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# $^{13}C$ NMR relaxation studies on cartilage and cartilage components

Lama Naji, Jörn Kaufmann, Daniel Huster, Jürgen Schiller, Klaus Arnold *

*Institute of Medical Physics and Biophysics, Medical Faculty, University of Leipzig, Liebigstraße 27, D-04103 Leipzig, Germany*

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**Abstract**

We have investigated the molecular motions of polysaccharides of bovine nasal and pig articular cartilage by measuring the $^{13}C$ NMR relaxation times ($T_1$ and $T_2$). Both types of cartilage differ significantly towards their collagen/glycosaminoglycan ratio, leading to different NMR spectra. As chondroitin sulfate is the main constituent of cartilage, aqueous solutions of related poly- and monosaccharides (*N*-acetylglucosamine and glucuronic acid) were also investigated. Although there are only slight differences in $T_1$ relaxation of the mono- and the polysaccharides, $T_2$ decreases about one order of magnitude, when glucuronic acid or *N*-acetylglucosamine and chondroitin sulfate are compared. It is concluded that the ring carbons are motion-restricted primarily by the embedment in the rigid pyranose structure and, thus, additional limitations of mobility do not more show a major effect. Significant differences were observed between bovine nasal and pig articular cartilage, resulting in a considerable line-broadening and a lower signal to noise ratio in the spectra of pig articular cartilage. This is most likely caused by the higher collagen content of articular cartilage in comparison to the polysaccharide-rich bovine nasal cartilage. 

*Keywords:* Cartilage; Chondroitin sulfate; Hyaluronan; Glycosaminoglycans; $^{13}C$ NMR; Relaxation

## 1. Introduction

Diseases like rheumatoid arthritis and osteoarthritis are a major cause of disability and early retirement in the industrialized countries and therefore of great socioeconomic significance [1]. Articular cartilage covers the ends of the bones and acts as a weight bearing, low friction, wear resistant tissue in synovial joints [2]. The extracellular matrix of articular cartilage (Fig. 1) consists of about 20% collagens and 6% proteoglycans (per wet weight) [3]. Cartilage cells, the chondrocytes, are few and far embedded in the extracellular matrix (Fig. 1(a)). Proteoglycan aggregates (Fig. 1(b)) possess a molecular weight of about 105 kDa and consist of hyaluronan [4,5] to which proteoglycan subunits are noncovalently bound by means of a special link protein [5].

Each proteoglycan subunit consists of a core protein, to which two different polysaccharides (keratan sulfate and chondroitin sulfate) are attached (Fig. 1(c)) [6]. These negatively charged glycosaminoglycans are responsible for the high swelling capacity of cartilage, while collagen determines the supermolecular cartilage structure but has only a minor influence on the osmotic activity [7,8]. It has been shown that mainly the polysaccha-

* Corresponding author. Tel.: +49-341-9715700; fax: +49-341-9715709.

*E-mail address:* arnold@server3.medizin.uni-leipzig.de (K. Arnold).

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ride moiety of cartilage is depleted during the course of rheumatic diseases, whereas the collagen is by far less affected [9–12].

Although there are many methods by which glycosaminoglycans can be characterized in solution, including, e.g., light scattering [13] or electron microscopy [14], methods suitable for the characterization of native cartilage are still lacking. Despite its comparably low sensitivity, $^{13}C$ NMR spectroscopy is a powerful method to obtain structural and dynamic information on polysaccharides [15]. The main advantage of $^{13}C$ NMR is its broad range of chemical shift, which enables the assignment of resonances even in complex compounds. This is the most important requirement for the determination of relaxation times, which provide a measure of mobility of the individual functional groups. A large number of relaxation studies of different polysaccharides ($T_1$ as well as $T_2$) have been already conducted [15]. There are very few reports on $^{13}C$ NMR spectroscopy of glycosaminoglycans [16,17] and cartilage [18,19]. So far, bovine nasal cartilage has been exclusively characterized, most probably since this cartilage type is relatively homogeneous and available in huge amounts [18,19]. Additionally, nasal cartilage contains in comparison with articular cartilage higher amounts of glycosaminoglycans [3,19], which are primarily detected by NMR. The similarity between the NMR spectra of aqueous glycosaminoglycan solutions and small cartilage pieces strongly indicates that polysaccharides possess a tremendous mobility even in the cartilage matrix [18].

