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Cationization of heparin for film applications

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ABSTRACT

Trimethylammonium-2-hydroxypropyl-(TMAHP) spacer was introduced into heparin (H) and the prepared films were characterized by elemental analysis, NMR, SEC-MALS, TG/DTG/DTA, AFM and mechanical tester. When quaternized at the ratio of H/NaOH/alkylating agent/H_2O = 0.1-1/0-2/0.1-1/50-500 mmol, H was substituted at A6 and A3 positions. The formation of double-substituted structures by substitution of free hydroxyl group of the previously introduced TMAHP substituent is evident. In the absence of NaOH (H/GTMAC/H_2O = 1:1:500) the most drastic decrease of M_n to 8.639 kg/mol and M_w/M_n at 1.48 was observed in comparison to H (M_n = 9532 g/mol with M_w/M_n = 1.38). The film mechanical properties were better on H (E = 4030 MPa; σ_b = 65 MPa; ε_b = 4.6%) than on quaternized specimens (E = 2500–3340 MPa; σ_b = 25–40 MPa; ε_b = 1.7–1.8%). The AFM images did not prove relation between mechanical properties and surface shape.

1. Introduction

Macromolecular chemistry of heparin is mostly related to O- or N-deacetylation, O- or N-sulfation, or re-sulfation (Casu, Naggi, & Torri, 2002; Naggi et al., 1987; Yates et al., 1996; Mulloy, 2012; Shriver, Capila, Venkataraman, & Sasisekharan, 2012). Apart from desulfation, the enzymatic and chemical modifications under alkaline conditions can result to depolymerization, 2,3-epoxidation, and β-elimination (Ragazzi et al., 1993; Linhardt, 1992; Naggi et al., 2001). In addition, semi-synthetic derivatives were also prepared by N-acetylation, esterification, amidation, O-acylation or reductive amination (Fernández, Hattan, & Kerns, 2006). The introduction of TMAHP spacer under water-alkaline conditions was studied on xylan, including sulfated xylan, as potential analogues of heparin (Šimkovic, Gedeon, Uhliariková, Mendichi, & Kirschnerová, 2011a, 2011b). The comparison of xylan properties with those of heparin could open new perspectives for their applications and also utilize heparin for all-polysaccharide composite new products (Šimkovic, 2013). The goal of the present work is to learn about possibilities of heparin cationization with glycidyltrimethylammonium chloride (GTMAC), (S)-(−)-(3-chloro-2-hydroxypropyl)-trimethylammonium chloride (SCHPAC) or (R)-(+)-(3-chloro-2-hydroxypropyl)-trimethylammonium chloride (RCHPAC) in presence or absence of NaOH, optimize the reactions conditions, to characterize the possible products and learn about their properties. These reactions have not been studied on heparin yet (Prado & Matulewicz, 2014). It could be expected that by introduction of quaternary groups into heparin some new properties not known at the present could be found. The new properties might result due to interaction of quaternary groups with sulphates groups or prevent the interaction of the heparin groups with the surrounding environment. The possible applications might be in the medical field with properties analogical to already studied composites (Kaminski, Zazakowny, Szczubialka, & Nowakowska, 2008; Kemp & Linhardt, 2010).

2. Experimental

2.1. Materials

Heparin (Serva, # 24590.02), glycidyltrimethylammonium chloride (GTMAC; Aldrich, # 50053-50ML), (S)-(−)-(3-chloro-2-hydroxypropyl)-trimethylammonium chloride (SCHPAC; Aldrich # 329177-5G), (R)-(+)-(3-chloro-2-hydroxy-propyl)-trimethylammonium chloride (RCHPAC; Aldrich # 32,916-9) and all other chemicals were used without further purification.

2.2. Chemical modification

The mixtures at ratios heparin/NaOH/alkylating agent/H_2O = 0.1–1/0–2/0.1–1/50–500 mmol were reacted at 1250 rpm/room

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temperature for 24 h. The reaction was stopped by dilution of the mixture and dialyzed (Spectra/Por®; 1 kDa MWCO) until pH dropped to 7.20 and freeze-dried or casted on Petri dishes and dried at RT till constant weight. The amounts of reactants used, elemental compositions of products, the total degree of quaternization, sulfation, acetyl content, amount of free hydroxyl groups and water in the average dimer unit as well as yields are listed in Table 1.

2.3. Analytical methods

High-resolution NMR measurements of samples (~50 mg) were performed in D_2O (ARMAR Cat-No. 014400.0010) at 25 °C on Varian 600 MHz UNITY INOVA spectrometer equipped with 5 mm cryogenic probe. ^1H and ^{13}C chemical shifts were referenced to internal 3-(trimethylsilyl)-propionic acid (TSP). The pH-in-D_2O (pD) values of solutions were adjusted to values higher than 7.2 (Nguyen & Rabenstein, 2011).

The elemental compositions of specimens were performed on FLASH 2000 Organic elemental analyzer (Thermo Fisher Scientific; furnace temperature: 950 °C; PTFE column, 6 mm o.d./5 mm i.d. × 2 m; 65 °C; helium as carrier and reference gas flow: 140 and 100 ml/min, respectively; oxygen flow: 250 ml/min; 720 s run time; 12 s sampling delay; 5 s injection end).

