

Self-diffusion of polymers in cartilage as studied by pulsed field gradient NMR

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Abstract

Pulsed field gradient (PFG) nuclear magnetic resonance (NMR) was used to investigate the self-diffusion behaviour of polymers in cartilage. Polyethylene glycol and dextran with different molecular weights and in different concentrations were used as model compounds to mimic the diffusion behaviour of metabolites of cartilage. The polymer self-diffusion depends extremely on the observation time: The short-time self-diffusion coefficients (diffusion time $\Delta \sim 15$ ms) are subjected to a rather non-specific obstruction effect that depends mainly on the molecular weights of the applied polymers as well as on the water content of the cartilage. The observed self-diffusion coefficients decrease with increasing molecular weights of the polymers and with a decreasing water content of the cartilage. In contrast, the long-time self-diffusion coefficients of the polymers in cartilage (diffusion time $\Delta \sim 600$ ms) reflect the structural properties of the tissue. Measurements at different water contents, different molecular weights of the polymers and varying observation times suggest that primarily the collagenous network of cartilage but also the entanglements of the polymer chains themselves are responsible for the observed restricted diffusion. Additionally, anomalous restricted diffusion was shown to occur already in concentrated polymer solutions. © 2002 Elsevier Science B.V. All rights reserved.

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1. Introduction

Inasmuch as cartilage contains only a small number of cells, the extracellular matrix determines the physicochemical properties of cartilage. This extracellular cartilage matrix consists of water, collagen and proteoglycans [1]. The proteoglycans are composed of a central protein core to

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which glycosaminoglycan side chains, especially chondroitin- and keratan sulfate are attached. Proteoglycans are linked to hyaluronic acid, forming the so-called aggrecans and these aggrecans are bound to the collagen network. The negatively charged glycosaminoglycans are responsible for the high swelling capacity of cartilage, while the collagen fibres determine the supermolecular cartilage structure but have only minor influence on the osmotic activity [2–6].

Since cartilage does not contain blood vessels at all, the diffusion of water, nutrients, metabolic waste products and molecules with regulatory functions (e.g. cytokines) plays a key role in cartilage function [7–10]. The metabolism of this tissue is extremely influenced by the diffusion behaviour of water and macromolecules [11,12].

Different methods of diffusion measurements are nowadays established. Pulsed field gradient (PFG) nuclear magnetic resonance (NMR) allows to determine the mean square displacement of molecules in a given diffusion time that is typically in the range of a few milliseconds up to seconds. PFG NMR monitors distances in the micrometer scale and has the considerable advantage—in contrast to tracer techniques—of being non-invasive [13–18].

A first comprehensive PFG NMR study of the bulk diffusion coefficients of water and small solutes as well as the spatially-resolved variation of the diffusivity of explanted cartilage was published by Burstein et al. [19]. Cartilage composition as well as the mechanical properties of cartilage specimens were changed in this investigation by the treatment with different enzymes and mechanical compression, respectively. Comprehensive data on the effect of different water contents and the observation time on the self-diffusion behaviour of water and cationic molecules in cartilage have also been obtained by Knauss et al. [20] and Ngwa et al. [21]. These authors [20,21] found that at short diffusion times (~ 13 ms) the self-diffusion coefficient of water and cations is primarily controlled by the water content of cartilage. The long-time diffusion (~ 500 ms), however, reflects structural properties of the cartilage within a 10 μm region. In these studies [20,21] it was demonstrated that PFG NMR is a suitable

method to determine a number of physiologically-relevant parameters in cartilage, for example the distance over which ‘free’ (i.e. non-restricted) diffusion occurs [20,21]. The present studies were performed to improve the understanding of the principles of the self-diffusion behaviour of macromolecules in cartilage.

