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Cation diffusion in cartilage measured by pulsed field gradient NMR

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Abstract In this study, the pulsed field gradient (PFG) nuclear magnetic resonance (NMR) technique was used for the investigation of (1) concentration and compression effects on cation self-diffusion, and (2) restricted diffusion of cations in cartilage. Since physiologically relevant cations like Na^+ are difficult to investigate owing to their very short relaxation times, the cations tetramethylammonium (TMA) and tetraethylammonium (TEA) were employed for diffusion studies in samples of explanted cartilage. Results indicated that the diffusion of monovalent cations shows strong similarities to observations already made in studies of the diffusion of water in cartilage: with increasing compression, i.e. decreasing water content, the diffusion coefficient of the cation decreases concomitantly. The diffusion coefficients also showed a decrease with increasing cation concentrations, basically reflecting the corresponding decrease in the water content. Both results could be explained by the well-established model of Mackie and Meares. This, together with the similarity of the diffusion coefficient D in cartilage relative to free solution (about 50%) for both cations, is consistent with the view that the water content and not the charge is the most important determinant of the intratissue diffusivity of monovalent cations. Diffusion studies with increasing observation times showed strong evidence of restricted diffusion, allowing the estimation of the geometry of barriers within cartilage.

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Introduction

In higher vertebrates, cartilage functions as a weight-bearing surface of articular joints. Transport of nutrients and metabolic waste products in cartilage occurs primarily by diffusion since it is avascular (Maroudas 1970; Maroudas 1979; Torzili et al. 1987). Similarly, therapeutic or diagnostic contrast agents must enter the tissue through a diffusive process. In general, cation diffusion studies may improve the understanding of ion transport in charged biopolymer systems. More particularly, diffusion studies in biopolymer matrices like cartilage play an important role in many biomedical as well as biophysical applications. The ability of cations to move (diffuse) through the cartilage matrix is valuable in studies on the normal function of cartilage, and also in cartilage altered by degenerative diseases such as rheumatoid arthritis or osteoarthritis (Burstein et al. 1993).

As a porous biopolymer consisting of a rigid collagenous fiber network supporting a hydrophilic proteoglycan polyelectrolyte gel (Buschmann and Grodzinsky 1995; Comper and Laurent 1978; Maroudas 1979; Maroudas et al. 1992), the extracellular matrix of cartilage consists of collagen II, water and proteoglycans. The proteoglycans are composed of a central protein core and glycosaminoglycan side chains, namely chondroitin and keratan sulfate covalently attached to the protein. These polysaccharides have pendant sulfate and carboxylate groups, resulting in a strong negative charge density under physiological conditions. The concentration of these anionic groups is referred to as the ''fixed charge density'', which exists in equilibrium with the positively charged counterions, especially sodium and potassium. The negative charges are responsible for the high swelling capacity of cartilage, while the collagen determines the supermolecular cartilage structure but has only a minor influence on the osmotic activity. The

collagen fibers impart their tensile strength to the matrix and act to resist the swelling of the proteoglycan gel (Urban et al. 1979). The osmotic activity of cartilage causes a water content of more than 70 wt%. The high swelling capacity and the elasticity are important properties of cartilage to ensure its biophysical function under varying external loads. Because of the importance of cartilage swelling, the relations between compression of cartilage, water content, and short-time as well as long-time diffusion of cations are studied in this paper.

The branched structure of proteoglycans localizes a considerable amount of charge calculated to be approximately 0.1 M within its domain (Comper and Laurent 1978). Because of the importance of negatively charged groups of glycosaminoglycan molecules for water binding and swelling, the concentration of cations should influence the cartilage properties. Solution studies on the connective tissue polysaccharides have demonstrated that they behave as typical polyelectrolytes, undergoing conformational changes that depend on the concentration of their microion environment and the type of counter ions (Comper and Laurent 1978). As the ionic strength is lowered, the anionic charges on the polysaccharide backbone have a greater tendency to repel one another and the polyion takes on an extended conformation. This process is reversible. It is predicted (Comper and Laurent 1978) that when the local charge concentration is as high as 0.6 M, marked effects on ion transport can be expected through polyion/mobile-ion interaction. Therefore, studies of cartilage at varying cation concentrations were also performed.

While cartilage is compressed, the fluid contained within the matrix is extruded into the joint space. This extruded fluid acts as a lubricant between contact points of opposing bone surfaces. Studies on compression effects on cartilage are of substantial interest towards understanding the mechanical properties of normal and degenerated articular cartilage. The molecular composition of cartilage is known to change with degenerative diseases such as those mentioned above (Burstein et al. 1993). Because the proteoglycan molecules are usually smaller in size and the fixed charge density is significantly reduced under pathological conditions (presumably by marked calcification), the viscoelastic behavior of cartilage is altered for load bearing, resulting in a loss of resilience of the cartilage.