The characterization of the molecular weight distribution (MWD) of samples was performed using a multi-angle laser light scattering (MALS) absolute detector on-line to a size exclusion chromatographic (SEC or GPC) system. A differential refractometer index (DRI) was used as concentration detector. An aqueous mobile phase (0.1 M CH_3COONH_4 at pH = 7.0) and two TSK gel PW_{XL} columns (G4000 and G3000 from Tosoh Bioscience) were used. The SEC-MALS experimental conditions were the following: 35 °C of temperature; 0.8 ml/min of flow rate; 100 μL of injection volume; about 3 mg/ml of sample concentration. The specific refractive index increment with respect to the mobile phase (dn/dc) was measured off-line by a Chromatix KMX-16 differential refractometer. The dn/dc values were 0.132 ml/g and 0.146 ml/g respectively for heparin and derivatives samples. The differences between dn/dc values of various derivatives were lower than the experimental uncertainty of the off-line measurement; consequently a constant value $dn/dc = 0.146$ ml/g was assumed for all derivatives. The "Recovered Mass" listed in Table 3 is the mass of sample eluting (i.e. recovered) from the SEC columns, expressed as % of the sample injected mass (sample concentration multiplied by injection volume). The recovered mass was calculated from the peak area of the DRI concentration detector after accurate calibration. All other analytical methods were described previously (Šimkovic et al., 2014).

Atom force microscopy (AFM) images in Fig. 3 were performed using BioScope Catalyst (Bruker, Santa Barbara, USA). Data were acquired using PeakForce QNM (Quantitative Nanomechanical Mapping) technique in air and evaluated by NanoScope Analysis 1.40 (Bruker). For this experiment samples were prepared by casting a 1–5% water solution on a disposable plate (WillCo Wells BV, Amsterdam, Netherlands). After evaporation of solvent (~24 h at RT) the plate with obtained film was placed into sample holder and AFM measurements were performed.

3. Results and discussion

3.1. Cationization

According to the elemental composition the average molar formula of heparin dimer unit (Fig. 1) could be calculated: $[C_{12}H_{13}O_8N(SO_3)_{2.5}Na_{3.5}(COCH_3)_{0.6}(OH)_{2.0}(H_2O)_5]_n$. The observed elemental analysis (C, 22.15; H, 3.68; N, 1.82; S, 11.14) is in agreement with theoretical values (C, 21.72; H, 3.67; N, 1.92; S,

Table 1
Reaction components (mmol), elemental composition and yields of products.

Sample/DQ[a]	H	NaOH	AA[b]	H_2O	Formula	Calculated/found [%]	Yield [%][c]
HQ1/0.30	0.1	–	0.1[d]	50	$[C_{12}H_{13}O_8N(SO_3)_{2.1}(Na)_{3.5}(OH)_{1.2}(CH_3CO)_{0.6}(C_6H_{14}ON)_{0.2}(C_6H_{15}ON)_{0.1}(HO^-)_{0.3}(H_2O)_{5.0}]_n$	C, 24.58/24.93; H, 4.25/4.24; N, 2.49/2.74; S, 9.18/9.41	93
HQ2/0.30	1	–	1[d]	500	$[C_{12}H_{13}O_8N(SO_3)_{2.1}(Na)_{3.4}(OH)_{1.7}(CH_3CO)_{0.6}(C_6H_{14}ON)_{0.2}(C_6H_{15}ON)_{0.1}(HO^-)_{0.3}(H_2O)_{4.0}]_n$	C, 25.28/25.53; H, 4.08/4.33; N, 2.56/3.05; S, 9.44/8.64	92
HQ3/1.10	1	2	1[d]	500	$[C_{12}H_{13}O_8N(SO_3)_{2.3}(Na)_{4.4}(OH)_{0.9}(CH_3CO)_{0.5}(C_6H_{14}ON)_{0.6}(C_6H_{15}ON)_{0.5}(HO^-)_{1.1}(H_2O)_{8.0}]_n$	C, 25.79/25.93; H, 5.21/4.55; N, 3.22/3.03; S, 8.07/8.84	85
HQ4/0.90	1	2	1[e]	500	$[C_{12}H_{13}O_8N(SO_3)_{2.4}(Na)_{4.3}(OH)_{0.9}(CH_3CO)_{0.4}(C_6H_{14}ON)_{0.5}(C_6H_{15}ON)_{0.4}(HO^-)_{0.9}(H_2O)_{8.0}]_n$	C, 24.54/24.62; H, 4.96/4.63; N, 2.99/2.83; S, 8.63/9.02	83
HQ5/4.00	0.1	2	1[e]	50	$[C_{12}H_{13}O_8N(SO_3)_{2.4}(Na)_{3.5}(CH_3CO)_{0.4}(C_6H_{14}ON)_{2.0}(C_6H_{15}ON)_{2.0}(HO^-)_{4.0}(H_2O)_8]_n$	C, 34.86/35.00; H, 7.28/6.76; N, 5.53/5.16; S, 6.06/6.79	79
HQ6/0.90	1	2	1[f]	500	$[C_{12}H_{13}O_8N(SO_3)_{2.3}(Na)_{3.9}(OH)_{1.1}(CH_3CO)_{0.6}(C_6H_{14}ON)_{0.5}(C_6H_{15}ON)_{0.4}(HO^-)_{0.9}(H_2O)_{9.0}]_n$	C, 25.27/25.59; H, 5.41/4.36; N, 3.01/2.95; S, 8.33/8.97	93

[a] Total degree of quaternization calculated from elemental analysis.

[b] Alkylating agent.

[c] (mmol of product according to calculated formula/mmol of heparin used according to calculated formula) × 100.

[d] GTMAC.

[e] (S)-(−)-(3-chloro-2-hydroxypropyl)-trimethylammonium chloride (SCHPAC).

[f] (R)-(+)-(3-chloro-2-hydroxy-propyl)-trimethylammonium chloride (RCHPAC).

