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Motional properties of *E. Coli* polysaccharide K5 in aqueous solution analyzed by NMR relaxation measurements

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Abstract

^{13}C NMR relaxation measurements at three different magnetic field strengths have been used to analyse the motional properties of a low molecular weight K5 polysaccharide (ΔUA-[$\rightarrow$4)-β-D-GlcNAc(1$\rightarrow$4)-β-D-GlcA(1$\rightarrow$]$_n$-GlcNAc$_{red}$) from *E. coli*. Two-dimensional double INEPT spectra with suppression of cross-correlation effects between dipolar and chemical shift anisotropy relaxation mechanisms were collected in order to determine carbon longitudinal and transverse relaxation times. The values of the overall correlation time and the rate of internal motions were obtained using the model free spectral densities. The data indicate that the overall motion of the molecule is non-isotropic and can be approximated with the symmetric top model with an axial ratio of $\sim$22. The magnitude of the generalized order parameter ($S^2 \sim 0.8$) and the internal motion correlation time ($\tau_e \sim 30$ ps) differ from those found for iduronic acid-containing glycosaminoglycans and suggest that the internal motions in K5 polysaccharide are more limited. © 1997 Elsevier Science Ltd.

Keywords: NMR relaxation; Capsular polysaccharide; Glycosaminoglycan; Heparin; Molecular dynamics

1. Introduction

Capsular polysaccharide from *E. Coli* K5 is a glycosaminoglycan with the structure $\rightarrow$4)-β-D-GlcA(1$\rightarrow$4)-β-D-GlcNAc(1$\rightarrow$ (Scheme 1), i.e. the same structure as the heparin precursor N-acetyl heparosan [1]. It is a model for the prevalent backbone structure of heparan sulfate and provides a substrate for the enzymes involved in biosynthesis of heparin and heparan sulfate [2]. K5 is interesting because it offers the possibility of producing heparin- and heparan sulfate-like compounds by chemical and enzymatic modification [3]. Sulfaminoheparosansulfates

Abbreviations: COSY, correlation spectroscopy; CPMG, Carr–Purcell–Meiboom–Gill; HMQC, heteronuclear multiple quantum coherence; INEPT, intensive nuclei enhanced by polarization transfer; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; TPPI, time-proportional phase incrementation; TOCSY, total correlation spectroscopy; ΔUA, 4,5-unsaturated uronic acid; U, uronic acid GlcNAc

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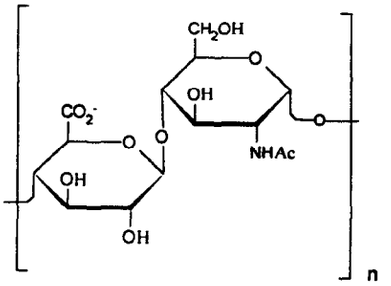

Scheme 1.

with heparin-like activity were prepared from K5 also using only chemical methods [4].

Although the primary structure is characterized [4,5], to our knowledge, nothing is known about the dynamics of this polymer in solution. NMR relaxation experiments [6–15], together with computational methods [16–24] can provide information on the nature of overall motions and the extent and the rate of internal motions [25–30]. The present work reports on the analysis of dynamics of low molecular weight polysaccharide K5 (LMW K5) and compares the data with those previously found for some other IdoA-containing glycosaminoglycans [31,32]. Since IdoA residues are usually present in more than one conformation in solution even when part of oligo- and polysaccharides [24] this comparison is of interest for an indirect evaluation of a possible influence of this conformational flexibility induced by IdoA on motional properties of glycosaminoglycan chains.

2. Experimental

Sample.—Natural, low molecular weight K5 polysaccharide ($M_W \approx 4000$) was prepared as previously described [1] and was a gift from Prof. K. Jann. The sample was treated with EDTA to remove all bivalent cations. The EDTA-cations complexes were removed by washing with NaCl solution with Microsep 1 K filter (Filtron Technology Corporation) and then washed with pure water. The EDTA removal was checked by one-dimensional ^{1}H NMR spectra. The compound ($\sim$ 15 mg) was lyophilized twice from D_2O and finally dissolved in 0.4 mL of D_2O (99.996 atom % D_2O, low paramagnetic impurities). The solution, in a 5 mm NMR tube, was purged with argon to remove oxygen and the tube was than sealed.