Although enzymatic treatment of cartilage slices resulted in enhanced spectral resolution of $^{13}C$ NMR spectra, it was estimated that already about 80% of the polysaccharides of intact cartilage are mobile enough to contribute to $^{13}C$ signal intensity [19]. Since these investigations were carried out using scalar decoupling only, there was no possibility to observe the by far less mobile collagen moiety of cartilage. More recently, however, the application of modern solid state NMR techniques in combination with high power decoupler fields also made the detection of collagen by means of $^{13}C$ NMR possible [20].

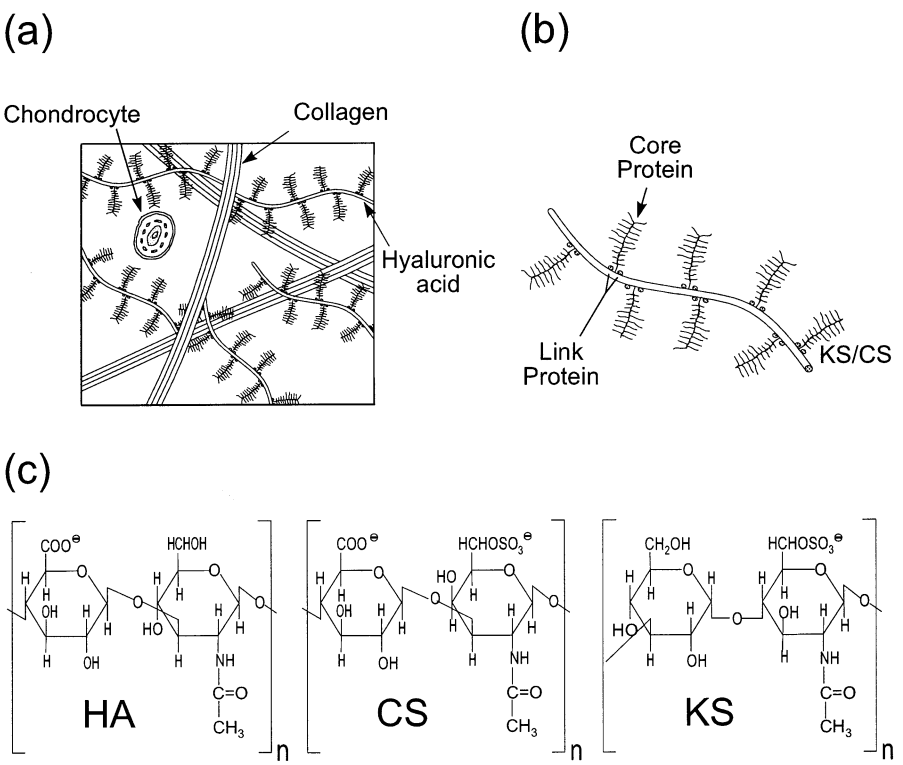


Fig. 1. Schematic representation of the molecular organization of articular cartilage. (a) Extracellular matrix, (b) proteoglycan subunit of cartilage, and (c) chemical structures of cartilage polysaccharides. Abbreviations used: HA, hyaluronan; CS, chondroitin sulfate; and KS, keratan sulfate.

Table 1
$T_1$ and $T_2$ $^{13}C$ relaxation times of some selected functional groups of polymers of cartilage and the related monomeric units, glucuronic acid (UA) and *N*-acetylglucosamine (N-Ac) [a]

