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The gel-forming behaviour of dextran in the presence of KCl: a quantitative ^{13}C and pulsed field gradient (PFG) NMR study

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Abstract

Although the gel forming ability of certain polysaccharides in the presence of ions is a well-known phenomenon, detailed physicochemical mechanisms of such processes are still unknown. In this investigation high resolution ^{13}C NMR as well as ^{1}H pulsed field gradient (PFG) NMR were used to investigate the mobility of dextran in the sol and in the gel state. Gel-formation of dextran can be easily induced by the addition of large amounts of potassium chloride. No major differences in the T_1 relaxation times of dextran in the sol and in the gel state could be observed. Accordingly, the analysis of the ^{13}C NMR spectroscopic data did not provide any indication of an observable line-broadening upon gel-formation. However, a KCl concentration dependent decrease of signal intensity in comparison to an internal standard was detected. On the other hand, the PFG NMR studies clearly indicated a gradual diminution of the self-diffusion coefficient of the dextran with increasing molecular weight as well as in the presence of potassium chloride. These measurements revealed in agreement with spectroscopic data that at least one potassium ion per monomer subunit (i.e. one glycopyranose residue) is necessary for gel formation.

Keywords: PFG NMR; ^{13}C NMR; T_1 relaxation; Dextran; Sol; Gel

1. Introduction

There is growing evidence that polysaccharides play an important role in a number of biochemical processes [1]. For instance, polysaccharides bound to proteins or lipids are known to stabilise the conformation of such biomolecules [2,3], whereas polysaccharides on the surface of cells are important for the recognition of other cells [4,5]. Additionally, charged polysaccharides prevent cells from aggregation and it was recently shown that tumour cells aggregate because of the lack of charged polysaccharides on their surface [6]. Therefore, polysaccharides are nowadays considered to be key molecules of cellular recognition because of their ability to change selectively their molecular structure.

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A number of naturally-occurring polysaccharides like alginate or κ-carrageenan show a more or less reversible sol–gel transformation [7,8]. The gel-formation can be induced by changing the temperature, the pH value or the ionic strength and is frequently accompanied by a macroscopically detectable change of the sample [9]. One of the most intriguing sol–gel transformations is the formation of alginate beads from alginate solutions in the presence of divalent cations like Ca^{2+} or Ba^{2+} [10].

Besides their physiological significance, polysaccharides are used as column filling materials in size-exclusion chromatography [11]. In this case, the separation of analyte molecules with different molecular weights and, therefore, different sizes is possible since the mesh size of the used gels can be easily controlled by the reaction conditions. One of the most widespread, uncharged polysaccharides is dextran [12]. Dextran is produced by different bacteria and yeasts and may be regarded as a linear condensation product of α-glucopyranose with branches primarily at the 1,2-1,3- or 1,4 position [12].

Although highly soluble in water, viscous polysaccharides like dextran are not able to form gels in a pure aqueous solution [13,14]. The gel-forming ability of dextran in the presence of high amounts of potassium ions, however, was already reported [15]. Since dextran does not possess any charged groups, that are often considered to be responsible for the gel-forming properties of, e.g. alginate, comprehensive studies were performed to clarify in more detail the mechanisms of dextran gel formation [13,16,17]. Although it is known that dextran gels are formed by interactions of the individual polysaccharide chains that are mediated by specific interactions between the hydroxyl groups and/or the bound water molecules, the basic gelation mechanism of dextran is so far unknown. It is remarkable, however, that the gel-formation can be induced exclusively by the addition of potassium ions, whereas other ions (monovalent as well as divalent cations) fail completely to induce this process. Therefore, it was concluded from X-ray data that the size of the potassium ions enables them to fit exactly into a 'hole' within the dextran backbone [18].

Watanabe et al. [17] have performed comprehensive pulsed field gradient (PFG) NMR diffusion studies on the diffusion behaviour of water as well as the polysaccharide chains within the dextran gel. From the restricted, i.e. the observation time-dependent water diffusion, they have elucidated important data on the pore size and the interbarrier distances within the dextran gel. The authors have also compared their diffusion data with the known crystal structure of dextran [17].

It is our intention to extend these previous investigations by the use of more sophisticated NMR methods: Since our diffusion NMR equipment allows the application of extremely strong magnetic field gradients (up to 25 T/m) [19], we are able to detect even components with very low self-diffusion coefficients.