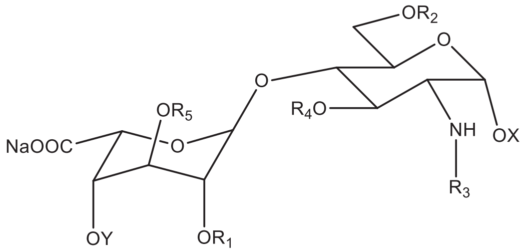

Fig. 1. Heparin (H) (R_1, R_2, and R_3 are $-SO_3Na$ groups or hydrogrn, R_2 and R_4 could be beside hydrogen also $-CH_2-CH(R_6)-CH_2-N^+(CH_3)_3$, R_3 could be beside hydrogen also acetyl, R_5 is hydrogen, R_6 is $-CH_2-CH(OH)-CH_2-N^+(CH_3)_3$ for HQ1–HQ6, while X and Y are rests of heparin macromolecule).

10.97) when considering the standard deviation of this method, the pH after dialysis of the sample, amount of water and the mixture of macromolecular structure possibilities of heparin. At the disaccharide molar mass of 729 g/mol there are in average two free hydroxyl groups, degree of sulfation at 2.5, degree of acetylation at 0.6 and five molecules of water incorporated.

This mixture of macromolecules is well characterized by NMR (Casu et al., 1995; Yates et al., 1996; Rudd et al., 2010; Torri & Guerrini, 2012; Keire, Buhse, & al-Hakim, 2013). According to HSQC integration of anomeric signals at pD 7.77 the N-sulfate-D-glucosamine 6-sulfate unit (A1, 5.39/99.47 ppm) is in ratio 0.76/1.00 with L-iduronate 2-sulfate unit (I1, 5.18/102.24 ppm) and surrounded by several minor signals. According to the known data the integrated minor signals could be assigned to $A_{NS\text{-}G}$ (5.55/100.73 ppm; 0.07), $A_{NS,\,S3}$ (5.50/99.10 ppm; 0.07), $A_{NS\text{-}(I)}$ (5.33/98.50 ppm; 0.07), $A_{NS\text{-}(I2S)}$ (5.33/99.90 ppm; 0.18) and $A_{NAc\text{-}(I/G)}$ (5.27/100.05 ppm; 0.11) (Torri & Guerrini, 2012; Keire et al., 2013). Additionally there are four other signals at 4.63/106.70 (0.10), 4.58/104.70 (0.06), 4.51/104.25 (0.12) and 4.42/105.90 (0.10) ppm which belong to D-glucuronic acid units of the heparin linkage region. At pD = 5.55 the two main integrated signals were at 5.38/99.50 ppm (A1, 1.23) and 5.22/102.02 ppm (I1, 1.00) ratio. While the chemical shifts have not changed, the ratio slightly increased at the expense of I1. At pD = 3.40 the ratio of A1 (5.41/100.60 ppm) to I1 (5.26/102.30 ppm) was 1.03/1.15. This indicates that chemical shifts and intensities of the anomeric signals are not much affected by the pD value. The average ratio of anomeric (Glc_{N6S} and Ido_{2S} residues) resonance intensities of the main signals, collected at three different experimental conditions, is 1.01 (A1)–1.05 (I1). The rest of the polysaccharide heparin A and I ring signals in the HSQC spectrum at pD 7.77 are listed in Table 2.

When cationized at H/GTMAC/H_2O = 0.1/0.1/50 molar ratio, HQ1 was obtained (Table 1). The total degree of quaternization was calculated from the summary formula $[C_{12}H_{13}O_8N(SO_3)_{2.1}Na_{3.5}(OH)_{1.7}(CH_3CO)_{0.6}(C_6H_{14}ON)_{0.2}(C_6H_{15}ON)_{0.1}(HO^-)_{0.3}(H_2O)_5]_n$, which was 0.3 in total according to elemental analysis as the sum of monosubstituted ($C_6H_{14}ON$) at 0.2 and disubstituted ($C_6H_{15}ON$) groups at 0.1. These quaternary ammonium groups were in HO^- cycles, which were also incorporated into the dimer formula. The A1/I1 ratio is 1.42 according to HSQC integration of anomeric signals (Table 2) and indicates that the original N-sulfate-D-glucosamine 6-sulfate units are in surplus, when compared to the ratio observed on pure heparin (0.76). The TMAHP-group chemical shifts are according to HSQC, COSY, TOCSY and HMBC evaluation at 3.55/70.30, 3.12–3.20/71.59 (CH_2), 4.46/67.84, 4.60/66.89, 4.92/65.00 and 5.30/70.96 (*C*HOH), 3.42–3.71/50.00 ($^+N\text{-}CH_2$) and 3.21/57.34, 3.25/57.17 and 3.28/56.55 ($^+N\text{-}CH_3$) ppm. Due to the fact that there were observed multiple signals related to $-CH_3$, $-CH-OH$ and $-CH_2-$ groups it is probable that another quaternary group is linked through the free hydroxyl on the TMAHP-group already bounded to heparin. The signals at 4.92 and 5.30 ppm according to their chemical shifts are probably the peaks which are related to secondary substituted TMAHP CHOH groups. According to COSY the signal at 5.30 ppm is in correlation with signal at 3.25 ppm ($N^+\text{-}CH_3$), while the signal at 4.92 ppm in the TOCSY spectrum correlates with signals at 3.57 and 3.48 ppm ($^+N\text{-}CH_3$). This result is also in agreement with elemental analysis calculations of molar formula (Table 1). A new signal not observed on heparin (4.39–4.46/70.23 ppm) likely originated from the substituted A6′ (Fig. 2a) was seen in the HSQC spectrum. This new resonance had similar chemical shifts as unsubstituted heparin A6 (4.46/70.23 ppm). A cross-peak at 4.46/76.12 ppm, corresponding to heparin-A6′-O-CH_2 interaction (Fig. 2b), was detected in HMBC spectrum. Another cross-peak present in the HMBC spectrum was at 3.57/74.97 ppm (Fig. 2b), which was assigned to heparin-A3′-O-CH_2- correlation with HSQC signal at 3.79/74.97 ppm. The presented data indicate that HQ1 was substituted at A6 and A3 with multiple substituents. They are distributed throughout the

Table 2
NMR data (ppm).