Because of the importance of cartilage swelling, the relationships between compression of cartilage and the resulting water content, the short-time as well as the long-time self-diffusion of the polymers polyethylene glycol (PEG) and dextran were studied. PEG and dextran were used as model polymers because they are commercially available in a great variety of different molecular weights and possess physiological relevance. One additional reason was the known structural differences between both polymers: PEG is a more flexible molecule, whereas dextran represents a more rigid one [22]. Therefore, for the more complex and longer lasting measurements of the long-time self-diffusion, exclusively PEG was used, since its higher flexibility also provides higher NMR sensitivity [24]. Although the diffusion behaviour of both polymers in cartilage has been already investigated [22], this is the very first study that uses PFG NMR methodology to measure the self-diffusion of polymers in cartilage. This technique is superior to other methods since actually the self-diffusion coefficients of molecules can be determined because no concentration gradients have to be used.

For establishing a highly defined water content, compression of cartilage was carried out by the osmotic stress technique [24], i.e. cartilage slices were incubated in polymer solutions of different concentrations resulting in different osmotic pressures. For means of comparison and to investigate if the polymers themselves might exhibit time-dependent diffusion properties, polymer diffusion was also studied in pure polymer solutions, i.e. in the absence of cartilage.

It will be shown that the molecular weight of the polymer as well as the water content of cartilage have the highest impact on the diffusion properties of the polymers. This resembles closely the previously reported diffusion behaviour of water [20] and cations [21] in cartilage: The short-time diffusion is mainly determined by the water

content of the cartilage, whereas the long-time diffusion reflects internal cartilage structures much better. However, both, the short-time and the long-time self-diffusion, depend also considerably on the molecular weight of the diffusing molecules.

2. Experimental

2.1. Materials

Bovine cartilage from the nasal septum was used for all experiments. Polyethylene glycol (PEG) with molecular weights of 600 and 20000 Da was obtained from Fluka (Neu-Ulm, Germany). PEG of 6000 and 40 000 Da was purchased from Serva (Heidelberg, Germany) and PEG with a molecular weight of 1500 Da was available from Ferak (Berlin, Germany). Dextran with molecular weights of 1500, 6000, 20 000, 40 000 and 70 000 Da were obtained from Fluka (Neu-Ulm, Germany). D₂O with an isotopic purity of 99.6% (Chemotrade, Germany) was used as solvent in all cases. All further chemicals were obtained in the highest available purity from Fluka.

2.2. Sample preparation

Cartilage specimens were separated from the surrounding soft tissue and nearly cubic or rectangular pieces of a size of approximately 2–3 mm were cut off. Cartilage samples were incubated in PEG/D₂O or dextran/D₂O solutions ranging from 0 (pure D₂O) to 50 weight percent (wt.%) of the corresponding polymer for 16 h [24]. Since the observation of the water self-diffusion coefficients was not of interest in this study, samples were prepared in D₂O instead of H₂O to attenuate the contribution of the water protons to the NMR signal. After incubation, the surface of the cartilage samples was carefully cleaned to remove even traces of the polymer solutions on the cartilage surface.

For NMR measurements the cartilage samples were filled in 8 mm (outer diameter) NMR sample tubes and sealed with a vespel stopper. Vespel (from DuPont) is a material that contains exclusively highly rigid protons and, therefore, does not provide any detectable NMR resonance. For the

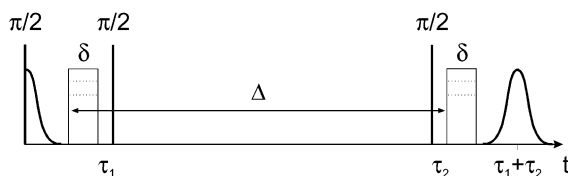


Fig. 1. Stimulated-echo pulse sequence with lengths of gradient pulses δ and the diffusion time Δ . This sequence was used for all measurements.

NMR characterization of the pure polymers, 150–200 μ l of the corresponding polymer solutions were filled in 8 mm (outer diameter) NMR sample tubes.

Immediately after NMR measurements the water content (as well as the PEG or dextran content) of the cartilage samples was determined by weighing, drying in a rapid evaporation system (Jouan RC-10-22, Germany) and re-weighing. For the determination of the polymer content, the weight of the cartilage samples filled with polymer was compared (after drying) with the weight of the samples that were just incubated in pure D₂O.

