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Preparation of water-soluble/insoluble derivatives of hyaluronic acid by cross-linking with epichlorohydrin in aqueous NaOH/NH_4OH solution ☆

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Abstract

Hyaluronan (HA; 0.1 or 1 mmol) was cross-linked by using epichlorohydrin (E; 0.005–0.25 mol) in the presence of NaOH (0.005–0.25 mol) and without or with NH_4OH (0.005 or 0.01 mol). The deacetylation of the *N*-acetyl-D-glucosamine units and a degradation of the product was confirmed in solution by NMR and the static light-scattering (LS) analysis. The products prepared with 0.005–0.1 mol of NaOH, at the presence of NH_4OH, and with 0.005–0.05 mol of E were highly water soluble. But at 0.075 mol of E and 0.1 mol of NaOH/5 mol of water present, the prepared products were water-insoluble. For their analysis, the solid-state ^{13}C CP-MAS NMR spectroscopy was employed. By this method, the presence of both hydroxypropyl and hydroxypropylamine groups, partially overlapped with HA signals, were observed. The $T_{1\rho}$(H) relaxation experiments supported the assumption that the linkages between HA and hydroxypropylamine or hydroxypropyl groups were formed, because all the carbon signals resulted in close $T_{1\rho}$(H) values.

Keywords: Hyaluronan; Cross-linking; Nuclear magnetic resonance; Light-scattering

1. Introduction

Hyaluronan (HA) is a biopolymer with many applications in human medicine (Ghosh, 1994; Prestwich, Marecek, Marecek, Vercruysse & Ziebell, 1998; Band, 1998). It is soluble in water independently on its molecular weight, but undergoes spontaneous depolymerization in solution, probably, due to some weak linkages related to *N*-acetylglucosamine in the chain (Kubo, Nakamura, Takagaki, Yoshiida & Endo, 1993). Polymer analog reactions, e.g. etherification, esterification, and/or introducing appropriate spacers, suitable for drug delivery applications (Chen, Jo & Park, 1995; Šoltés, Mendichi, Machová, Steiner, Alföldi, Sasinková, Bystrický & Balog, 1999), are currently of great interest. Usually, the products maintain water solubility, when, e.g. a part of carboxyls remain unmodified (Wada, Chirachanchai, Izawa, Inaki & Takemoto, 1994a), or even after cross-linking, when HA oligosaccharides were used (Pouyani & Prestwich, 1994). When cross-linked through amide functionality (Pouyani, Harbison & Prestwich, 1994; Kuo, Wen & Prestwich, 1991), insoluble materials are prepared.

In the present study, novel HA derivatives were prepared by using epichlorohydrin (E) in the presence of NaOH and NH_4OH. Similar modification reactions were used for the derivatization of starch (Šimkovic, Laszlo & Thompson, 1996) and polygalacturonic acid (Šimkovic, 1997). The advantage of the prepared derivatives were the insolublity in water in relation to their ion exchanging properties. However, when HA is cross-linked (Yui, Okano & Sakuri, 1992; Sakurai, Ueno & Okuyama, 1987) or deacetylated with NaOH in water (Wada et al., 1994b), a degradation (Ghosh, Kobal, Zanette & Reed, 1993) can occur. Under previous condition, a water-soluble organic solvent mixture was used to prevent HA from decomposition (Sakurai et al., 1987). With a goal to prepare water-insoluble HA derivatives, we did not experiment with organic solvent mixtures, and our cross-linking conditions could be

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Table 1
Reaction conditions of HA modification in the absence of NH_4OH

Sample	Moles of reactants			Yield[a] (%)	N[b] (%)	E/NaOH
	E	NaOH	H_2O			
1	0	0.1	1	72[c]	2.62	–
2	0	0.1	0.5	68[c,d]	2.37	–
3	0.01	0.1	0.5	90[c,d]	2.60	0.1
4	0.01	0.01	0.5	103[c]	2.38	1
5	0.01	0.1	5	75[c]	2.77	0.1
6	0.05	0.1	5	105[c]	2.35	0.5
7	0.075	0.1	5	106[e]	3.34	0.75
8	0.10	0.1	5	128[e]	3.24	1
9	0.125	0.1	5	112[e]	3.37	1.25
10	0.15	0.1	5	116[e]	2.77	1.50
11[f]	0.10	0.1	5	100[e]	2.75	1
12[f]	0.25	0.25	1	1576[e]	0.12	1