We will show in this paper that diffusion methods are superior in comparison to other NMR techniques. Although the ^{13}C spin-lattice relaxation times (T_1) are often used as a measure of the molecular mobility of certain functional groups within polymers [20,21], we have found in this study that the detection of resolved resonances of dextran in the gel state is not possible since resonances are broadened beyond the detection limits. Therefore, the most pronounced effect is a significant diminution of the signal intensity of individual dextran resonances.

2. Materials and methods

2.1. Chemicals

Dextran from *Leuconostoc Mesenteroides* of different molecular weights (1.5, 15, 40 and 73 kDa) and dextran standards for chromatographic purposes (5, 12, 25, 50, 80 and 150 kDa) with a narrow molecular weight distribution as well as glycine and potassium chloride were purchased in the highest available purity from Fluka Feinchemikalien GmbH (Neu-Ulm, Germany) and used without further purification. The same holds for chemicals for NMR spectroscopy (deuterated water with an isotopic purity of 99.95% and 3-(tri-methylsilyl)-l-propionate-2,2,3,3-d_4 sodium salt (TSP-d_4) that served as ^{13}C frequency standard).

2.2. Sample preparation

Sample preparations were performed according to [17]. All dextran sols were simply prepared by dissolving a fixed quantity of dextran in the corresponding amount of deuterated water (D_2O). The pH of all resulting dextran solutions was in the range of 6.5 up to 8.2. Dextran gels were prepared by dissolving the calculated amount of dextran in potassium chloride solutions of known concentration (0.5, 1, 1.5, 2 and 2.5 M). All dextran/potassium chloride mixtures were vigorously shaken under warming to approximately 80 °C until they appeared completely homogeneous. Afterwards, samples were cooled down in a refrigerator (4 °C) to induce the gel formation. Because of the known time-dependence of gel formation on the molecular weight of the dextran [17], all samples were left in the refrigerator for two days to achieve complete gelation. In the case of the quantitative ^{13}C NMR measurements, a known quantity of glycine (0.1 M final concentration) was additionally dissolved in the applied KCl solutions.

In some PFG NMR experiments, potential effects of the residual water (by the exchange of the $-OH$ groups of the polysaccharide with the deuterated water) were minimised by drying the sample in a rapid evaporation system (Jouan RC 10–22) at 60 °C and re-dissolving the residual powder in D_2O. It was shown by mass spectrometry (MALDI-TOF MS) [22] that this procedure did not result in changes of the molecular weight of the dextran.

2.3. ^1H Pulsed field gradient (PFG) NMR measurements

For ^1H PFG NMR self diffusion measurements all dextran samples were filled in 7.5 mm (outer diameter) NMR sample tubes and sealed with a stopper. All measurements were performed on the home-built spectrometer FEGRIS 400 operating at a proton NMR frequency of 400 MHz [19].

The stimulated echo pulse sequence [$\pi/2$-τ_1-$\pi/2$-($\tau_2-\tau_1$)-$\pi/2$-τ_1-echo] was used. The two field gradients are applied after the first and the third $\pi/2$ r.f. pulse, respectively [23]. The time τ_1 between the first two r.f. pulses was always 3 ms. The field gradient g was varied between 0 and 25 T/m at a fixed pulse width δ (between 0.2 and 2 ms). The observed quantity in PFG NMR is the attenuation of the spin echo amplitude Ψ due to the applied field gradients [24,25],

$$\Psi = \exp[-(\gamma\delta g)^2 D(\Delta - 1/3\ \delta)] \quad (1)$$

where γ denotes the gyromagnetic ratio of the proton, δ the width, g the magnitude of the applied field gradient pulses and Δ the diffusion time (i.e. the distance between the two field gradient pulses), respectively.

In the majority of experiments, the observation (i.e. diffusion) time Δ was chosen to be at least one order of magnitude larger than the width of the field gradient pulses. Thus, the condition $\Delta \gg \delta/3$ is fulfilled and $\Delta\delta/3$ may be replaced by Δ [23]. Each data point is an average of at least four independent measurements. All investigations were performed at 298 K.