2.3. PFG NMR measurements

The self-diffusion coefficients D of polymers in cartilage were measured by pulsed field gradient (PFG) NMR. The measured quantity in PFG NMR is the spin-echo amplitude $A(g\delta, \Delta)$. The attenuation of the amplitude $\Psi = A(g\delta, \Delta)/A_0$ in dependence on the applied pulsed field gradients is given by the Stejskal–Tanner equation:

$$\Psi = \exp \left[-q^2 D \left(\Delta - \frac{1}{3} \delta \right) \right] \quad (1)$$

$q = \gamma \delta g$ is a generalised scattering vector [15–18] with γ denoting the gyromagnetic ratio of the proton, δ the width and g the magnitude of the applied field gradient pulses. Δ represents the diffusion time and D is the apparent self-diffusion coefficient. The stimulated echo sequence as shown in Fig. 1 was exclusively used [20].

One important advantage of PFG NMR is the possibility to vary the observation time Δ (i.e. the time where diffusion is monitored) and, therefore, to observe restricted diffusion. In this work Δ was

varied between 3 and 645 ms. The lower limit is caused by limitations of our device and the upper limit is due to T_1 relaxation effects of the cartilage molecules. In the case of free diffusion the mean square displacement $\langle z^2 \rangle$ of the diffusing species obeys the Einstein equation, i.e. $\langle z^2 \rangle$ increases with the observation time:

$$\langle z^2 \rangle = 2D\Delta \quad (2)$$

If the diffusion is restricted the mean square displacement $\langle z^2 \rangle$ increases with less than the first power of the diffusion time [25]. The self-diffusion coefficient now depends on the observation time $D = D(\Delta)$. In the case of complete restriction, the stimulated-echo attenuation reflects the dimension of the restricting geometry rather than dynamic processes [15].

The PFG NMR self-diffusion measurements were performed on the home-built spectrometer FEGRIS 400 at a proton resonance frequency of 400 MHz [26,27]. The magnitude of the field gradient g was varied between 0 and 25 T/m. The pulse width δ was 0.5 ms in all cases. The observation time Δ was varied between 3 and 645 ms and all diffusion measurements were carried out at room temperature (293 K).

One should note that our PFG NMR equipment does not allow the spectroscopic differentiation between the polymer protons and the residual water protons within the cartilage samples by differences in the chemical shifts. Therefore, only species differing towards their self-diffusion coefficients for more than one order of magnitude can be unequivocally analysed. This is, however, a fulfilled criterion of our system and the contribution of the residual water could also be minimised by the use of D_2O instead of H_2O .

Due to the high field gradient stability of our PFG NMR equipment standard errors are estimated to be lower than $\pm 2\%$ if a certain sample is investigated several times. Although all measurements were at least performed in triplicate no error bars are given since errors would be in the range of the symbol size. Deviations related to biological diversity of the cartilage are much more pronounced and, therefore, cartilage from different animals was mixed to minimise these deviations.

2.4. Data analysis

In PEG only the terminal protons of the hydroxyl group are exchanging with the solvent and, therefore, only a negligible residual water signal is observed. In the case of a single diffusing species the attenuation of the stimulated-echo amplitude was approximated by the Kohlrausch–Williams–Watts function [28]:

$$\Psi = \exp(-Dx)^\beta \quad \text{with } x = g^2 \left(\Delta - \frac{1}{3} \delta \right) \quad (3)$$

D is the apparent self-diffusion coefficient and β describes the extent of the non-exponential decrease of the echo attenuation. In our experiments β is very close to 1 and, therefore, this parameter was not further considered. If there were two different diffusing species the attenuation was approximated by a biexponential function:

$$\Psi = a \exp(D_1 x) + (1 - a) \exp(-D_2 x) \quad (4)$$

D_1 and D_2 are the self-diffusion coefficients of species 1 and 2, respectively, and a is the contribution of the species having the self-diffusion coefficient D_1 in relation to the total signal.