NMR Spectroscopy.—NMR spectra were recorded at three different magnetic field strengths, namely, 14 T, 11.7 T and 7 T, on Bruker AMX 600, AMX 500 and DRX 300 spectrometers, respectively. The temperature of the sample in the spectrometer probe was maintained at 303 K in all measurements.

Two-dimensional gradient enhanced HSQC [33] spectrum was recorded using z-gradient rectangular pulses of 20, 5, −20 and 5 G/cm, respectively. Two-dimensional double INEPT experiments, with suppression of the effects of cross-correlation between dipolar and chemical-shift anisotropy relaxation mechanisms, were used for measurements of ^{13}C T_1 and T_2 relaxation times [34]. A modified double INEPT sequence, with pulsed field gradients, was used in order to determine spin–spin relaxation times at 7 T [35]. The data matrix was 512 × 1024 points and the quadrature detection in ω_1 was achieved with TPPI. The following values of relaxation interval for measurement of T_1 were used: 20, 60, 100, 180, 260 and 340 ms at 14 and 7 T, as well as 40, 80 140, 200, 280, 380, 500 and 600 ms at 11.7 T. In the T_2 experiments, the CPMG pulse trains were 3.1, 9.4, 18.8, 37.5, 56.3, and 75.0 ms long. The evaluation of the proton–carbon NOEs were performed by the 2D sequence [34], recording the spectra in both the presence and the absence of proton saturation. Proton saturation was achieved by the application of 120 pulses spaced at 20 ms intervals for 7 s prior to the first carbon pulse.

A shifted sine-bell-squared function was applied before Fourier transformation and the cross-peak volumes were measured in each T_1 and T_2 experiment using the UXNMR software package running on a Silicon Graphics Indy work station. The fitting of the cross-peak intensities and the calculation of the motional parameters from the experimental data was carried out as previously reported [31,32].

3. Results

^{1}H One-dimensional (1D) and two-dimensional (2D) homonuclear high-resolution NMR spectra of low molecular weight (LMW) K5 polysaccharide were collected in order to confirm the primary structure in aqueous solution. The proton 600 MHz spectrum, measured at 303 K, is presented in Fig. 1. The complete assignment of the proton signals is based on two-dimensional COSY and TOCSY experiments. The values of chemical shifts and three-bond proton–proton coupling constants were found to be in agreement with the data published [4,5] for the main resonances. As seen in the conventional ^{1}H spectrum,

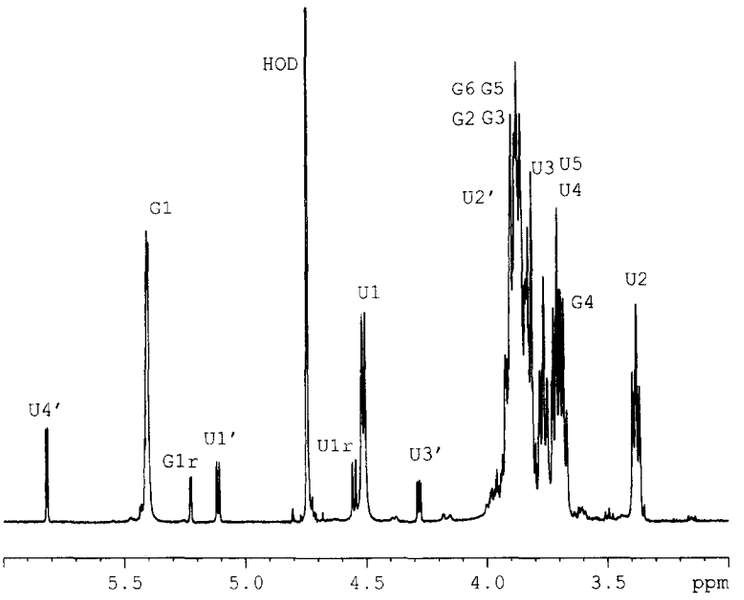

Fig. 1. The ^{1}H 600 MHz NMR spectrum of LMW K5 in aqueous solution at 303 K. The values of chemical shifts are referenced to external TSP.