2.4. ^{13}C NMR spectra and relaxation time measurements

Carbon-13 NMR spectra were acquired on a Bruker DRX-600 spectrometer, operating at a ^{13}C resonance frequency of 150.918 MHz. Spectra were obtained with a 5 mm broadband probe using proton broadband decoupling of 3.5 kHz, a spectral width of 100 ppm, a pulse length of 6 μs for the $\pi/2$ pulse, and a relaxation delay of 3 s. A line broadening of 10 Hz was applied prior to Fourier transformation [26]. All measurements were performed at physiological temperature (310 K) using the deuterated water as deuterium 'lock' for field stabilisation. Spectra were referenced by the use of TSP-d_4 (trimethylsilyl-d_4-propionate), and the resonance of the trimethylsilyl group was set to 1.70 ppm [27]. In order to obtain a reasonable signal to noise ratio, 256 transients were accumulated.

Quantitative investigations were performed using the inverse gated ^1H decoupling technique. Here, composite pulse decoupling is applied only during the acquisition time but not during the interval between two pulses [28]. Therefore,

decoupled ^{13}C NMR spectra without nuclear Overhauser effect (NOE) can be obtained. These spectra were acquired in the presence of 0.1 M glycine as internal concentration standard.

In a few cases ^{13}C NMR spectra of dextran solutions and dextran gels were also acquired with a 4 mm HR-MAS ('high-resolution magic angle spinning') probe. The spin rate was 5000 Hz and the spectra were acquired at a temperature of 310 K. All experimental parameters were the same as described above for ^{13}C high-resolution NMR spectroscopy [29]. The spin-lattice relaxation times (T_1) were measured using the standard inversion recovery (IR) method (π-τ-$\pi/2$-acq). A repetition time of at least $5\times T_1$ (of the slowest relaxating carbon atom in the sample) was used. Typically, 10–15 experiments were performed with varying τ-values. Signal intensities were subsequently obtained by the integration of the individual peaks. The corresponding relaxation times were determined by fitting the function

$$M_z(\tau) = M_0[1-2\exp(-\tau/T_1)] \quad (2)$$

to the intensities of the individual resonances [28].

The quality of the individual fit functions was assessed by the χ^2 test. χ^2 was in the range of approximately 0.001 for all accepted fits. The standard errors of all relaxation times were estimated to be approximately $\pm 5\%$. Details of relaxation time measurements were already previously described [26].

3. Results and discussion

3.1. ^{13}C NMR and T_1 relaxation time measurements

^{13}C NMR spectra can be routinely recorded and allow a clear differentiation of individual dextran resonances. For higher molecular weight dextrans there are only six resonances that correspond to the six carbon atoms of each monomer subunit. The assignment of all dextran resonances to the individual carbon atoms is given in Fig. 1 [30,31]. The ^{13}C NMR spectra of different dextran samples resemble each other closely and do not depend on the molecular weight, neither the concentration of the dextran nor the KCl concentration. The T_1 relaxation times of the individual carbon atoms of the dextran are in a very similar range (between approx. 350 and 500 ms), too, and do not depend on the molecular weight or the KCl concentration (data not shown).

In order to investigate whether the gel-formation is accompanied by such a marked broadening of lines that the corresponding dextran resonances are not detectable anymore, the so-called 'inverse gated' decoupling technique [28] was used for recording quantitative ^{13}C NMR spectra. Under these conditions, proton-decoupled spectra are still obtained, but effects of the NOE on the signal intensities are eliminated.

Fig. 1 shows the effect of increasing potassium chloride concentrations on the ^{13}C NMR spectra of dextran (MW = 73 kDa). The provided integral intensities of the C-l ^{13}C resonance were obtained by comparison with internal glycine that was added to all samples in a fixed concentration (0.1 M). Glycine was chosen since it is easily soluble in water and provides only a single resonance (marked with an asterisk) in the spectral region of interest that does not interfere with the dextran resonances.

It is obvious from Fig. 1 that increasing amounts of KCl do neither change the chemical shifts nor influence the intensity ratios of the individual dextran resonances. However, if one compares the integral intensities of the dextran resonances in reference to the glycine peak, a marked decrease is obvious when the KCl concentration rises. Although this is valid for all resonances, we have focused our main interest on the analysis of the C-l resonance. This resonance is well separated from the remaining resonances and, therefore, its integral intensity can be given with the best accuracy. It is obvious that up to a KCl concentration of approximately 1.5 M only a relatively slight decrease of the integral intensities occurs, whereas this intensity loss is more marked at KC1 concentrations higher than 1.5 M. This result is in good agreement with the previous finding that gel formation can only be induced when the KC1 concentration exceeds a 'critical' concentration [17].