| Group/compound | Glucuronic acid | *N*-acetylglucosamine | Chondroitin sulfate | Hyaluronan |
|---|---|---|---|---|
| *N*-acetyl (–$CH_3$) | | 2.31/0.59 (23.1; α) [b]<br>2.15/0.51 (23.3; β) | 1.53/0.09 (23.7) | 1.82/0.04 (23.7) |
| C-1 (UA) | 0.97/0.47 (93.0; α)<br>1.37/0.52 (96.8; β) | | 0.45/0.04 (104.9) | 0.43/0.03 (104.4) |
| C-1 (N-Ac) | | 0.91/0.25 (91.9; α)<br>0.83/0.32 (96.0; β) | 0.43/0.05 (102.1) | 0.51/0.03 (101.7) |
| C-2 (UA) | 0.94/0.40 (72.3; α)<br>0.78/0.40 (75.0; β) | | 0.42/0.04 (73.5) | 0.54/0.03 (73.8) |
| C-2 (N-Ac) | | 0.90/0.25 (55.2; α)<br>0.71/0.24 (57.8; β) | 0.50/0.04 (52.7) | 0.47/0.03 (56.2) |
| C-6 (N-Ac) | | 0.54/0.18 (61.7; α)<br>0.43/0.20 (61.9; β) | 0.31/0.04 (62.2) | 0.34/0.02 (61.7) |
| COOH | 6.70/0.71 (177.4; α)<br>9.70/0.77 (176.5; β) | | 2.09/0.04 (175.5) | 2.30/0.04 (175.2) |
| C=O (N-Ac) | | 4.11/1.09 (175.5; α)<br>4.00/0.77 (175.7; β) | 1.54/0.03 (176.1) | 2.22/0.05 (176.1) |

[a] The first values always give the $T_1$, whereas the second values denote the corresponding $T_2$ relaxation times. All relaxation times are given in seconds. Standard errors are about $\pm 5\%$.

[b] The values in brackets give the corresponding chemical shift (ppm) and indicate the α or the β anomeric form. In the case of chondroitin sulfate, only chondroitin-4-sulfate is considered.

There is still a complete lack of $^{13}C$ NMR investigations on the physiologically more relevant articular cartilage. The analysis of pig articular cartilage by $^{13}C$ NMR as well as relaxometry ($T_1/T_2$ determinations) are the main topics of the present paper. These data are additionally compared with high-field $^{13}C$ NMR spectra recorded at 150 MHz and relaxation times of bovine nasal cartilage and selected glycosaminoglycans.

## 2. Results

To obtain a better understanding of the molecular motions of carbohydrates in cartilage, $T_1$ and $T_2$ $^{13}C$ NMR relaxation times of these isolated monomeric and polymeric carbohydrates were determined by the 'inversion recovery' method. The corresponding values as well as the chemical shifts and the according assignments are given in Table 1. Since there are only minor differences for the carbons (C-1–C-5) in the relatively rigid carbohydrate rings [15], only selected data are given.

Considering the monosaccharides, there is a very similar relaxation behavior of all pyranose resonances. As the *N*-acetyl side chain is less restricted in its motion, the $T_1$ and $T_2$ values of the carbonyl and the methyl group are considerably larger than for all the remaining carbon atoms. However, the carboxylate group of the glucuronic acid residue possesses the longest relaxation time, which is even longer than the corresponding time of the carbonyl resonance in *N*-acetylglucosamine. It is most likely that the vicinity of the $^{14}N$-isotope ($I = 1$) shortens this relaxation time. Furthermore, the relaxation time of the C-6 in *N*-acetylglucosamine is considerably shorter than for the remaining carbons, due to the presence of two hydrogens in the –$CH_2OH$-group. This holds for all carbohydrates in this investigation. It is well known that the number of directly bound hydrogens strongly influences the relaxation behavior of the corresponding carbon atom [21].