3. Results

Fig. 2 shows the dependence of the self-diffusion coefficients of PEG (a) and dextran (b) in bovine nasal cartilage on the molecular weight of the polymer. It is obvious that for both polymers the self-diffusion coefficients decrease with increasing molecular weights and decreasing D_2O contents, i.e. higher polymer concentrations. In both cases, this dependence is exponential and can be described by the following equation:

$$D \approx M^{-m} \quad (5)$$

where M represents the molecular weight and m is a constant exponent for different polymers. The exponent m varies slightly in dependence on the corresponding molecular weight but $0.8 < m < 1.0$ is a suitable value for both polymers.

By the fact that the diffusion properties of both polymers can be described by the same mathematical function, it is evident that the chemical struc-

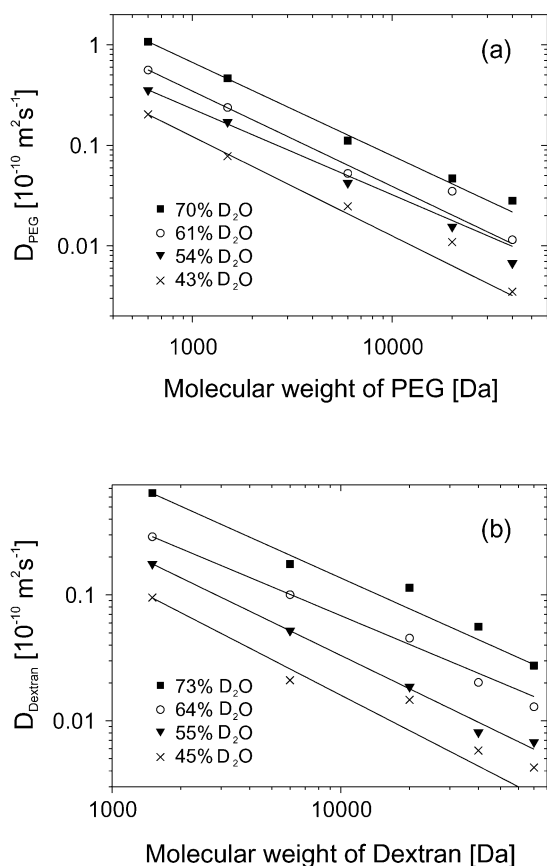


Fig. 2. Self-diffusion coefficients of polyethyleneglycol (PEG) (a) and dextran (b) in cartilage as a function of the corresponding molecular weights of the polymers. D_2O contents of the individual samples were adjusted by the osmotic stress technique. A diffusion time of $\Delta = 15 \text{ ms}$ was used in all cases.

ture of the polymer does not have a major impact on their diffusion properties. One should, however, notice that at the same polymer concentration and the same molecular weight of both polymers, the dextran possesses slightly higher self-diffusion coefficients than the PEG. This is surprising since the viscosity of the dextran solution as well as the rigidity of the dextran molecule is considerably higher compared with PEG [23].

To investigate whether the obstacles (e.g. the collagen fibrils or the cartilage cells) present in cartilage influence the self-diffusion behaviour to a major extent [20], both polymers were also investigated in pure, aqueous solutions to exclude

influences of the environment on the mobility of the polymers. Surprisingly, the determined self-diffusion coefficients of PEG and dextran in cartilage resemble very closely the data obtained in the pure solution (Fig. 3). There is also an exponential dependence of the self-diffusion coefficient on the molecular weight as it is requested by Eq. (5) with similar values of m ($0.8 < m < 1.0$). Callaghan et al. [29] have also found such an exponential dependence for the diffusion of dextran in water but with a slightly different parameter ($m = 0.57$).