the resolution of the signals is poor and most of the resonances in the GlcNAc residue, i.e. G2, G3 and G5, G6 are strongly coupled although a high field was used in the experiments. Similarly, tight coupling was detected for the U3 and U4 signals in the glucuronic acid residue. The resonances with the smaller intensities U1′ (5.11 ppm), U3′ (4.28 ppm) and U4′ (5.82 ppm), compared with the other signals, originate from the non-reducing 4,5-unsaturated uronic acid residue. The remaining resonance of this residue overlaps with those corresponding to the ring protons ($\delta_{U2'} = 3.79$ ppm). The signal at 5.23 ppm (G1r) is that of the anomeric proton from the terminal GlcNAc, the resonance at 4.55 ppm (U1r) belongs to the anomeric proton of the uronic acid unit linked to the terminal GlcNAc. G2 from the terminal GlcNAc is at 3.89 ppm, U2 of the neighbouring residue is at 3.33 ppm. The resonances of the other ring protons on both residues at the reducing end were found in the bulk region between 3.6–3.9 ppm. The integration of the resonances in the anomeric region gave the ratio of 1:10 between the anomeric proton of the non-reducing unit (U1′) and the anomeric protons of the main two signals. The molecule is therefore composed of 10 disaccharide (in average) units (UA–GlcNAc), one terminal unsaturated residue (U′) and one terminal GlcNAc (G1r). However, since the sample was obtained by isolation it has a polydisperse nature.

The 2D HSQC spectrum, collected at 14 T is shown in Fig. 2. The expansion into the second dimension improved the spectral resolution considerably, however, the U3 and U4 cross-peaks still par-

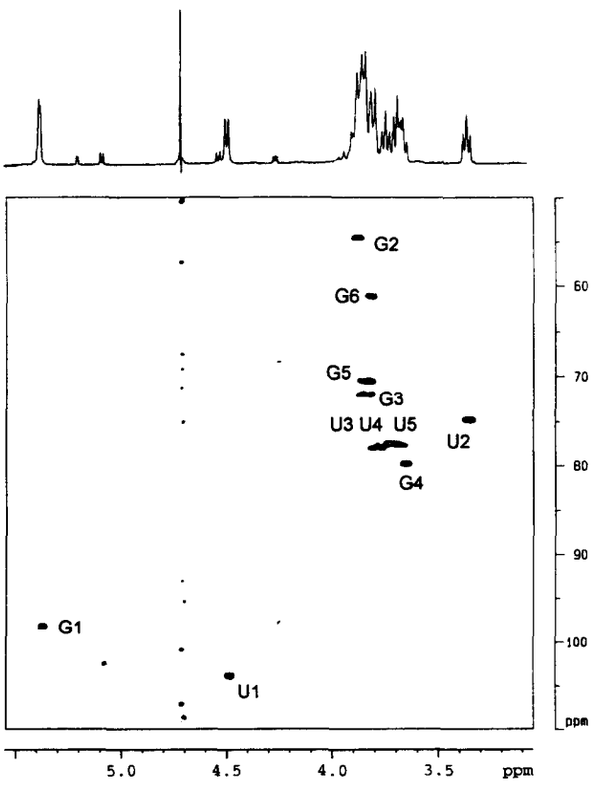

Fig. 2. Two-dimensional HSQC spectrum collected at 500 MHz.

Table 1
^{13}C chemical shifts (relative to external TSP, $\delta_{TSP} = 0$ ppm) for LMW K5 in aqueous solution at 303 K

Carbon	U1	U2	U3	U4	U5	G1	G2	G3	G4	G5	G6	U1′	U2′	U3′	U4′
δ(ppm)	105.19	76.00	78.91	78.70	79.11	99.48	55.84	73.19	81.09	71.74	62.20	103.78	81.04	69.90	110.42

tially overlap. The cross-peaks from the unsaturated residue are also well-resolved but the relaxation rates for these carbons could not be evaluated with confidence in relaxation measurements because of their lower intensities (except for the T_1 experiment at 11.7 T where the experimental time was considerably longer). The values of the ^{13}C chemical shifts, measured from the HSQC spectrum, are given in Table 1; the chemical shifts for the main resonances are comparable with those previously published [4].

In order to characterize the dynamics of LMW K5 in aqueous solution we followed the methodology used in our previous studies [31]. In the present case, however, the ^{13}C relaxation rates and heteronuclear NOEs were used exclusively since the measurement of proton homonuclear cross-relaxation rates was precluded due to severe overlap and strong couplings. The sets of the 2D double INEPT experiments were collected at various magnetic fields strengths. Fig. 3 illustrates the contour plots of subspectra of two different T_1 experiments collected at 11.7 T with the relaxation interval of 60 ms and 260 ms, respectively.