Sample/unit	H_1/C_1	H_2/C_2	H_3/C_3	H_4/C_4	H_5/C_5	H_6/C_6
H at pD 7.77						
A[a]	5.41/99.60	3.28/60.82	3.67/72.46	3.78/78.85	4.03/72.11	4.42–4.27/68.93
I[b]	5.18/102.22	4.35/78.81	4.21/72.12	4.12/78.84	4.83/72.05	–/177.86
A1[a]/I1[b] = 0.76[c]						
HQ1 at pD 7.8						
A[a]	5.39/99.90	3.27/60.87	3.70–3.73/72.52	3.81/79.17	4.06/72.08	4.23–4.39/69.21
Q[d,e]	–	–	3.79/74.97	–	–	4.39–4.46/70.23
I[b]	5.34/102.10	4.37/78.22	4.23/71.59	4.11/78.86	.88/72.25	–
A1[a]/I1[b] = 1.42[c]						
HQ2 at pD 6.98						
A[a]	5.42/99.46	3.26/60.80	3.69/72.50	3.81/79.35	4.04/72.17	4.22, 4.41/69.07
Q[d,e]	5.41/101.60	–	3.86/73.20	–	–	4.27, 4.41/70.38
I[b]	5.23/101.78	4.35/78.71	4.21/72.09	4.11/78.79	4.95/72.12	–
A1[a]/I1[b] = 0.69[c]						
HQ3 at pD 8.17						
A[a]	5.42/99.46	3.28/60.80	3.69/72.50	3.80/78.76	4.05/71.96	4.23–4.46/69.37
Q[d,e]	5.41/101.70	–	3.79/75.13	–	–	4.41, 4.45/70.40
I[b]	5.28/101.89	4.35/78.59	4.22/71.80	4.11/78.76	4.85/72.14	–
A1[a]/I1[b] = 1.13[c]						

[a] N-sulfate/acetyl-D-glucosamine 6-sulfate unit.
[b] L-Iduronate 2-sulfate unit.
[c] Ratio of areas of integrated anomeric signals.
[d] Qaternized D-glucosamine unit at A6.
[e] Qaternized D-glucosamine unit at A3.