The similarity of the short-time diffusion behaviour of polymers in cartilage and in pure solution indicates that—at least at short diffusion times ($= 15 \text{ ms}$)—the supermolecular structure of cartilage

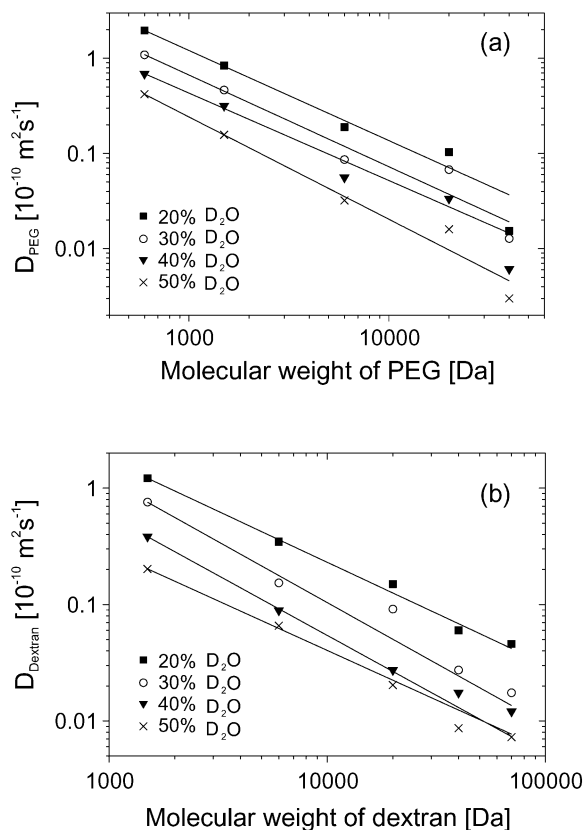


Fig. 3. Self-diffusion coefficients of PEG (a) and dextran (b) in solution (D_2O) as a function of the molecular weight of the polymers. D_2O contents are indicated in the figure. A diffusion time of $\Delta = 15 \text{ ms}$ was used in all cases.

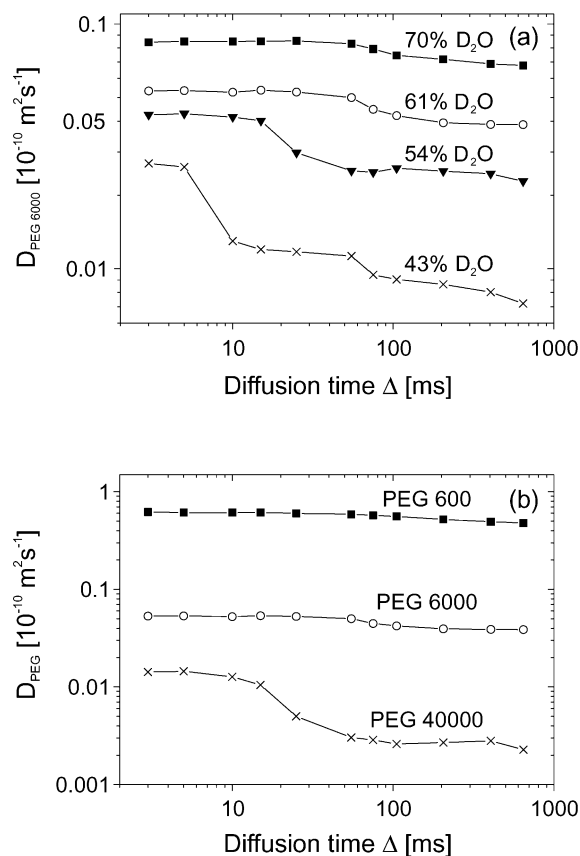


Fig. 4. Self-diffusion coefficients of (a) PEG 6000 at varying polymer concentrations and (b) PEG samples with different molecular weights (at a fixed polymer concentration of 40%) in cartilage as a function of the diffusion time.

has only negligible influence on the self-diffusion of the polymers. The influence of internal cartilage structures becomes, however, much stronger at longer diffusion times (Fig. 4).

Fig. 4a shows the dependence of the self-diffusion coefficients of PEG 6000 in cartilage at different D_2O contents on the diffusion time. At each of the four different D_2O contents restricted diffusion occurs but this effect is most pronounced at lower D_2O contents, i.e. at the highest polymer concentrations.