Relaxation times are decreased in the case of the polysaccharides due to longer rotational correlation times of larger molecules. However, marked differences between the $T_1$ and $T_2$ relaxation times occur. Whereas the $T_1$ relaxation times of the polysaccharides are about the half of the values determined for the

Table 2
Influence of sample water concentration on line-width of some selected carbons of an aqueous solution of chondroitin sulfate [a]

| Functional group | $^{13}C$ NMR line-width at half-height (Hz) | | | | |
|---|---|---|---|---|---|
| | 2.5% | 8% | 10% | 15% | 25% |
| C=O | 17 | 23 | 24 | 29 | 33 |
| Ring | 25 | 32 | 32 | 35 | 37 |
| $-CH_3$ | 18 | 24 | 24 | 33 | 41 |

[a] All line-widths are given in Hz, whereas the chondroitin sulfate concentration is given in wt.%.

monosaccharides, the $T_2$ values decrease by far stronger: The $T_2$-relaxation times of the polymers are more than one order of magnitude smaller than $T_1$. Polymeric molecules do not perform isotropic tumbling anymore, but rather anisotropic motions that account to these differences [15]. It has to be mentioned that our data do not agree with the $T_2$-data obtained by Sterk and co-workers [22]. These authors found upon the analysis of, e.g., hyaluronan solutions $T_1$ values comparable to our data, but their $T_2$-values, especially for the *N*-acetyl side chains, were by far larger. Although these discrepancies are not yet completely understood they might arise from differences in the molecular weights of the used hyaluronan as these differences cannot be explained by the different field-strengths of the used NMR spectrometer.

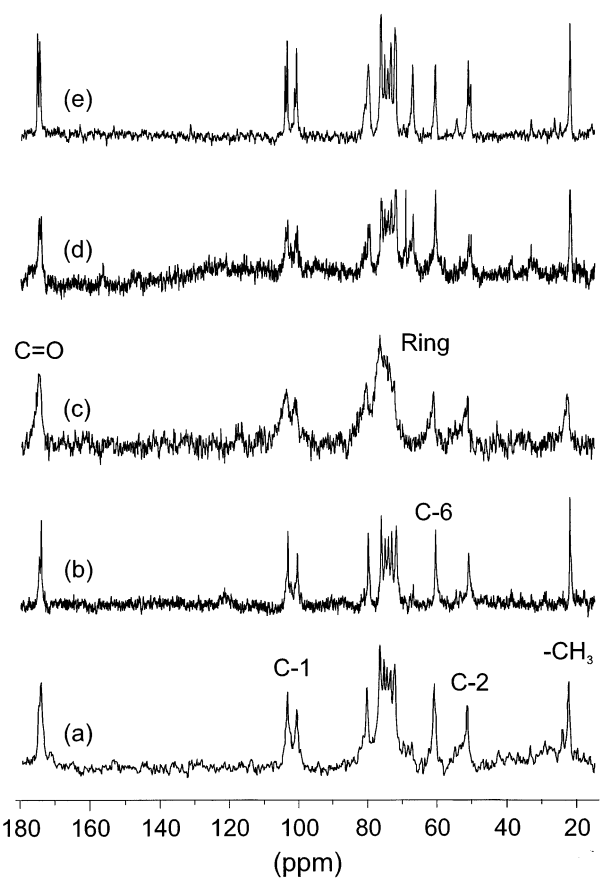


Fig. 2. $^{13}C$ NMR spectra of bovine nasal cartilage (a,b) and pig articular cartilage (c,d) and chondroitin sulfate (8 wt.% in $D_2O$; e). Both types of cartilage were allowed to free swelling in $D_2O$. Spectra (b,d) were recorded under conditions of HRMAS (see Section 4).

In Table 2 the influence of the water content on the line-width of some selected resonances of chondroitin sulfate is shown. Line-width analysis was performed to obtain data, which are comparable to the data found by other scientists [22]. It is evident from Table 2 that the line-width of the *N*-acetyl side chain is more affected by concentrating the sample than the pyranose resonances. This once again confirms our hypothesis that the ring carbons are much more motion-restricted in the rigid pyranose ring and, thus, additional restrictions by concentrating the sample do not more play an important role. Unfortunately, if the water content is reduced to about 50% no more separated carbon resonances can be obtained even under conditions of HRMAS. Therefore, under these conditions, solid state NMR with high-power decoupling must be applied.