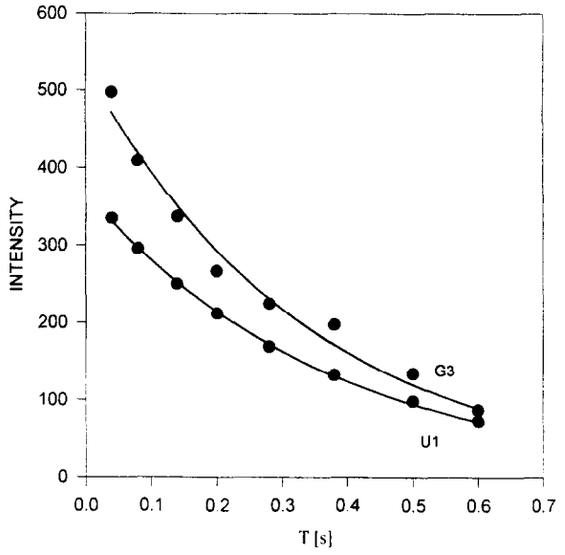

Fig. 4. ^{13}C T_1 magnetization decay curves for two selected signals. The points represent intensities (arbitrary units) of the cross-peaks, the solid lines are single-exponential fits of the data.

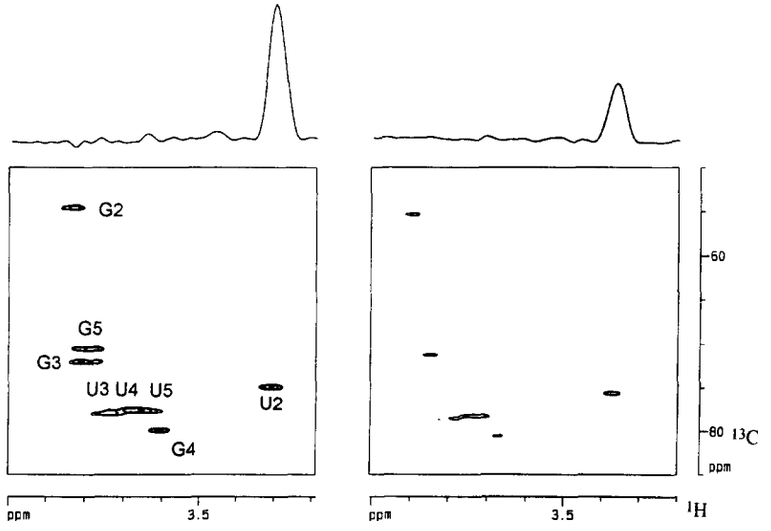

Fig. 3. Contour plots of the ring carbon region of the spectra recorded with the pulse sequence for T_1 measurement with different lengths of the relaxation interval: 60 ms (left) and 260 ms (right). The cross-section along the ω_2 axis through the U2 signal is shown above the contour plot.

Table 2
^{13}C spin–lattice and spin–spin relaxation times (in seconds) for LMW K5 collected at various magnetic field strengths in aqueous solutions at 303 K. ^{1}H–^{13}C NOEs measured at 11.7 T are presented in the last column

	T_1 (14.0 T)	T_1 (11.7 T)	T_1 (7 T)	T_2 (11.7 T)	T_2 (7.0 T)	^{1}H–^{13}C NOE (%)
U1	0.40	0.37	0.24	0.12	0.11	160
U2	0.41	0.40	0.26	0.12	0.10	169
U5	0.43	0.39	0.27	0.12		
G1	0.37	0.33	0.23	0.10	0.10	155
G2	0.45	0.39	0.26	0.11	0.09	160
G3	0.34	0.34	0.26	0.13		174
G4	0.36	0.35	0.25	0.10	0.09	147
G5	0.39	0.36	0.24	0.11		163
U1′		0.42				
U3′		0.44				

^{13}C spin–lattice and spin–spin relaxation times were obtained from the fitting to the experimental points. Typical magnetization decay curves together with the plot of cross-peak intensities are presented in Fig. 4.