[a] Calculated from the weight of HA used for the reaction (0.1 mmol).
[b] Nitrogen content.
[c] Water-soluble product.
[d] 0.5 mmol of HA used.
[e] Water-insoluble product.
[f] 1 mmol of HA used.

schematically ascribed by the equation:

$$a\mathrm{H_3CCO{-}NH{-}HA{-}COOH} + b\mathrm{NaOH} + c\mathrm{E} + d\mathrm{NH_4OH} + e\mathrm{H_2O} \rightarrow r\mathrm{HA{-}NH{-}CH_2{-}CH(OH){-}CH_2{-}R} + s\mathrm{HA{-}NH{-}CH_2{-}CH(OH){-}CH_2{-}NR_2} + t\mathrm{NaCl} + u\mathrm{NaOH} + v\mathrm{NH_3} + x\mathrm{H_2O}$$

where a–x are the amounts of reaction components and R represents hydrogen, hydroxyl, hydroxypropyl, hydroxypropylamine group, and HA or deacetylated HA, linked/unlinked to another HA. The addition of NH_4OH was used to find out if water-soluble products with higher molecular weight than the starting material could be prepared in this way. The LS method was applied to determine the changes in molecular weights of the water-soluble HA fraction and NMR in D_2O, for the determination of the degree of deacetylation prior to the derivatization as well as the linkage of substituents in solution. Solid-state NMR spectroscopy and elemental analysis were used for the confirmation of the functionality of the insoluble products. Our goal was to explain exactly what products are formed and what is the evidence for the formation of linkages between HA and the hydroxypropyl/hydroxypropylamine groups on the macromolecular level. A partial goal was also to prepare insoluble derivatives with a lower production cost with varied amounts of HA. This might be more suitable for some applications than blends of HA with synthetic polymers, where components remain water-soluble even after blending (Cascone, Di Silvio, Sim & Downes, 1994).

Table 2
Reaction conditions of HA modification in the presence of NH_4OH

Sample	Moles of reactants				Yield[a] (%)	N[b] (%)	E/NaOH
	E	NH_4OH	NaOH	H_2O			
1	0.005	0.005	0.005	5	117[c]	7.80	1
2	0.005	0.005	0.005	0.5	94[c,d]	3.23	1
3	0.01	0.005	0.005	0.5	90[c,d]	2.60	2
4	0.01	0.01	0.1	5	91[e]	4.12	0.1
5	0.03	0.01	0.1	5	91[e]	3.78	0.3
6	0.05	0.01	0.1	5	90[e]	3.82	0.5
7	0.10	0.01	0.1	5	148[e]	4.65	1
8	0.125	0.01	0.1	5	137[e]	4.26	1.25
9	0.15	0.01	0.1	5	114[e]	3.93	1.50
10[f]	0.10	0.01	0.1	5	149[e]	4.29	1
11	0.10	0.10	0.1	5	174[e]	11.33	1

[a] Calculated from the weight of HA used for reaction (0.1 mmol).
[b] Nitrogen content.
[c] Water-soluble product.
[d] 0.5 mmol of HA used.
[e] Water-insoluble product.
[f] 1 mmol of HA used.

2. Experimental

2.1. Materials

Hyaluronan (CONTIPRO, Ústí nad Orlicí, Czech Republic; $M_w = 647$ kDa, $D = 1.9$; Šoltés et al., 1999). All other chemicals were of commercial grade and were used without further purification.

2.2. Sample preparation

The derivatization reactions were performed at room temperature in closed glass vials in ratios of components as listed in Tables 1 and 2. The reactions were stopped after 24 h of stirring, and the polysaccharides were isolated by sample dialysis (12–14 kDa; MWCO) and lyophilization. In experiments, where 0.5 mmol of HA were used, the samples were deacetylated in NaOH solutions for 24 h at room temperature and, subsequently, E and NH_4OH were added and the mixture was stirred for an additional 24 h. The yields were calculated on the dry basis on the amount of HA used.