Fig. 2 shows the comparison between the $^{13}C$ NMR spectra of bovine nasal (a,b) and pig articular cartilage (c,d). Spectra (a) and (c) were recorded using a standard high-resolution NMR probe and (b) and (d) were recorded using MAS conditions (5000 Hz). For comparison in (e) the $^{13}C$ NMR spectrum of an aqueous chondroitin sulfate sample (8% w/w) is also shown. The spectra resemble each other closely, whereby nasal cartilage (a) gives (at least without MAS) a by far higher re-

Table 3
Comparison of $T_1$ and $T_2$ relaxation times of some different, well-resolved resonances of pig articular and bovine nasal cartilage [a]

| Chemical shift (ppm) | Assignment | Articular, $T_1$ (s) | Nasal, $T_1$ (s) | Nasal, $T_2$ (ms) |
|---|---|---|---|---|
| 175.6 | *N*-acetyl C=O | 1.68 | 2.11 | 44 |
| 175 | carboxylate | nr | 2.37 | 39 |
| 104.3 | GlucUA-1 | 0.42 | 0.91 | 21 |
| 101.6 | GalNAc-1 | 0.55 | 0.81 | 15 |
| 81.1 | ring | 0.77 | 0.84 | 24 |
| 77.2 | ring | 0.53 | 0.68 | 19 |
| 76.2 | ring | 0.53 | 0.56 | 18 |
| 52.7 | GalNAc-2 | 0.82 | 0.45 | 27 |
| 23.7 | *N*-acetyl $-CH_3$ | 1.34 | 2.20 | 33 |

[a] $T_2$ was determined only in the case of bovine nasal cartilage. Standard errors are about $\pm 5\%$. nr = not resolved resonance.

solved spectrum than pig articular cartilage (c).

According to its higher collagen and lower glycosaminoglycan content, the spectra of pig articular cartilage exhibit a lower signal to noise ratio. HRMAS considerably improves the quality of the $^{13}C$ NMR spectrum of pig articular cartilage (d), whereas the effect towards the nasal cartilage is less pronounced. Thus, nasal cartilage is a more homogeneous tissue than pig articular cartilage and, therefore, is characterized by a lower line-width.

The close similarity between bovine nasal cartilage and the chondroitin sulfate spectrum clearly reflects the fact that cartilage is mainly composed of chondroitin sulfate [18,19,23], whereas keratan sulfate and hyaluronan only play a minor role. Despite the considerable broadening of lines, relaxation times of cartilage are very similar to the values determined for chondroitin sulfate. In Table 3 the $^{13}C$ NMR relaxation times of pig articular cartilage and bovine nasal cartilage (both swollen in an excess of water) are compared. Only the $T_1$ values of pig articular cartilage could be measured on a single sample without marked degradation of the sample because the required measuring times are extremely long in the case of pig articular cartilage (about 2 days).

In comparison to the relaxation times of hyaluronan and chondroitin sulfate in aqueous solution, the relaxation times of both types of cartilage are in the same order of magnitude, what indicates that the collagen moiety does not markedly influence the relaxation properties of cartilage glycosaminoglycans. Both cartilage resonances exhibit a rather broad underground, which is not observed in the spectra of pure chondroitin sulfate. This is in agreement with Darke and co-workers [24].

To exclude the possibility that paramagnetic impurities like ferrous or cuprous ions influence relaxation in articular cartilage, the influence of ferrous ions on an aqueous chondroitin sulfate solution was tested. It is known that divalent cations are strongly bound to glycosaminoglycans, whereas there is no marked interaction with monovalent ions [25,26]. In Fig. 3 the $^{13}C$ NMR spectra of an aqueous chondroitin sulfate solution (8% w/w) are shown with 5 mM EDTA (a), 1 (b), 2 (c) and 5 mM $FeCl_2$ (d). Obviously,