^{13}C T_1, T_2 relaxation times and heteronuclear NOEs are summarized in Table 2. The computed standard deviations of the measured data were within the interval of 5–10% in most cases; these values were bigger for the data at 14 T due to the lower signal/noise ratio. The collected T_1 relaxation times differ from each other depending on the magnetic field strength used in the measurements, T_1 being largest at 14 T ($T_1 \sim 0.4$ s). The variations were also observed in the T_1 values among the carbons belonging to the UA and GlcNAc units. These data suggest that the GlcNAc residue exhibits different local motions but this trend is not clear at 7 T and, in addition, the variations among the carbons within the same unit were also considerable at higher fields. The only significant differences, which were observed in the spin–lattice relaxation times, were those for the UA1′ and UA3′ signals of the terminal residue where T_1 was 0.42 s and 0.44s, respectively at 11.7 T. The spin–spin relaxation times, measured at 11.7 T and 7 T, showed only a small field dependence. Since the signal/noise ratio at 7 T was lower than in T_1 experiments the reliable determination of the relaxation times for some carbons was not possible.

4. Discussion

The dynamics of oligo- and poly-saccharides can be analyzed from ^{1}H and ^{13}C relaxation times measured at various magnetic fields and different temperatures. The nature of overall motion and the rates of overall and internal motions can be estimated using an appropriate spectral density functions from the analysis of the experimental data. In fact, several studies of oligosaccharide dynamics characterized the overall tumbling as isotropic [8,13,20], however, the model for anisotropic rotational diffusion has also been used to describe the overall motion even for relatively small carbohydrate molecules [19,31]. The rates of internal motions have been found to be on a picosecond timescale, typically 20–70 ps. For oligosaccharides, it seems quite typical that, when applying the model-free approach, the generalized order parameters decrease from the central residue towards the terminal units. This can be explained by the rather simplistic view that the outer residues have more motional freedom. For example, the order parameters, S^2, were found to be almost identical for the three central residues in the recent studies of two pentasaccharides [31,36]; S^2 of the terminal units being smaller (~ 0.6) in the latter study [36] compared to those estimated in the pentasaccharide which corresponds to the active site of heparin for antithrombin III. The reason for this minor difference is probably the different stereochemistry of the glycosidic bonds linking the terminal residues and the inner ones. In the former pentasaccharide, which has the structure $GlcNSO_3$,$6SO_3$-(α1-4)-GlcA-(β1-4)-$GlcNSO_3$,3,$6SO_3$-(α1-4)-IdoA-(α1-4)-$GlcNSO_3$, $6SO_3$–OMe, both linkages have α configuration, thus the C-1–O-1 bond is axially oriented, whereas equatorial–equatorial linkages are found in the latter with the structure 2,6-di-O-[Glc(β1–4)GlcNAc]Man [36]. In both cases the rate of internal motion correlation time was within $\sim$ 20–60 ps. On the other hand, the type of the glycosidic linkage did not affect the longitudinal relaxation rates, in aqueous solution, for a series of oligomers of maltose and isomaltose [37]. Though isomaltose seems to be a more flexible molecule than maltose, according to the computa-

tional analysis, spin–lattice relaxation times in both molecules were found to be the same within experimental error. This trend was observed at various temperatures and could be due to the fact that longitudinal relaxation times are sensitive to high frequencies (rapid internal motions). Thus, depending on the extent (S^2) and the rate (τ_e) of internal motions, the spectral densities can be modulated so that the resulting values of spin–lattice relaxation rates are comparable although the overall tumbling values can be slightly different. Fast internal motions, at the rate of 20–60 ps, and the high S^2 values have also been obtained by fitting the experimental data in cyclodextrin molecules at various temperatures [38] and are comparable with the rate of internal motions obtained for small oligosaccharides by molecular dynamics calculations [39,40]. It therefore seems characteristic that the rate of internal motions is of the order of tens of picoseconds for oligosaccharides in aqueous solution.

As mentioned earlier, a similar methodology to that in our previous studies [31,32] was applied in order to derive the motional parameters for LMW K5 in water solution. The isotropic overall motion with or without internal motions, as well as the symmetric top model of the rigid molecule could not interpret the relaxation data. Satisfactory agreement was obtained only by applying the model-free spectral densities and assuming the overall anisotropic rotational diffusion with axial symmetry [41]. The following motional parameters were obtained by the fitting procedure: $\tau_{\parallel} = 0.35$ ns, $\tau_{\perp} = 7.8$ ns, $\tau_e = 30$ ps, $S^2 = 0.79$, $\theta = 88$. The computed and the experimental relaxation data are summarized in Table 3. It is noteworthy that the fitting procedure is quite sensitive to the choice of motional parameters. The values of spin–spin relaxation times are particularly sensitive to the nature of overall tumbling and the values of the rotational correlation times, thus also on the axial ratio $\tau_{\perp}/\tau_{\parallel}$, owing to their dependence on lower frequencies, $J^0(0)$.