We assume that the collagens of cartilage form marked intracartilaginous barriers and are, therefore, the main reasons of restricted diffusion [20]. The application of higher osmotic pressures on

cartilage—in the same manner like mechanical pressures—lead to decreased distances of the diffusion barriers [30,31], i.e. of the collagen fibres. Therefore, differences in the diffusion behaviour of the polymers can be well explained by changes of the cartilage structures.

Additionally, there is also a marked influence of the molecular weight of the polymer on the self-diffusion properties. Fig. 4b shows the dependence of the self-diffusion coefficients of PEG 600, 6000 and 40 000 in cartilage on the observation time. The D_2O content of the cartilage was adjusted to 60% in all these cases. This was achieved by the incubation of all cartilage samples in differently concentrated PEG solutions [20]. Even if these effects seem to be very small if one considers PEG 600 and PEG 6000, one should also take into consideration that the data are represented in logarithmic scaling of the y-axis which weakens effects considerably.

Restricted diffusion occurs in the case of all applied PEGs but with an increasing molecular weight the onset, i.e. the time point where restricted diffusion can be observed is shifted to smaller diffusion times. This indicates that larger diffusing species experience geometrical obstacles earlier than smaller molecules. As expected, this effect is most pronounced when the polymer with the highest molecular weight is used (in this study PEG 40000 (x)).

However, it is so far not clear whether restricted diffusion does exclusively depend on the geometry of the barriers in the cartilage or it may also depend on entanglements of the polymer chains themselves. This is the reason why the potential occurrence of restricted diffusion was also investigated on the hand of the pure polymer solutions. Fig. 5a shows the dependence of the self-diffusion coefficients of pure, differently concentrated PEG 6000 solutions on the diffusion time. Accordingly, Fig. 5b shows this dependence on the hand of pure aqueous solutions of PEG 600, 6000 and 40000 at a fixed polymer concentration of 30 wt.%. One should note that this time-dependence of diffusion is not caused by barriers as the ‘classical’ restricted diffusion. Therefore, the term ‘anomalous restricted diffusion’ instead of restricted diffusion has been recently introduced [28,32].

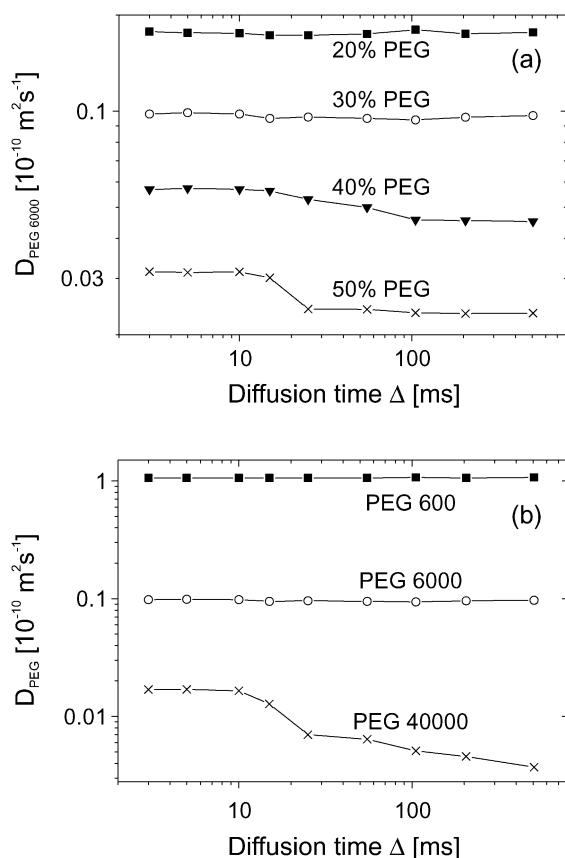


Fig. 5. Dependence of the self-diffusion coefficients of PEG 6000 in aqueous solution (D_2O) on the diffusion time (a). The different PEG concentrations are indicated in the figure. In (b) PEG samples with different molecular weights were used (30% polymer) in each case.