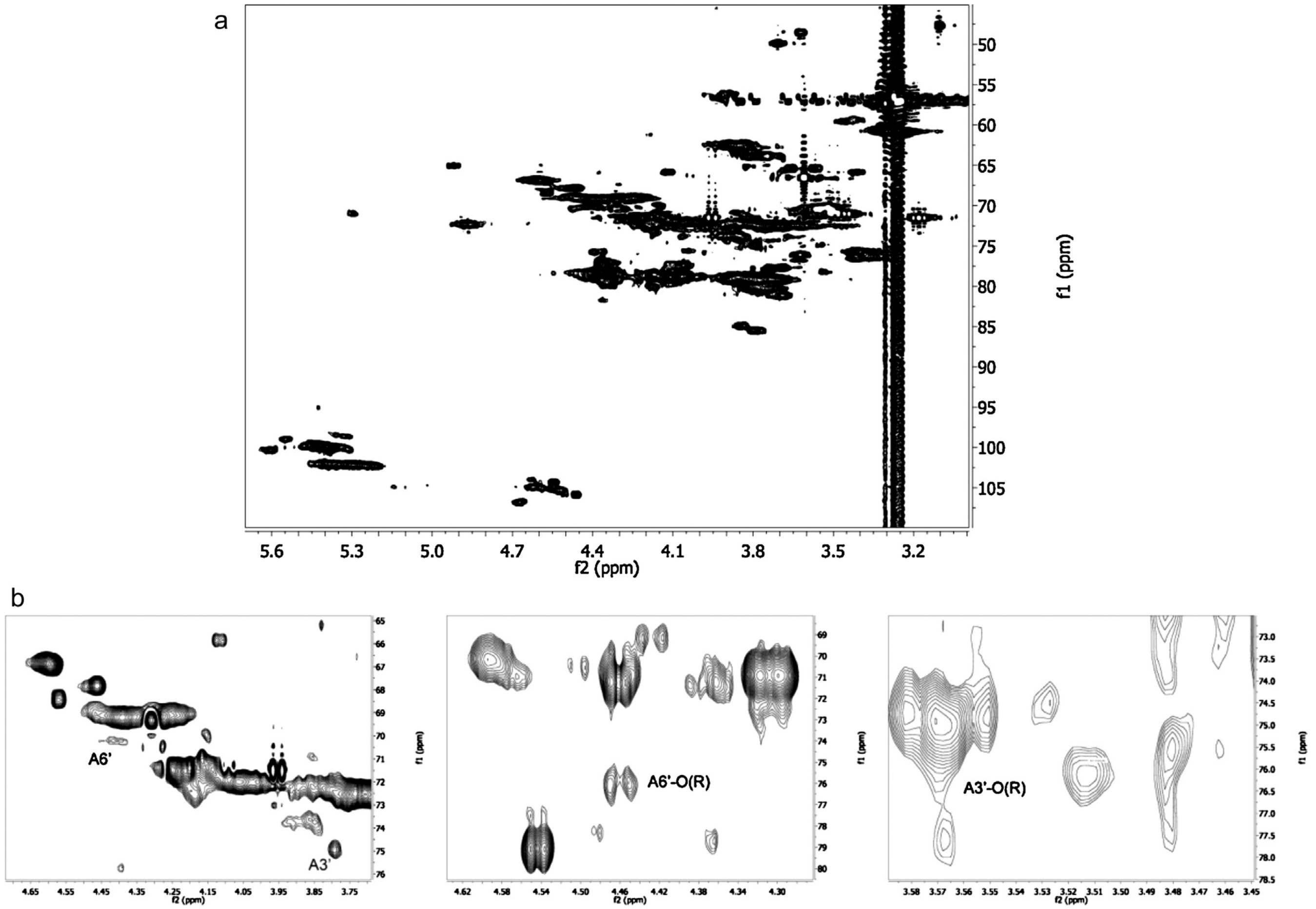

Fig. 2. The complete HSQC (a) and enlarged areas of HSQC (left) and HMBC spectra of assigned A6′ and A3′ signals (b) in HQ1 sample.

heparin macromolecule (Fig. 1). No A2 substitution was observed, which is in agreement with the identical degree of acetylation calculated from elemental analysis. This value was identical for used heparin and HQ1 sample (Table 1). The anomeric minor signals observed on heparin remained at same intensity also on HQ1 as well as intensities of the unsulfated A6 signals of heparin. For that reason the possible formation of epoxy uronic, galacturonic acid or formation of other structures mentioned in the literature could not be discussed (Jaseja et al., 1989; Casu, Naggi, & Torri, 2014).

At H/GTMAC/H_2O = 1/1/500, ten times bigger amounts of the components were used due to film preparation from HQ2. The total degree of quaternization 0.3 was calculated from elemental analysis as mentioned above (Table 1). This is the same value as for HQ1. Also the degree of acetylation and sulfation are according to elemental analysis the same as the values determined on HQ1. For HQ2 the molar mass 711.9 g/mol of disaccharide repeating unit is $[C_{12}H_{13}O_8N(SO_3)_{2.1}Na_{3.4}(OH)_{1.7}(CH_3CO)_{0.6}(C_6H_{14}ON)_{0.2}(C_6H_{15}ON)_{0.1}(HO^-)_{0.3}(H_2O)_{4.0}]_n$. For this sample a new anomeric signal at 5.41/101.60 ppm was observed (Table 2). The A1/I1 ratio 0.72/1.05 determined from HSQC integration was 0.69 which is similar to the value determined on heparin and much lower than determined on HQ1. This might be due to the fact that bigger amount of sample (50–60 mg) was used for analysis for HQ2 experiment than on HQ1 (20–30 mg), which might affect the resolution of the peaks. In addition, there were three *CH*OH signals at 4.30/69.10, 4.47/67.72 and 4.60/66.75 ppm and four lines of CH_2 signals at 3.62/66.43, 3.56/70.40, 3.52/70.88 and 3.62/75.97 ppm and CH_3 signal at 3.26/57.01 ppm related to the TMAHP-group. A doublet resonances (4.46 resp. 4.48/76.50 ppm) originating from heparin-A6′-CH_2-group correlation were clearly seen in HMBC spectrum. Also the detection of the new signal (3.86/73.20 ppm) in HSQC which originates from A3′. Similarly as for HQ1, the peak at 3.57/72.65 ppm was observed in HMBC spectrum. The signal at 3.57 ppm was also observed in COSY and TOCSY spectra of HQ2 and was absent in NMR spectrum of native heparin (Table 2).

At the molar ratio of H/NaOH/GTMAC/H_2O = 1/2/1/500, HQ3 disaccharide with molar mass at 911.8 g/mol and the molar formula: $[C_{12}H_{13}O_8N(SO_3)_{2.3}Na_{4.4}(OH)_{0.9}(CH_3CO)_{0.5}(C_6H_{14}ON)_{0.6}(C_6H_{15}ON)_{0.5}(HO^-)_{1.1}(H_2O)_{8.0}]_n$ was calculated with the total degree of quaternization 1.1. In this case the degree of substitution for monomerically at 0.6 and double substituted (0.5) quaternary groups are much higher than for HQ1 and HQ2 (Table 1). The averaged sulfation degree calculated increased from 2.0 (HQ1), 2.1(HQ2) to 2.3, which we assume is due to different reaction conditions in individual experiments. It indicates that the quaternary ammonium base causes more desulfation than in observed by the combination of NaOH and alkylating agent. The decrease of acetylation degree from 0.6 to 0.5 indicates minimal effect on that value at room temperature in the presence of NaOH. Similarly like for HQ2 also in this case an anomeric signal at 5.41/101.70 ppm was present in the HSQC spectrum. The observed ratio of the integrated intensities of anomeric peaks A1/I1 was 1.13. Also for this case bigger amount of sample was used for the analysis. The spectra revealed also several other signals. For example, doublet (4.41 and 4.45/70.10 ppm) indicated the presence of new A6′ signal whereas the signal at 3.79/75.13 ppm was assigned to A3′. In the HMBC spectrum there was again a doublet at 4.45 and 4.47/76.37 ppm due to *H*-A6′-O-CH_2- correlation and another doublet at 3.56 and 3.58/75.23 ppm due to A3′-O-CH_2-interaction.

Table 3
SEC-MALS data of samples.

Sample	M_p [g/mol]	M_n [g/mol]	M_w [g/mol]	M_w/M_n	Recovered mass [%][a]
H	10,782	9534	13,164	1.38	90
HQ1	13,822	11,305	15,300	1.35	81
HQ2	11,643	8639	12,762	1.48	88
HQ3	11,234	9523	12,480	1.31	91
HQ4	11,776	9957	13,122	1.32	90
HQ5	14,460	12,051	16,362	1.36	96
HQ6	11,407	9254	12,770	1.38	90

[a] "Recovered Mass" is the mass of sample (g) eluting (i.e. recovered) from the SEC columns, expressed as % of the sample injected mass (sample concentration times injection volume). The Recovered Mass is calculated from the area of the DRI concentration detector after accurate calibration.