2.3. Methods

High-resolution NMR spectra were measured on Bruker AMX 500 and DPX 300 instruments in aqueous solution at 313 K. Standard pulse sequences were used to obtain ^1H spectra. The solid-state NMR spectra were obtained by the DSX300 NMR spectrometer. The samples (100–150 mg) were spun in a zirconia rotors at 4 kHz and typically 1000–2000 scans were collected for each spectrum. Other experimental conditions (Košíková, Hricovíni & Simonutti, 1996) as well as SEC-MALLS methods (Mendichi, Audisio,

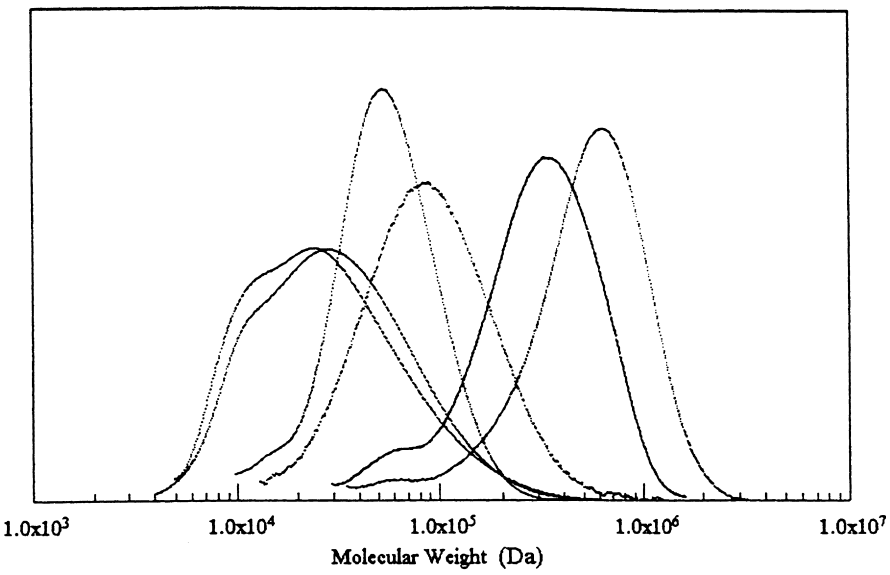

Fig. 1. Comparison of the HA molecular weight distribution curves. From right to left: native sample, $M_w = 647$ kDa; sample 1, Table 2, $M_w = 360$ kDa; sample 1, Table 1, $M_w = 111$ kDa; sample 2, Table 1, $M_w = 63$ kDa; dialyzed and lyophylized sample without chemical treatment, $M_w = 47$ kDa; sample 2, Table 2, $M_w = 40$ kDa.

Maffei Facino, Carini, Giacometti Schieroni & Saibene, 1995) were described previously.

3. Results and discussion

The conditions of experiments carried out without NH_4OH are listed in Table 1. The following amounts of compounds were used: 0.1–1 mmol of HA; 0.01–0.25 mol of NaOH; 0.01–0.25 mol of E; and 0.5–5 mol of H_2O. The yield of the product was 72% obtained by treating HA (0.1 mmol) only with NaOH (0.1 mol; sample 1). According to the acetyl content determined by the NMR method (Wada et al., 1994b), this sample contains 93% of acetyls. It indicates that the 7% of the deacetylated amido group could be involved in derivatization. When only 0.5 mol of water was used and HA deacetylated for 24 h, the yield decreased to 68% and the *N*-acetyl content was 84% (sample 2).