A more quantitative analysis is given in Fig. 2. In this figure, the intensities of three selected carbon atoms (relative to the intensity of a pure

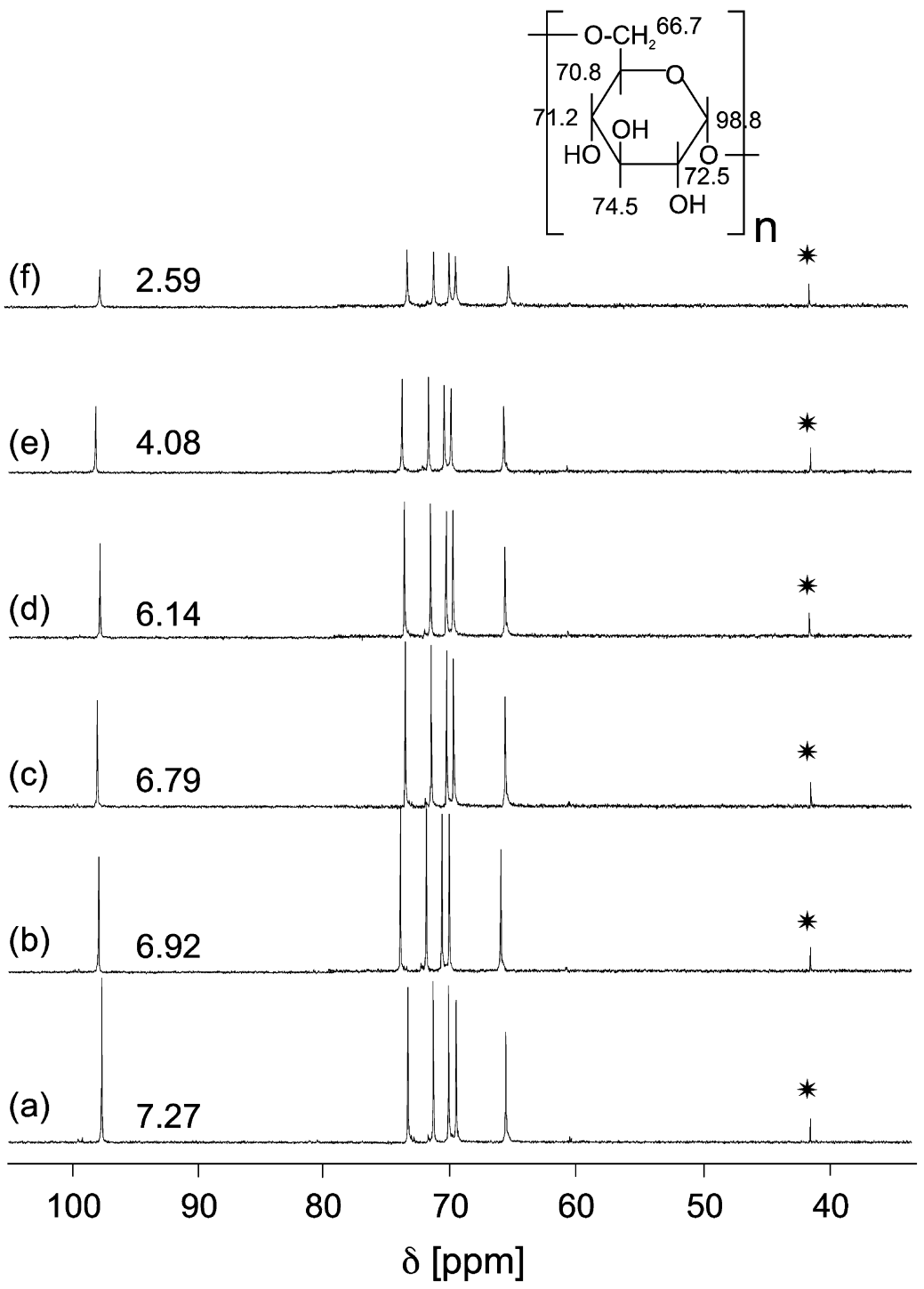

Fig. 1. Quantitative 'inverse gated decoupled' ^{13}C NMR spectra of 20 wt.% dextran solutions (73 kDa molecular weight) in D_2O. Increasing amounts of KCl [(a) pure dextran; (b) 0.5 M; (c) 1 M; (d) 1.5M, (e) 2.0 M and (f) 2.5 M KCl] lead to a loss of signal intensity of the dextran resonances in comparison to a known internal standard [0.1 M glycine, $\delta = 42.8$ ppm (marked with an asterisk)]. The intensity diminution is indicated by the integral intensity of the C-l resonance of dextran (the integral intensity of the glycine resonance was set to 1 in all cases).

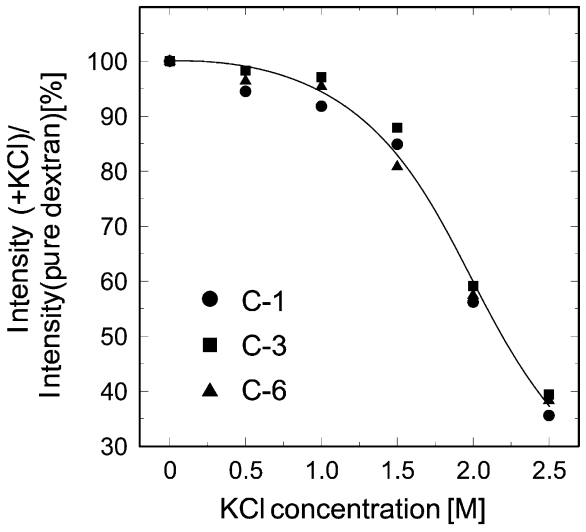

Fig. 2. Dependence of signal intensity (as percentage in comparison to a pure dextran sample without KCl) of three selected carbon resonances of dextran in dependence on the KCl concentration.

dextran solution without any KCl) are plotted against the KCl concentration. Only at KCl concentrations higher than approximately 1 M, the reduction of integral intensities is significant, whereas at lower KCl concentrations there are just slight differences in comparison to the measurements carried out in the absence of KCl. Even at the highest KCl concentration (2.5 M) approximately 40% of the dextran is still in the sol state. In order to convert all the dextran into the gel state much higher concentrations of KCl would be required. However, the solubility of KCl is limited, and, therefore, increasing the concentration is not simple. Since the ratio between dextran and KCl is most important, one might also think about lowering dextran concentration. However, a certain minimum concentration of dextran is necessary for the induction of gel-formation [17].