When SCHPAC was used instead of GTMAC at the same ratio as for HQ3, HQ4 with molar mass 889.90 g/mol and calculated formula: $[C_{12}H_{13}O_8N(SO_3)_{2.4}(Na)_{4.3}(OH)_{0.9}(CH_3CO)_{0.4}(C_6H_{14}ON)_{0.5}(C_6H_{15}ON)_{0.4}(HO^-)_{0.9}(H_2O)_{8.0}]_n$ was prepared with slightly smaller total degree of quaternization 0.9. The ratio of A1/I1 HSQC integrated signals was at 0.83/1.10 = 0.75 at the same value as for HQ2 and HQ3. The anomeric signal at 5.41/102.00 ppm originating from A1 was also present (data not shown in Table 2). Also in this case a new anomeric signal A1′ was observed. In the HSQC spectrum, there were also new signals at 4.41/70.16 and 3.80/74.29 ppm which were analogically assigned to A6′ and A3′ groups of HQ4 as for HQ1–HQ3. There were no other differences observed in the NMR spectra due to the use of chiral alkylating agent.

To verify that there is no different behaviour observed SCHPAC was used. In this case HQ5 with molar mass of disaccharide unit calculated at 1266.7 g/mol was prepared at H/NaOH/SCHPAC/H_2O = 0.1/2/1/50 ratio of reactants. The calculated summary formula was: $[C_{12}H_{13}O_8N(SO_3)_{2.4}(Na)_{3.5}(CH_3CO)_{0.4}(C_6H_{14}ON)_{2.0}(C_6H_{15}ON)_{2.0}(HO^-)_{4.0}(H_2O)_8]_n$ based on elemental analysis (Table 1). For this sample the total degree of quaternization 4.0 was calculated. In this case, however, the anomeric resonance intensities could not be determined due to low intensities of the signals. High dynamic spectral range as well as considerable peaks overlap prevented also the assignment of the substituent. For that reason also the presentation of mono-dimensional proton NMR spectra could not be used for the demonstration of the difference in derivatization. Also the integration of anomeric signals even for HQ1 up HQ4 might be affected by this factor. The formation of ammonium salts between reagent and heparin carboxyl was excluded by the absence of additional carboxyl signal in ^{13}C NMR spectra of the derivatives beside the signal at 177.86 ppm observed also in used heparin. On the other side the formation of ammonium salt between heparin sulfate and the reagent or the introduced substituent is not indicated from the elemental analysis as the amounts of sodium are corresponding to amounts of sulfates and carboxyls present in heparin.

The use of RCHPAC at the same molar ratio of reactants as for HQ4 resulted in formation of HQ6 with calculated repeating disaccharide unit mass at 883.3 g/mol and formula: $[C_{12}H_{13}O_8N(SO_3)_{2.3}(Na)_{3.9}(OH)_{1.1}(CH_3CO)_{0.6}(C_6H_{14}ON)_{0.5}(C_6H_{15}ON)_{0.4}(HO^-)_{0.9}(H_2O)_{9.0}]_n$ based on elemental analysis (Table 1). The total degree of substitution is the same as for HQ4. The molar ratio of HSQC integrated A1/I1 = 0.88, which is slightly bigger than for HQ4, probably due to different concentrations used for HQ6 than for HQ4.

It could be concluded that the quaternization with GTMAC, in the presence of NaOH, caused substitution at A6 and A3 positions. For all the used conditions double-substituted products were obtained with modified hydroxyls of already introduced TMAHP groups. Signals due to β-elimination (Linhardt, 1992; Ragazzi et al., 1993) were not observed. Additional observed difference was a lower degree of quaternization for HQ4 and HQ6 in comparison to HQ3. The reason for these differences is likely due to a formation of racemic glycidyltrimethylammonium intermediate, which than alkylates the corresponding hydroxyl group. It seems that alkylating agent presence is affecting the decrease of sulfation degree in experiments (HQ1 and HQ2) when the sodium hydroxide was absent. In the presence of NaOH only small decrease of degree of acetylation took place (HQ3–HQ6), while the degree of sulfation remained similar to the value calculated on heparin formula. Also no substitution at A2 position was observed which was confirmed by similar degree of acetylation calculated at the range from 0.6 for heparin, HQ1, HQ2 and HQ6 and to 0.5 for HQ3 and 0.4 determined for HQ4.

3.2. SEC-MALS analysis

The film form of heparin and modified samples were analyzed using the SEC-MALS system and 0.1 M CH_3COONH_4 at pH = 7.0 as solvent. The recovered mass of unmodified heparin was 90% (Table 3). In general no meaningful molecular aggregation was noted for all analyzed samples according to the light scattering signal. The mass at the peak of the chromatogram (M_p) value was used because is a meaningful molar mass value not particularly influenced by an eventual molecular aggregation of the sample.

While the heparin sample had number average molar mass M_n = 9532 g/mol and weight average molar mass M_w = 13164 g/mol with polydispersity index M_w/M_n = 1.38; the values of M_p for modified samples were higher than for the starting heparin. According to the measured data the most drastic conditions were used for HQ2 at the absence of NaOH when M_n = 8639 g/mol, M_w/M_n = 1.48 and 88% recovered mass were observed. The presence of NaOH at H/NaOH/GTMAC/H_2O ratio 1/2/1/500 mmol resulted in HQ3 with M_p = 11,234, M_n = 9523 g/mol, M_w/M_n = 1.31 and 91% of recovered mass. The use of SCHPAC and RCHPAC resulted in higher M_n values and similar polydispersity compared with HQ3. The highest molar mass values were obtained at the highest degree of quaternization which confirms the effect of the substituent on M_p and M_n and simultaneously the highest determined recovered mass of 96% was measured.

