the antiviral activity. The higher the degree of sulfation the lower is the concentration of modified HA necessary to inhibit the HSV-1 induced cytopathic effect by 50%. This is obvious by comparing the IC_{50} values of high-sulfated derivatives HA-S 1 and HA-S 3 ($DS_S \geq 3$) with the ones of low-sulfated derivatives HA-S 2, HA-S 4 ($DS_S \leq 2$). The antiviral activity of the high-sulfated hyaluronans is higher than that of the control PFA by about one order of magnitude. Furthermore, the results indicate that the molecular weight of the HA polymers does not play a significant role for the antiviral activity of the polymers containing similar DS_S values (see for example HA-S 1 in comparison with HA-S 3). The 50% inhibitory concentration of CM-HA-S with a high DS_S of 3.2 (CM-HA-S 1) was 4.7 μg/ml. This value is slightly higher than the IC_{50} of the hyaluronan sulfate HA-S 1 with a comparable DS_S of 3.0 (IC_{50} = 0.5 μg/ml). The low-sulfated carboxymethyl hyaluronan derivatives CM-HA-S 3 and CM-HA-S 5 with DS_S = 0.9 and 1.1, respectively, have no antiviral activity, whereas the low-sulfated HA-S 2 with a DS_S of about 1.5 exhibited an only moderate antiviral activity with IC_{50} = 37.5 μg/ml. This result suggests that a DS_S of about 1.5 is necessary to achieve any antiviral effect. The introduction of carboxymethyl groups into hyaluronan or hyaluronan sulfates at a DS_{CM} range of up to 0.8 does not distinctly affect the antiviral activity. To figure out the reason of this surprising result further investigations are necessary.

Summarizing, it can be stated that the antiviral activity of carboxymethylated hyaluronan sulfates is comparable to those only sulfated and that the high-sulfated derivatives with and without carboxymethyl groups show a higher antiviral activity than PFA, the used reference substance.

The carboxymethyl groups in the sulfated polymer enable an interaction with other biologically active substances and therefore the creation of molecules with interesting biological properties.

The stability of sulfated hyaluronan derivatives in solution was proved by examining their antiherpetic activity after long-time storage. Solutions were stored at 4 °C as well as at room temperature up to 10–14 weeks, in one case (HA-S 1) even for 6 months. At different time points, samples were taken and applied to CPE-inhibitory assays to check their antiherpetic activity. The antiviral test results (data not shown) demonstrate that modified HA derivatives are completely stable in solution at 4 °C as well as at room temperature up to 10–14 weeks. When stored up to 6 months, a slight loss of the antiherpetic activity is found.

4. Conclusion

The chemical modification of HA offers a promising approach to glycosaminoglycan derivatives with useful biological activities preserving the excellent cytocompatibility of the starting materials. In this paper we studied the introduction of carboxymethyl and sulfate groups into HA by a stepwise reaction sequence. Carboxymethylation was performed by reaction of high and low molecular weight HA with MCA under alkaline conditions. DS_{CM} values of up to 0.2–0.5 were obtained after a single carboxymethylation process. Repeated carboxymethylation resulted only in a slight increase of the DS_{CM} values of about 0.2–0.5. The subsequent sulfation of the CM-HA derivatives was performed using two different procedures. The treatment of CM-HA with SO_3-DMF in the presence of *p*-toluene sulfonic acid resulted in DS_S values of about 2.8. Another method for the sulfation of HA and CM-HA is their transformation into tributylammonium salts and subsequent sulfation. The degree of sulfation can be properly controlled by the type and quantity of the used sulfation agent. Sulfated derivatives with DS_S values between 0.9 (with SO_3-pyridine) and 3.3 (with SO_3-DMF) are available. A preferred sulfation of the primary OH groups at the C-6′ position of the ASU could be observed. As expected the HA starting materials undergo a remarkable decrease in their molecular weight during the modification reactions. Starting from HA of a M_W of 1×10^6 g/mol the determined M_W values of the final CM-HA-S derivatives were found in the range between 10,000 and 40,000 g/mol.

HA-S exhibits a strong antiherpetic activity that correlates with the degree of sulfation. The antiviral effect remains stable even after long time storage of aqueous solutions of sulfated hyaluronan at 4 °C as well as at room temperature. An effect of introduced carboxymethyl groups into HA or HA-S on the antiherpetic activity could not be identified.

The established synthesis methodology for preparing HA-S derivatives with controllable degree of sulfate groups and with different anionic substitutents represents a valuable basis for the initiation of more extensive structure–activity relations studies concerning further important biological properties of

glycosaminoglycans like their antithrombogenic activities or their capability to interact with growth factors.

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