In the case of PEG 6000 (Fig. 5a) restricted polymer diffusion cannot be observed at concentrations of 20% and 30% but for concentrations higher than 40%. At 50% concentration, the point where restriction can be observed, is shifted to

smaller diffusion times. This indicates that the motion of polymers gets more and more limited when the concentration increases. In the case of PEG 40000 (Fig. 5b) restricted diffusion can already be observed at a concentration of 30% PEG whereas for PEG 600 restricted diffusion cannot be observed, equally what concentration is used.

If the Einstein equation [Eq. (2)] is applied to the measured data, the mean square displacement $\langle z^2 \rangle^{1/2}$ indicates the pathway of the free diffusion of the polymer chains (Table 1). Since the restricted diffusion depends also on the chain lengths of the polymers themselves (Fig. 5) the diffusion values of PEG 6000 and 40 000 are not high enough to reflect the space between the barriers within the cartilage. Only the calculated value for the diffusion of PEG 600 (2 μm at a D_2O content of 60%) is in agreement with the distance between the collagen chains within the cartilage [20], because in the case of PEG 600 with that molecular weight restricted diffusion in pure solution could not be observed (Fig. 5b). Therefore, polymers with lower molecular weights seem to be more suitable for studying the internal structures of the cartilage.

4. Discussion

It is well known that rheumatic diseases are accompanied by the degradation of the native cartilage polymers under the formation of smaller products [33]. To simulate the motion properties of these smaller molecules, the diffusion of defined polymers with different molecular weights in cartilage was investigated. Bovine nasal cartilage was used for that investigation since this kind of cartilage is available in higher amounts and deviations from sample to sample are less pronounced

Table 1

Diffusion time Δ_0 (the value of Δ until restricted diffusion occurs) and the corresponding mean square displacement $\langle z^2 \rangle^{1/2}$ at different water contents of cartilage and different molecular weights of the applied PEG

	PEG 6000 (70% H_2O)	PEG 6000 (61% H_2O)	PEG 6000 (54% H_2O)	PEG 6000 (43% H_2O)	PEG 600 (60% H_2O)	PEG 40000 (60% H_2O)
Δ_0 (ms)	55	25	15	5	55	10
$\langle z^2 \rangle^{1/2}$ (μm)	0.7	0.4	0.3	0.1	2	0.1

than in the case of the physiologically more relevant articular cartilage [34].

PFG NMR was used to measure the corresponding self-diffusion coefficients since this technique offers besides the lack of the necessity to use radioactively labelled compounds also the advantage that diffusion processes can be observed over different time scales [35]. This is very useful for the investigation of the influence of internal cartilage structures on the mobility of different polymers. Additionally, PFG NMR does not require concentration gradients and, therefore, actually self-diffusion coefficients are determined. Although different investigations on the diffusion of PEG [36–38] and dextran [39] in polymer gels or artificial membranes were published, this is the first study of the diffusion behaviour of dextran and PEG in cartilage by PFG NMR. Using this approach we were not able to confirm the results of previous diffusion studies of the diffusivity of dextran and PEG in cartilage [22]. These authors found that the diffusion of dextran in cartilage is retarded in the cartilage matrix in comparison with the pure aqueous polymer solution, whereas the opposite holds for PEG [22]. In our study, however, the same tendencies were found for both polymers.

The first important observation of our study was that the short-time ($\Delta = 15$ ms) self-diffusion coefficients of PEG and dextran are strongly dependent on the molecular weight of the polymer as well as the water content of cartilage. A dependence of the diffusion coefficients on the molecular weights was expected and reflects the decreased mobility of polymers if their molecular weight increases. However, it is assumed that the dependence of the diffusion coefficients on the water content is a consequence of a rather unspecific obstruction effect caused by the impenetrable collagen chains within the cartilage. An analogous behaviour was already reported by other authors [19–21]. It has also been shown that the water as well as the cation diffusion (at short diffusion times) is exclusively determined by the water content of the sample, equally if one considers the diffusion in cartilage or in pure polymer solutions.