For HQ1 the lowest recovered mass value 81% was observed. The HQ2 sample with the lowest M_n value had the second lowest value of mass recovered. Generally it could be stated that the recovered mass of starting heparin and also of modified samples was high. We ascribe the decrease of mass recovered to the high water content of polysaccharides which is even increasing with introduction of ion-exchanging groups.

3.3. TG/DTG/DTA analysis

When analyzed in the powder form (as delivered by manufacturer) in air using TG/DTG/DTA method under dynamic conditions

Table 4
TG/DTG/DTA data of studied films.

Sample	OT[a]	DTA peaks		DTG peaks		Residue [%] at [°C]				
	[°C]	[°C]	Δ [K/mg]	[°C]	[mg/min]	200	300	400	500	1000
H[b]	212	251	0.1104	250	7.70	88	60	48	41	34
		335	0.1410	288	0.49					
		424	0.1514	424	0.43					
		538	0.1069	568	0.59					
		573	0.1615							
HF[c]	197	241	0.0370	236	2.98	86	58	51	45	31
		340	0.0520	278	0.51					
		581	0.1304	579	0.36					
		620	0.1322							
HQ2	183	255	0.0045	252	5.93	87	65	48	42	25
				308	4.50					
		418	0.0095	412	1.03					
		561	0.0158	557	1.87					
HQ3	154	165	0.0003	173	0.86	88	61	48	42	24
		192	0.0003	200	1.15					
		253	0.0020	250	7.54					
		359	0.0024	305	3.66					
		425	0.0024							
		600	0.0051	598	1.62					
HQ4	174	247	0.0021	197	0.78	87	61	49	42	25
				245	8.25					
				305	3.35					
		377	0.0024							
		429	0.0023							
		486	0.0027	479	0.98					
		603	0.0053	577	1.82					
HQ6	157	247	0.0014	243	8.48	87	60	48	41	24
				305	3.60					
		374	0.0017							
		454 0.0016								
		483	0.0018	475	0.90					
		604	0.0041	575	1.86					

[a] Onset temperature.
[b] Heparin powder.
[c] Heparin film.

at 10 K/min heating rate heparin had the onset temperature (OT) at 221 °C (Table 4). The first DTA and DTG peaks were at 251 and 250 °C. In total the sample degraded in five exotherms and four DTG peaks as listed in Table 4. The residues at 200, 300, 400, 500 and 1000 °C were 88, 60, 48, 41 and 34% respectively. When analyzed in film form on heparin specimens used for mechanical testing (Šimkovic et al., 2014) the OT decreased to 197 °C and the first exotherm was at 241 °C with only three additional exotherms 340, 581 and 620 °C with slightly lower heat effect than observed on powder sample. There were only three DTG peaks at 236, 278 and 579 °C with the highest rate of thermo-oxidation at 2.98 mg/min for the first peak. The residues were 86, 58, 51, 45 and 31%, at 200, 300, 400, 500 and 1000 °C respectively, which were slightly lower values up to 400 °C when the film was more stable than the powder form, but at 1000 °C film resulted in only 3% lower residue than powder. It indicates that the sulfate groups remained in sodium form during the film preparation.

By the quaternization the OT of HQ2 decreased to 183 °C in comparison to heparin film, while the thermal stability did not decreased much with residues higher than for H up to 300 °C and with 25% residue at 1000 °C. With increase DS from 0.30 to 1.10 the OT of HQ3 decreased to 154 °C, but the thermal stability of HQ3 was similar like for HQ2 with similar residues during the whole degradation process with residue of 24% at 1000 °C. Similarly like in previous studies by the derivatization of the polysaccharide the thermal stability of the produced specimens decreased probably due to decreased amount of hydrogen bonds formed by hydroxyls, which stabilized the macromolecule (Šimkovic et al., 2014).

3.4. Mechanical properties of films

The film made out of heparin was white and partially transparent with modulus at 4030 MPa, tensile strength of 65 MPa and elongation at the break at 4.6% (Table 5). These are values slightly lower than observed on xylan (Šimkovic et al., 2014). The significantly thicker film in the case of heparin could be used due to easy preparation of test sample by cutting even with thickness of 0.1 mm indicating its relatively good toughness. On the other hand, successful cutting of modified heparin was possible at lower thickness only due to damage of thicker samples in the course of cutting. This confirms certain embrittlement due to modification indicated by reduced elongation at break values. With the quaternization the mechanical properties were diminished. On HQ2 when the film was the thickest (0.04 mm) and at the absence of NaOH similar data were measured as on HQ4 and HQ6. The values determined were similar for both HQ4 and HQ6 prepared by using the two chiral quaternizing agents. Similarly like observed previously on xylan (Šimkovic et al., 2014), lower values were observed after quaternization. We think it was due to decreased amount of free hydroxyl groups able to form hydrogen bonds. But even at higher degree of substitution the values were comparable with data observed on other polysaccharides (Kanmani & Rhim, 2014).

3.5. AFM analysis

The AFM height image of film prepared from heparin gives maximal horizontal distance ($R_{\max}$ = 37 nm) for 15 × 15 μm area of top surfaces (Fig. 3a), while for HQ4 the value is 51.8 nm (Fig. 3b). These values were fluctuating for two independent images of HQ6 (22.6

Table 5
Mechanical properties of studied heparin films.