Water soluble derivatives were prepared (samples 3–6) in the presence of up to 0.05 mol of E, and the absence of NH_4OH. The yields and nitrogen content in these samples were slightly increasing with the increased amount of E, reaching the maximum at E/NaOH = 1 (sample 8). At the ratios of E/NaOH > 1, these values decreased probably due to the absence of sufficient amount of NaOH. The increase of nitrogen content in products, for samples where NH_4OH was not used (E/NaOH < 1), supports the expected deacetylation of the *N*-acetyl groups, as well as the formation of the hydroxypropyl HA derivatives, linked predominantly through the formed HA amino groups. These groups react preferably with E (prior to hydroxyl anions of HA), which is in surplus to HA amount. At the ten-times increased amount of HA (sample 11), keeping with the optimal E/NaOH ratio, the lower yield indicates a lower degree of sample derivatization and deacetylation. At the excess of E (sample 12) and E/NaOH = 1, HA is in the minority (as could be seen from the sample yield and nitrogen content). It indicates that E reacts preferentially with another molecule of E than with HA, and in this way the poly(hydroxypropyl) structures are formed. The fact that the sample was water insoluble, supports the linkage formation between HA and poly(hydroxypropyl) components.

The nitrogen content in the product was 7.8% (Table 2, samples 1) at 0.1 mmol of HA and the presence of NH_4OH (5 mmol), indicating the presence of a mixture of poly(hydroxypropylamine) derivatives and HA which was water-soluble. The high nitrogen content was obtained at low concentrations of E, NH_4OH and NaOH used (sample 1 in comparison to samples 2–10). Also samples 2 and 3 were water-soluble. This might be due to the low concentration of NaOH, when the HA derivatization did not take place and only poly(hydroxypropylamine) components not linked to HA were present in solution. The use of 0.5 mmol of HA resulted in lower yields and nitrogen content (samples 2 and 3), which indicates that poly(hydroxypropylamine) blocks are not formed due to NaOH consumption for the solubilization of HA at the smaller amount of water. With the 0.1 mol NaOH and 0.01 mol NH_4OH used (samples 4–10), all the samples were water-insoluble and the yield and nitrogen content reached the maximum again at E/NaOH = 1 (sample 7). At the higher values of this ratio (samples 8 and 9), the yields and nitrogen content were not further increased, probably, because of the shortage of NaOH as well as due to limited amounts of *N*-deacetylated groups on HA. The result obtained at ten times greater amount of HA was identical with that obtained at

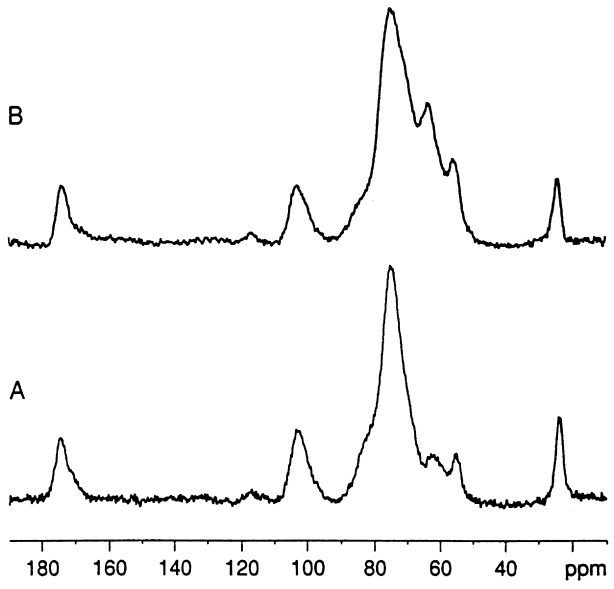

Fig. 2. ^{13}C CP-MAS NMR spectra of samples 9 (Table 1(A)) and 7 (Table 2(B)).

0.1 mmol of HA (compare samples 7 and 10). As seen from the numerical values (sample 11), the yield increased significantly, and the product was again water-insoluble when applying 0.1 mol of NH_4OH at E/NaOH = 1. The highest content of nitrogen indicates the formation of poly(hydroxypropylamine) blocks. The results indicate that insoluble materials with different HA content could be prepared by the variation of reaction components.