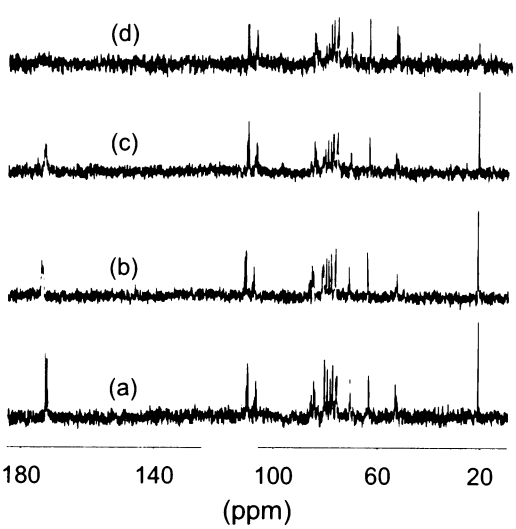


Fig. 3. Influence of paramagnetic ions on the shape of the $^{13}C$ NMR spectra of an aqueous 8% chondroitin sulfate solution. (a) It was recorded in the presence of 5 mM EDTA, whereas in (b) 1, (c) 2 and in (d) 5 mM $FeCl_2$ was artificially added.

increasing amounts of ferrous ions lead to a nearly selective broadening of the charged carboxylate group as well as the C=O resonance of the *N*-acetyl side chain, whereas the other resonances remain nearly unchanged. This is caused by the charge of the uronic acid group or the complexing properties of the carbonyl group of the *N*-acetyl residue, which enables both carbonyl groups to interact strongly with paramagnetic ions.

Additionally, the EDTA experiment (Fig. 3(a)) indicates that previously present impurities of paramagnetic ions do not contribute to the line-broadening, since there is no difference between both chondroitin sulfate spectra in the presence and in the absence of EDTA. The carbonyl resonance is clearly detectable in each cartilage spectrum and, thus, one must conclude that paramagnetic ions do not influence the spectral and relaxation properties of the articular cartilage itself. This might be of some importance for studying the binding behavior of NMR contrast agents within the cartilage.

## 3. Discussion

In the present investigation we have recorded the $^{13}C$ NMR spectra and measured the corresponding relaxation times of pig articular and bovine nasal cartilage as well as isolated solutions of important glycosaminoglycans of cartilage (chondroitin sulfate and hyaluronan). Although $^{13}C$ NMR spectroscopy provides higher-resolved NMR spectra than proton NMR spectroscopy, the spectra of articular cartilage are strongly dominated by broad resonances. An attempt to overcome these problems is the application of the so-called HRMAS–NMR technique. This minimizes the line-broadening effects of chemical shift anisotropy and dipolar coupling considerably and, therefore, by far sharper NMR resonances can be obtained. To our knowledge cartilage has not yet been investigated by HRMAS and the present paper is the very first example.

Under standard high-resolution as well as HRMAS conditions; however, exclusively the carbohydrate moiety of cartilage contributes to the $^{13}C$ NMR resonances [18,19], whereas the rigid collagen fibrils are broadened by dipolar couplings beyond the detection limit.

Additionally, our data clearly indicate that there are only slight differences in the relaxation behavior of monosaccharides on the one hand and polysaccharides on the other hand. Even the relaxation times of chondroitin sulfate within the cartilage agree very well with the corresponding data in solution. Since relaxation times are also often estimated by the analysis of the line-width of the NMR resonances [22], we have also used this approach to estimate the influence of the water content on the shape of the spectra. As expected, decreasing the water content broadens considerably the resonances of the carbon atoms of the polysaccharides, whereby the ring atoms as well as the *N*-acetyl side-chains are affected.

From the obtained data, we conclude that the pyranose carbons of carbohydrates are motion-restricted primarily by embedding in the rigid ring structure, whereas additional limitations of mobility (cartilage polysaccharides in comparison to solution conditions) do not more show major effects. Additionally to the $T_1$ relaxation times, $T_2$ relaxation was also determined. These values are by far shorter than $T_1$ but again the values for the solution and for the cartilage are in a comparable order of magnitude.

As cartilage was obtained from different sources, we also addressed the question of what role paramagnetic impurities may play towards the relaxation behavior. However, their role must be regarded to be very small since no major differences were found upon EDTA treatment. Vice versa, the addition of ferrous ions selectively broadened the carbonyl resonances, which assigns them as the major ion binding sites. This is in good agreement with previous results reported by other authors [25,26].