Sample	E (MPa)	Thickness (mm)	σ_b (MPa)	ε_b (%)
H	4030 ± 300	0.10	65 ± 4	4.6 ± 0.7
HQ2	3300 ± 300	0.04	35 ± 5	1.4 ± 0.8
HQ3	2500 ± 350	0.03	25 ± 8	2.0 ± 0.5
HQ4	3340 ± 300	0.03	37 ± 9	1.7 ± 0.3
HQ6	3250 ± 50	0.03	40 ± 5	1.8 ± 0.4

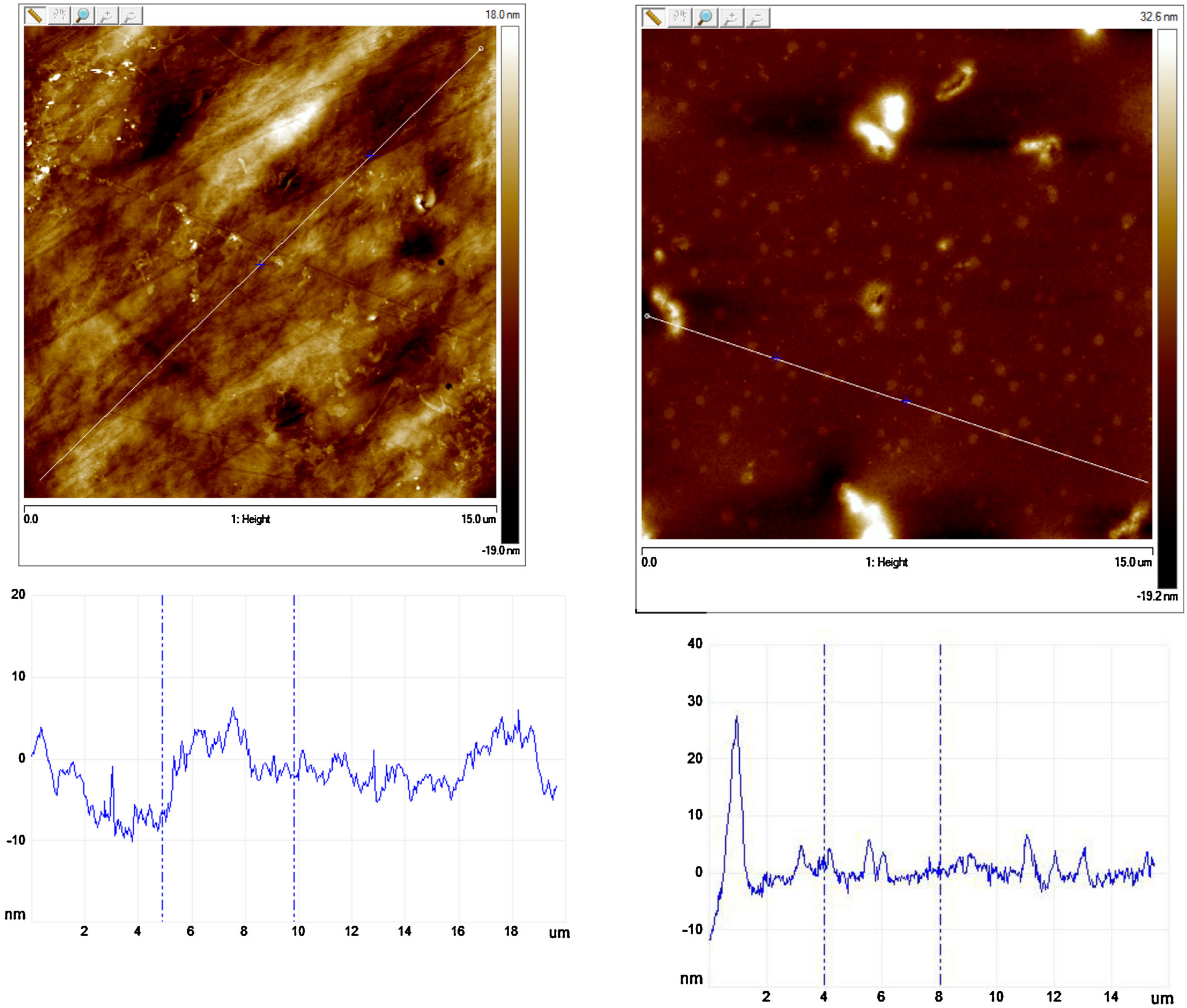

Fig. 3. AFM height images with section analysis of H (left) and HQ4 (right) films.

or 6.8 nm) to 5.9 nm for HQ2 film and 34.7 nm for HQ3 sample. It indicates that there is no relation between film mechanical properties and their upper surface homogeneity, which is probably more dependent upon the history of drying process.

4. Conclusions

TMAHP heparin derivatives were prepared by alkylation with three alkylating agents in water without or with NaOH presence. According to the elemental analysis the total degree of quaternization values were from 0.30 to 4.00, for trimethylammoniumhydroxyprophyl-groups, directly or indirectly, linked to heparin, while in all cases double substitution of the substituent was achieved. The substitution took place at A3, and A6 of the D-glucosamine unit according to NMR results. The introduction of cationic group into I3 position could not be proved. The β-elimination of glycosidic bond at C-4 of L-iduronic acid was also not observed. The presence of GTMAC slightly lowered the degree of sulfation but did not affect the degree of acetylation. The presence of NaOH resulted in increased degree of quaternization but did not dramatically influence the degree of sulfation, acetylation and the molar masses of products. With increasing degree of quaternization also the numerical molar mass values of samples increased. The introduction of the substituent group resulted in decrease of mechanical properties and thermal stability of prepared films. But even at a higher degree of substitution the mechanical properties were comparable with values obtained on other polysaccharides. According to AFM measurements there is no relation between the homogeneity of upper film surface and its mechanical properties.

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