The results of the light scattering analysis are in Fig. 1. The unmodified HA had $M_w = 647$ kDa ($D = 1.9$). When the sample was dialyzed and lyophylized in the same way as modified samples but without chemical treatment, its M_w decreased to 47 kDa ($D = 2.28$). Sample 1 (Table 1) had $M_w = 111$ kDa ($D = 1.73$) when 0.1 mmol of HA was deacetylated. It indicates that unmodified HA was degraded during the dialysis process when NaOH was absent, more than sample 1 partially deacetylated by NaOH. Also, with the presence of chemicals used for modification or on HA-derivatives, the degradation was not so dramatic than in their absence. This is a known phenomenon not fully explained yet (Kubo et al., 1993). The modification at 5 mmol of NaOH and NH_4OH which resulted in the soluble product (Table 2, sample 1) had $M_w = 360$ kDa ($D = 1.57$). In this experiment, 0.1 mmol of HA was used. When 0.5 mmol of HA was deacetylated under the same conditions as above (Table 1, sample 2), $M_w = 62$ kDa ($D = 1.37$) was observed. At E/NaOH = 2 (Table 2, sample 2), when 0.5 mmol of HA was used, $M_w = 40$ kDa ($D = 2.06$) was measured. It shows that in all experiments, some degradation took place and no increase of molecular weight was observed prior to the cross-linking to such an extent that the samples changed into water-insoluble derivatives. At the presence of NH_4OH (Table 2, sample1), the sample seems to be less degraded than during the cyclodextrin modification, where the same starting material was used (Šoltés et al., 1999).

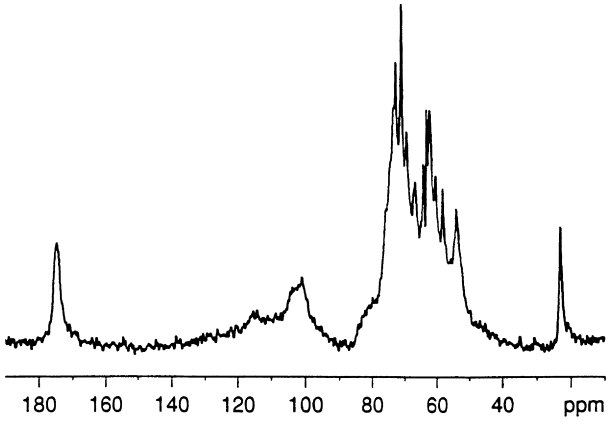

Fig. 3. ^{13}C MAS NMR spectrum of hydrated form (30% w/w of water) of sample 7.

The deacetylated sample 1 (Table 1) had 91% of *N*-acetyls as inferred from 300 MHz ^1H NMR in D_2O solution at 283 K. However, the method (Wada et al., 1994a) used at 293 K was not precise enough, because the anomeric signals and HOD signal partially overlapped. Comparable amount of *N*-acetyls (93%) in the same sample was obtained independently on 500 MHz ^1H NMR instrument at 298 K. It is noteworthy that no connectivities between HA signals and hydroxypropyl groups were found in the water-soluble products of samples modified with E and with or without NH_4OH (Table 1, samples 3–6; Table 2, samples 1,2 and 10). This is probably due to the fact that the four-bound connectivities are rare for NMR observation and also problematic on polymers. If linked through glucosamine nitrogen, the two closest hydrogens of hydroxypropyl group and H-2 of glucosamine unit are in a four bound distance. According to the experiments in water–organic solvent mixtures, when up to 40 cross-linkages took place in 1000 repeatinig disaccharide units and HA remained water-soluble (Sakurai et al., 1987), these quantities of linkage could not be detected in polysaccharide by NMR in solution.