Table 3
Computed and experimental relaxation times and NOEs for LMW K5 in aqueous solution at 303 K. The averaged values and standard deviations are given for the experimental data. The computed data were obtained with the following motional parameters: $\tau_{\parallel} = 0.35$ ns, $\tau_{\perp} = 7.8$ ns, $\tau_e = 30$ ps, $S^2 = 0.79$, $\theta = 88$

Parameter	Magnetic field (T)	Experimental data (s)	Computed data (s)
T_1	14	0.40 ± 0.037	0.43
T_1	11.7	0.38 ± 0.035	0.36
T_1	7	0.25 ± 0.014	0.24
T_2	11.7	0.11 ± 0.011	0.11
T_2	7	0.10 ± 0.008	0.11
$^1H-^{13}C$ NOE	11.7	161 ± 8.86	174

The derived correlation times indicate relatively fast overall motion of the molecule where the correlation time $\tau_{\parallel}$ is comparable with that found for the pentasaccharide molecule at 298 K [31]. This is probably a consequence of the low concentration of the solution of the saccharide in the present study. However, the value of $\tau_{\perp}$ is considerably longer because of the different molecular size. Compared to the other glycosaminoglycans, such as heparin epoxide [32], the axial ratio is quite high (~ 22), whereas in the epoxide derivative, with molecular weight $\sim 12{,}000$ D, this ratio is ~ 50. Furthermore, the internal motion correlation time, τ_e, and the S^2 values also differ from each other in these two polymers. The value of τ_e estimated in heparin epoxide was ~ 150 ps and $S^2 \sim 0.65$, which suggest there is a higher extent of internal motion in the molecule and agree, in terms of flexibility, with the results of the computational analysis [42]. In LMW K5, the effect of internal motions seems limited according to the derived dynamic parameters. Since it is expected that the flexibility results in a more disordered molecular shape, the higher axial ratio in K5 is probably the consequence of a more rigid structure compared to the epoxide derivative. The data obtained for the other glycosaminoglycan, heparin [43], where the values of dynamic parameters were found to be comparable with those in heparin epoxide, might support the above observations. However, as mentioned, the data are derived for the low molecular weight polysaccharide (~ 4000 D) and are compared to naturally occurring heparin as well as heparin epoxide, which have much higher molecular weights. Such a comparison is not obvious and should be viewed with caution. Moreover, since ^{13}C relaxation data are affected by molecular motions with a certain timescale and the model-free approach can be used to derive the rates of internal motions faster that the overall molecular tumbling the presence of very slow motions (slower than overall tumbling) cannot be excluded. Thus, this still seems rather a simplistic, preliminary conclusion and requires further verification.

It is also of interest that in spite of the presence of fewer charged groups than in previously investigated polymers, for instance heparin (where the sulfate groups are present in both glucosamine and iduronic acid residues), their structures appear to be more

flexible. This seems surprising since it is expected [17] that for polyelectrolyte molecules the rigidity is proportional to the number of charges present in the structure.

Using heteronuclear relaxation measurements at three magnetic fields we found that the overall motion of K5 polysaccharide in aqueous solution is anisotropic and can be approximated with the symmetric top model. The relatively high values of the order parameter, $S^2 \sim 0.8$, could be a consequence of local motions of a single conformer within an energy well rather than the motions characteristic for interconversion of different conformers where smaller order parameters would be expected. This situation seems different from other glycosaminoglycans, such as heparin and heparin epoxide, where the values of generalized order parameters were smaller and the values of τ_e corresponding to longer time-scales were found [32,43]. The reason for these differences might reflect the presence of different uronic acids, which constitute the structure of these polysaccharides. Since iduronic acid remains flexible even in a polymer chain [24], interconverting between the $^1C_4$ and $^2S_0$ conformers, the presence of glucuronic acid in K5 polysaccharide results in a more rigid structure for this molecule.

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