Finally, the question of what moiety of polysaccharides of cartilage is detectable by NMR is not yet answered. Using proton and $^{13}C$ NMR spectroscopy, Darke and co-workers [24] found that, e.g., hyaluronan samples contain, in all cases, components with very short relaxation times, resulting in consider-

able line-widths. The concentration of such components is often underestimated by NMR.

## 4. Experimental

*Chemicals and cartilage samples.*—All chemicals were obtained in the highest commercially available purity and used without further purification. The sodium salts of hyaluronan (HA) and chondroitin sulfate (CS), both from bovine trachea with a molecular weight of about $5 \times 10^4$ g/mol, and the monomers, GlcNAc and GlcUA, as well as ferrous chloride, EDTA and merthiolate (ethylmercurithiosalicylate) were purchased from Fluka Feinchemikalien (Neu-Ulm, Germany). Deuterated water with an isotopic purity of 99.9% was supplied by Chemotrade, Leipzig. All carbohydrates were dissolved in deuterated water to give a final concentration of 8% (w/w) and were allowed to equilibrate for 1 day at room temperature. To prevent growth of microorganisms, the used $D_2O$ contained 200 mg/L merthiolate [27].

Bovine nasal and pig articular cartilage were obtained from a local slaughter house immediately after slaughter from healthy juvenile animals (about 1-year-old). Both types of cartilage were separated from the surrounding tissue, and nearly cubic or rectangular pieces of a size of about 2 mm were cut off. The cartilage pieces were allowed to swell in an excess of $D_2O$.

*$^{13}C$ NMR spectra.*—Carbon NMR spectra were acquired on a Bruker DRX-600 spectrometer, operating at a resonance frequency of 150.918 MHz for $^{13}C$. Spectra were obtained with a 5 mm broadband probe using proton broadband decoupling of 3.5 kHz, a spectral width of 200 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. All measurements were carried out at physiological temperature (310 K) using the deuterated solvent as a deuterium lock for field stabilization. Spectra were referenced by the use of TSP (trimethylsilyl-$d_4$-propionate), where the resonance of the trimethylsilyl group was set to 1.70 ppm [28]. For each polymer spectrum 1024 transients were accumulated, whereas for the monosaccharides, 16 accumulations were sufficient to obtain a reasonable signal to noise ratio. At least 2000 transients were necessary for the different cartilage samples. In some cases $^{13}C$ NMR spectra of cartilage were also acquired with a 4 mm HRMAS 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 high-resolution NMR spectroscopy.

*$T_1$ and $T_2$ $^{13}C$ NMR relaxation measurements.*—The spin-lattice relaxation times ($T_1$) were measured using the standard inversion recovery (IR) method ($\pi-\tau-\pi/2$-acq) [29]. 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 $\tau$-values. Signal intensities were obtained by integration of the peaks in the NMR spectra. The corresponding relaxation times were determined by fitting the intensities of the spectra to an exponential decay function according to the following equation:

$$M_z(\tau) = M_0\left(1 - 2\,\mathrm{e}^{-\frac{\tau}{T_1}}\right)$$

The quality of the individual fit functions was assessed by the $\chi^2$ test. $\chi^2$ was in the range of about 0.001 for all accepted fits.

$T_2$ (spin–spin) relaxation time measurements were performed using a standard CPMG sequence ($\pi/2$-$\pi-\pi-\tau$-acq) [30], whereby the echo time was incremented. $T_2$ was obtained by fitting signal intensities to an exponential decay:

$$M_y(\tau) = B\,\mathrm{e}^{-\frac{\tau}{T_2}}$$

$\chi^2$ was in the range of about 0.001 for all accepted fits.

In a few cases $T_2$ was also determined by the line-width $\Delta\nu_{1/2}$ assuming a Lorentzian line shape:

$$\Delta\nu_{1/2} = \frac{1}{\pi T_2}$$

The standard errors of all given relaxation times were estimated to be about $\pm$ 5%. De-

tails of relaxation-time measurements are given in [28].

As relaxation measurements lasted for about 55 h, all samples were investigated by $^{1}H$ NMR spectroscopy subsequent to $^{13}C$ NMR measurements. No significant differences were detectable in the spectra compared with that prior to measurement.

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