We conclude that the sol–gel transition results in a marked diminution of the mobility of the dextran chains and that dextran resonances in the gel state, i.e. above a 'critical' KCl concentration are not detectable by high resolution ^{13}C NMR spectroscopy. Therefore, the most pronounced effect is a decrease of the intensities of the individual dextran resonances. We also tried to apply solid state NMR spectroscopy since cross-polarisation (CP) ^{13}C NMR spectra should provide important information about the more rigid moieties of dextran that cannot be observed under standard high resolution NMR conditions. Unfortunately, we failed to record solid state NMR spectra since it was impossible—most probably because of the high KCl concentration that is required—to tune the probe properly to the dextran sample. This was, however, not a serious problem in high resolution NMR. Using for means of comparison a concentrated glucose sample, we have also shown that high KCl concentrations as such do not lead to major changes of signal intensities. This, however, only holds, when the pulse lengths are carefully re-determined each time when the ion concentration is changed.

One useful compromise between solution and solid state NMR for samples between the solid and the liquid state is the application of the 'high resolution' magic-angle-spinning (HR-MAS) technique [29]. Here, in contrast to standard solid state NMR no strong decoupling is used. The solid-like sample is, however, spun at high speeds under the magic angle of 54.7°. This minimises the line-broadening effects of chemical shift anisotropy and dipolar coupling and, therefore, 'sharper' NMR resonances are obtained. As shown in Fig. 3, the application of HR-MAS slightly diminishes the line-widths of the dextran resonances in the gel state resulting in a higher integral intensity. This is shown on the hand of the C-l resonance under HR-MAS conditions (3b) in comparison to the spectrum under 'conventional' NMR conditions (3a). Unfortunately, to obtain a reasonable signal to noise ratio, an increased number of scans is necessary in the case of HR-MAS since the 4 mm MAS rotor does only contain low amounts of dextran. Therefore—even if HR MAS may provide some improvements in signal intensities—it is evident that the diminution of dextran signal intensities is the most pronounced change upon gel-formation.