The dependence of the self-diffusion coefficients of polymers in cartilage on the water content at

short diffusion times may be used for the estimation of the water content of an arbitrary sample by PFG NMR independently of its structure and function. Of course, this is also possible by measuring the self-diffusion of water [20]. However, by the method presented in this paper, this estimation can be additionally combined with the investigation of the diffusion behaviour of polymers in cartilage to gain further insights into the diffusion behaviour of macromolecules in cartilage.

In this study it was also found that under identical experimental conditions (same molecular weight and water content) the diffusion of dextran is faster than the diffusion of PEG. Although differences in diffusivities of both polymers were already discussed [39], this is surprising since dextran is known to be a more rigid molecule and to yield higher viscosity in solutions [23]. We assume that the higher flexibility of the PEG leads to a higher degree of entanglements of the polymer chains resulting in lower self-diffusion coefficients in the gel-like structure of cartilage.

In contrast to short-time, the long-time diffusion of polymers in cartilage was found to be restricted, i.e. to be dependent on the corresponding diffusion time. The extent of diffusion restriction is the more pronounced the lower the water content of the cartilage. The observed restriction is caused by the inner structures of the cartilage [20] but in the case of PEG 6000 and PEG 40 000 also by the diffusing polymer chains themselves. Restricted diffusion in pure polymer solutions was already described by Fleischer et al. [28] using styrene–methylmethacrylate copolymers in semidilute acetone solutions. Since the restriction of diffusion cannot be explained by the presence of barriers in that case, the term ‘anomalous restricted diffusion’ is normally used here. This phenomenon was also found for aqueous solutions of poly(ethylene oxide)–poly(propylene oxide)–poly(ethylene oxide) block copolymers [32] and was explained by sol–gel transitions that may easily occur in that system since it contains hydrophilic [poly(ethylene oxide)] as well as hydrophobic [poly(propylene oxide)] moieties. The reason why even in pure PEG solutions restricted diffusion occurs cannot be explained so far.

Since the effect of restriction is more and more pronounced when the molecular weight of the polymer rises, the long-time diffusion of PEG 6000 and 40 000 cannot be used for the calculation of the structural properties of cartilage. However, the long-time diffusion of PEG 600 does not show any restriction in solution and, therefore, the long-time diffusion of this polymer reflects structural properties of cartilage. Our data revealed barrier distances of approximately 2 μm , which agree with the distance of the collagen chains in cartilage at a water content of 60%. Similar data based on the measurement of the diffusion of water and cations were already reported by Knauss et al. [20] and Ngwa et al. [21]. This means that our measurements of the diffusion of polymers in cartilage provide useful and complementary information.

Even if there are clear differences between the diffusion of the PEG in pure aqueous solution and in cartilage that can be used for the characterization of cartilage, further studies on the basic mechanisms of the anomalous restricted diffusion in PEG are obviously necessary. Polymer segment displacement as well as center of mass displacement may both contribute to the observed restricted diffusion [28]. At the present stage, we do, however, not want to speculate about these individual contributions. Further experiments to clarify these problems in more details are currently being performed in our laboratory.

5. Conclusions

It was found that at short diffusion times the self-diffusion coefficients of PEG and dextran in cartilage are controlled by: (a) their molecular weights; and (b) by the water content of the cartilage. The polymers behave quite similar in solution. Therefore, the inner structure of the cartilage does not influence the short-time diffusion of polymers to a high extent.

The dependence of the self-diffusion coefficients of polymers in cartilage on the water content at short diffusion times may be used for the estimation of the water content of an arbitrary sample with PFG NMR independently of its structure and composition. Evidence of restricted diffusion within the cartilage but also in polymer solution was

obtained in experiments where the diffusion coefficient was measured as a function of the applied diffusion time.

The long-time diffusion of PEG 600 shows no restriction in solution and reflects structural properties of the cartilage within approximately 2 μm , which can be identified with the distance between the collagen chains. In contrast, for PEG 6000 and 40000 the influence of the restriction caused by the polymer chains themselves (anomalous restricted diffusion) makes the calculation of structural properties of cartilage impossible.

Acknowledgments

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