The solid-state ^{13}C CP-MAS NMR spectrum (Fig. 2A) of the sample cross-linked with E and without the use of NH_4OH (Table 1, sample 9), had signals at 174.1 (C6′ of GlcA and carbonyl carbon of acetyl group of GlcNAc); 102.3 (C1 and C1′); 82.3 (C3); 74.6 (C3′, C4′, C5, C6′); 68.3 (C2′, C4); 62.3 (C6); 55.7 (C2); and 23.0 ppm (CH_3 of acetyl) (Pouyani & Prestwich, 1994; Pouyani et al., 1994). The sample cross-linked with E in the presence of NH_4OH (Table 2, sample 7; Fig. 2B) showed signals at 174.2, 103.0, 82.9, 73.7, 70.3, 62.3, 54.3 and 23.0 ppm originating from HA. However, the intensities of resonances were found different when compared to those observed in sample 9. The higher content of cross-linking agent E in sample 7 resulted in more intense signals between 52 and 74 ppm changing the overall pattern of the spectrum. Some of the

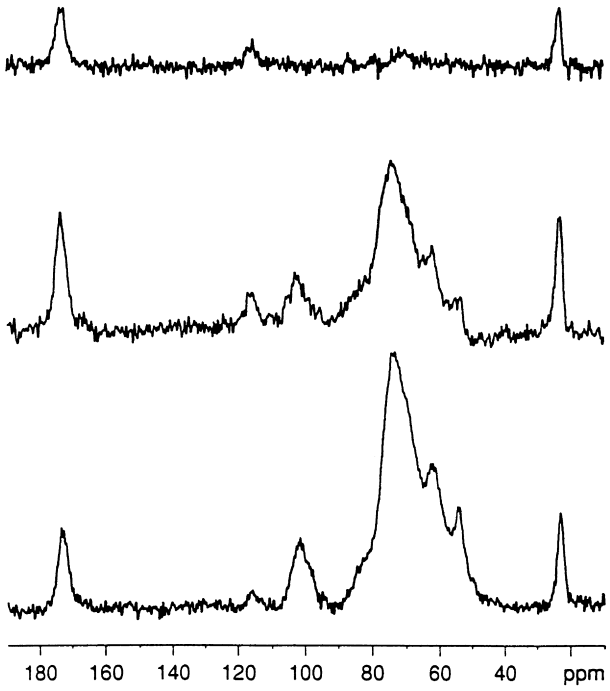

Fig. 4. Dipolar dephasing experiments on sample 7 collected with three different dephasing delays: 10 (bottom trace); 20 (central trace); and 30 μs (upper trace).

corresponding signals from E were seen at 61.7, 59.1 and 54.3 ppm in sample 9. The other signals from hydroxypropylamine group overlapped with HA signals. Such overlapping was evident also in sample 7, as well as in sample 12

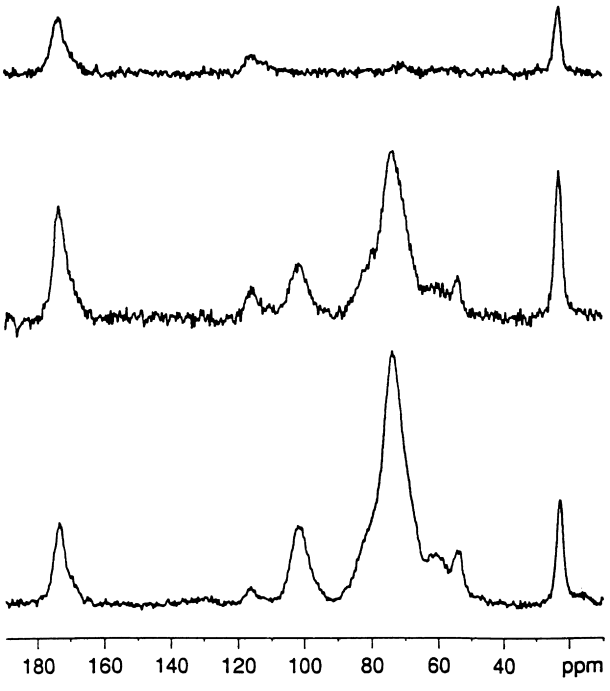

Fig. 5. Dipolar dephasing experiments on sample 9 collected with three different dephasing delays: 10 (bottom trace); 20 (central trace); and 30 μs (upper trace).