3.2. Diffusion NMR measurements

One major advantage for the application of the pulsed field gradient (PFG) self-diffusion meas-

urement technique to polymer solutions is the two orders of magnitude slower polymer self-diffusion in comparison to the water diffusion. Thus, dextran diffusion can be easily discriminated from the water diffusion—even without spectral resolution—and a single measurement is sufficient to obtain both data. The water self-diffusion coefficients in dextran solutions were already given elsewhere [23]. In Fig. 4, the polymer self-diffusion coefficients of dextran solutions of different concentrations are given. Highly refined dextran for chromatographic purposes was used in these measurements to minimise problems with broad distributions of the molecular weights.

As expected, the polymer self-diffusion coefficients (D) strongly depend on the molecular weight and decrease strongly with increasing molecular weight: D depends on the molecular weight M according to $D \sim M^{-1}$, where m is normally in the range between 0.8 and 1. Therefore, different dextran samples can be easily differentiated.

The sol–gel transition of dextran can also be accurately analysed by means of PFG diffusion

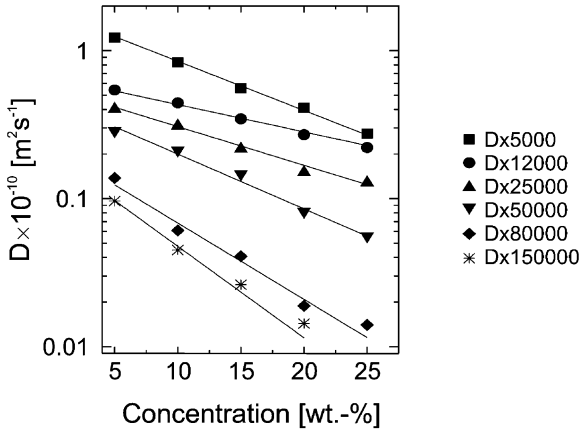

Fig. 4. Polymer self-diffusion coefficients D of different molecular weight dextrans in dependence on the dextran concentration (provided in wt.%). To minimise influences of the distribution of the molecular weight, highly defined dextrans for chromatographic purposes were used. Molecular weights of individual dextrans are given in the figure.

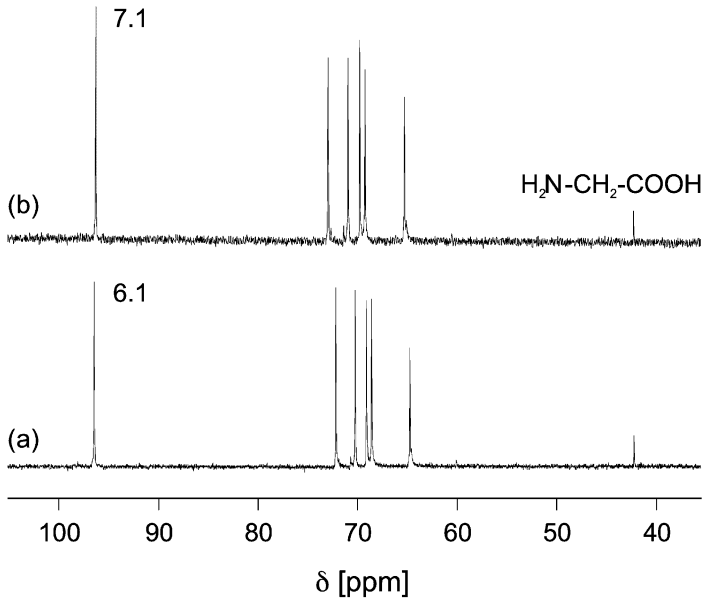

Fig. 3. 150.9 MHz ^{13}C NMR spectra of a 20 wt.% dextran solution of a molecular weight of 73 kDa in D_2O in the presence of 1.5 M KCl, (a) was recorded with a 5 mm 'standard' NMR probe, whereas (b) was recorded under high resolution magic angle spinning (HR-MAS) conditions (5000 Hz). The same experimental parameters were used for both spectra.

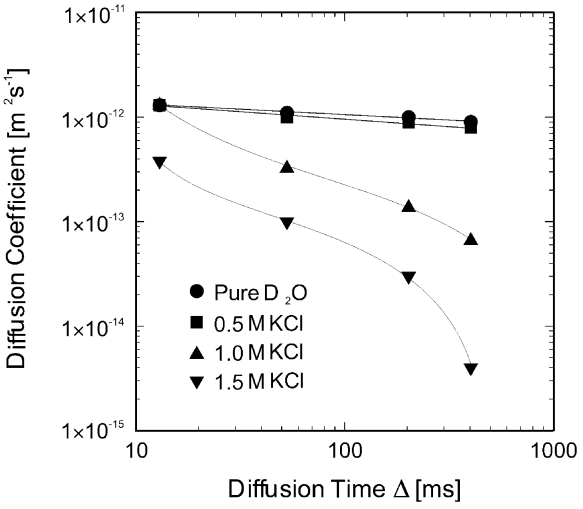

Fig. 5. Polymer self-diffusion coefficients of a 20 wt.% dextran solution of a molecular weight of 73 kDa in D_2O in dependence on the diffusion time. The concentration of KCl was varied as indicated in the figure. The lines represent 'spline' curves that were not obtained by fitting the data by a mathematical model.

measurements. In Fig. 5 the time-dependent polymer self-diffusion coefficients of dextran of a molecular weight of 73 kDa (20 wt.% in D_2O) are shown for varying KCl concentrations.

It is obvious that in the absence of KCl or at moderate concentrations of KCl (0.5 M) there is only a very small dependence on diffusion time, whereas this dependence is much more pronounced at higher KCl concentrations. It is evident that the diffusion coefficient of the dextran in the presence of 1.5 M KCl is about two orders of magnitude lower when $\Delta = 403$ ms is used.

In agreement with the NMR spectroscopic measurements, only higher KCl concentrations provide a marked effect, whereas the influence of lower KCl concentrations is weak. The marked decrease of the diffusion coefficients at longer diffusion times is a clear indication of steric restriction that occurs upon gel formation. The barriers within the dextran gel network lead to diminished diffusion pathways at longer observation times.

Thus, PFG NMR measurements allow a precise determination of the extent of the sol–gel transition, since already comparably low KCl concentrations lead to a considerable effect, while for the detection by NMR spectroscopy higher KCl concentrations would be required.

Therefore, the measurement of the polymer self-diffusion is a convenient method to obtain valuable information on the sol–gel transition. We assume that the gelation effect is caused by the formation of linkages between different polysaccharide chains and such a mechanism was already suggested by the analysis of the corresponding X-ray data [18]. The KCl concentration is the most important parameter, whereas the dextran concentration has a much smaller effect on gelation and, accordingly, on polymer diffusion. We have recently studied the diffusion behaviour of two selected polymers—polyethylenglycol and dextran—in explants of native cartilage [32]. We had expected that the inclusion of the polymers into the cartilage should lead to a decrease of the diffusivity of the polymers in comparison to the polymers in pure solution. However, we found in this case only a very slight diminution of the corresponding self-diffusion coefficients of the polymers in comparison to the pure solutions [32].

4. Conclusions

In this study, the sol–gel transformation of concentrated dextran solutions in the presence of KCl was investigated by ^{13}C NMR spectroscopy and ^{13}C T_1 relaxation time measurements as well as pulsed field gradient NMR. Whereas no changes could be observed in the ^{13}C T_1 relaxation times, the quantitative ^{13}C NMR spectroscopy could be used to monitor the sol–gel transition as a function of the KCl concentration. However, self-diffusion studies of the dextran by ^{1}H PFG NMR represent the method of choice for monitoring the sol–gel transition. Using this technique, the sol–gel transition can be followed more accurately and more sensitively in comparison to other methods. Above a critical KCl concentration, the polymer self-diffusion coefficients of dextran show a pronounced decrease. Dextran diffusion in the gel state is more than two orders of magnitude slower in comparison to the sol state and depends strongly on the observation time.

Acknowledgments

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