(Table 1; spectrum not shown). Due to the more complex nature (reaction of HA with E in the presence of NH_4OH), ^{13}C MAS spectrum with dipolar decoupling of the hydrated form of sample 7 (30% w/w of water) was collected (Fig. 3). Considerably, higher resolution of the spectrum is visible mainly in the region 54–75 ppm which allowed more reliable assignment of hydroxypropylamine signals. Since the resonances originating from more rigid sites of molecule have lower intensities than those from more flexible parts of the latter component, is detected more reliably. Thus, the signals from poly(hydroxypropylamine) were seen mainly in the spectrum. The complexity of the spectrum reflects various types of groups and linkages in the product molecule. As observed before (Šimkovic et al., 1996), although partially overlapped with HA resonances, the signals at 72.6, 70.9, 64.1, 63.1, 62.0, 58.1 and 53.8 ppm originate from $-O-CH_2-$, $-N-CH_2-$ and $-CH(OH)-$ groups of the hydroxypropyl and hydroxypropylamine bridges. The signal at 69.7 ppm might originate from C-6 of GlcNAc of HA linked to hydroxypropyl group; comparable to chemical shifts in the Glc residue of β-cyclodextrin cross-linked with E were detected recently (Crini et al., 1998). Furthermore, the experiment also suggests the existence of two components in the compound in its hydrated form. HA (resonances with lower intensities) represents a slightly more rigid part, whereas poly(hydroxypropylamine) component appears as more flexible.

Dipolar-dephasing spectra of both samples 7 and 9 (both non-hydrated) are shown in Figs. 4 and 5. Although the experiment was designed to monitor quaternary carbons (Opella, Frey & Cross, 1979), local motion in the molecule can partially average $^{13}C/^{1}H$ dipolar couplings, the signals from more mobile parts can be detected as well. Thus, the signals from more rigid parts of the molecule decrease faster as a function of the dephasing delay than those from the more flexible parts. The upper traces in Figs. 4 and 5 (spectra collected with a dephasing delay of 30 μs) show that, in addition of methyl and carbonyl groups, residual signals originating from cross-linking agent were detected as well, and confirm that there are no differences in the mobilities of signals relating to the HA part and the hydroxypropylamine part of the molecule. Further analysis was performed using $T_{1\rho}(H)$ relaxation times. The $T_{1\rho}(H)$ values were found uniform in both samples 9 (2.4–2.9 ms) and 7 (3.3–4.3 ms) for all the signals. The values indicate that spin diffusion is complete and homogeneous due to spatial proximity of spins within the domains of small dimensions, and thus support the assumption that the linkages between HA and hydroxypropyl or hydroxypropylamine groups were formed in these two samples. More quantitative analysis, based on the measured $T_{1\rho}(H)$ relaxation time values, gave the scale of mixing of 4.5×10^{-9} m for sample 9 and 5.4×10^{-9} m for sample 7. These numerical values of distances of mixing are consistent with the former supposition that HA–E linkage was created in the presence of NH_4OH. The numerical values further indicate that both

domains in samples 7 and 9 are comparable in their dimensions, and it appears that no considerable differences were introduced in the domain sizes in molecules when using E as the cross-linking agent in the presence of NH_4OH. These support the linkage formation between HA and hydroxypropyl or hydroxypropylamine groups for both HA derivatives.

4. Conclusion

HA could be cross-linked in an aqueous solution in the presence of NaOH with E in the presence or absence of NH_4OH. By increasing the amount of E and NH_4OH, the content of HA could be varied. The products prepared were water insoluble, when 0.1 mol of NaOH in 5 mol of water were used. In the presence of 0.05 mol of NaOH and NH_4OH, HA derivative was water-soluble. There are indications that at these concentrations, E reacted with deacetylated amine groups. The molecular weight of the water-soluble products decreased from 647 (unmodified HA) to 360.2 kDa, prior to cross-linking, or to 111 kDa due to deacetylation, or to 47.33 kDa due to sample(s) degradation. As observed by $T_{1\rho}$(H) relaxation experiments in insoluble products, no considerable changes were introduced in the domain sizes in molecules, when using E as the derivatization agent in the presence of NH_4OH. These experiments also indicate that the linkages between HA and hydroxypropyl or hydroxypropylamine groups were formed for all the water-insoluble samples.

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