Evidence of restricted diffusion of water has been found within cartilage in studies where the diffusion coefficient D was measured as a function of diffusion time (Burstein et al. 1993). The self-diffusion becomes an apparent self-diffusion, owing to its time dependence. The apparent self-diffusion of water in pig articular and bovine nasal cartilage as a function of diffusion time Δ at various water contents has also been measured (Knauss et al. 1999), and gave strong evidence that short-time diffusion is solely determined by the water content of cartilage. Therefore, these results can be used for the determination of the water content in cartilage as well as other related systems. In contrast, long-time diffusion is sensitive to the internal cartilage structure. Therefore, studies on the effect of different observation times on the diffusion of cations are also important.

Pulsed field gradient (PFG) nuclear magnetic resonance (NMR) was employed in this study. This technique provides information on the translational mobility of fluid molecules. In porous media (like cartilage) the diffusion path of fluid molecules in the pore space is affected by interactions with the pore wall. PFG NMR measurements are sensitive to structural peculiarities of the confining porous medium. The sample cations tetramethylammonium (TMA) and tetraethylammonium (TEA) were used since physiologically relevant cations like Na^+ exhibit a very short relaxation time that confers serious problems for NMR measurements. One further advantage is that neither TMA nor TEA possesses exchangeable protons and therefore no interference with the water protons may occur. NMR techniques provide several advantages for the study of diffusive transport in cartilage, including the fact that NMR is nondestructive. Thus consecutive measurements can be made on a given sample. Additionally, no concentration gradients are required, i.e. actually the self-diffusion is measured.

Considering all these, and in the spirit of improving the understanding of cation diffusion in a charged biopolymer system like cartilage, our objectives in this study were to use PFG NMR for the following:

1. To measure the diffusion of TMA and TEA in free solution and in cartilage, as a useful comparison to results with physiologically relevant cations and the results of previous studies of water diffusion in cartilage.
2. To examine the effects of compression and different cation concentrations on the diffusion coefficient. These effects are perturbations that mimic physiological/pathological states.
3. To investigate the dependence (if any) of the measured diffusion coefficients on observation times.

Theory

In the case of unrestricted diffusion, the mean square displacement $< z^2 >$ of the diffusing species obeys Einstein's equation:

$$< z^2 >= 2D\Delta \tag{1}$$

for one dimension, where Δ is the observation (diffusion) time and D is the diffusion coefficient. If the diffusion is restricted, $< z^2 >$ increases with less than the first power of diffusion time (Knauss et al. 1999).

The measured quantity in PFG NMR is the attenuation of the spin echo signal ψ as a function of the magnetic field gradient pulse. For successful diffusion measurements, "NMR active" atoms, with a nonzero gyromagnetic ratio γ, have to occur with sufficiently

large density and with a typical minimum concentration in the order of 0.1 mol L^{-1} (Kärger et al. 1998). In this technique, the field gradient is applied for a short time Δ, which imparts a phase to the magnetic moment of each nucleus as a function of the position within the sample. After a specified time δ, which allows the nuclei to diffuse within the sample, a second magnetic field gradient is applied, which imparts a phase opposite that of the first gradient, again as a function of position (Kärger and Ruthven 1992).

The stimulated-echo pulse sequence used in these experiments (Knauss et al. 1999) generates an NMR signal whose intensity is proportional to the total (net) magnetization. The application of the field gradient pulses leads to signal attenuation, i.e. the vector sum of the contributions of the individual spins. The smaller the diffusion coefficient, the higher is the required gradient strength.

Under the assumption that the nuclei are moving with free Brownian motion described by a single D, it has been shown that the ratio ψ of the NMR signal with the diffusion-sensitizing magnetic field gradients and that without field gradients can be written as (Stejskal and Tanner 1970):

$$\ln(\psi) = -q^2 D(\Delta - \delta/3) \tag{2}$$

where $q = \gamma \delta g$ is a generalized scattering vector (Callaghan 1991; Kärger et al. 1988). Here δ is the time during which the gradient g is applied (the pulse width) and γ is the gyromagnetic ratio of the observed nucleus. The experiment is typically performed such that ψ is measured for different values of g while all other variables are held constant. D is subsequently calculated from a fit of the data to Eq. 2. It is important to realize that, in this technique, motion is measured along the direction of the magnetic field gradient, which can be set arbitrarily, i.e. this experiment describes the diffusion only in a single direction.

Model of Mackie and Meares

This model (Mackie and Meares 1955), used for the interpretation of experimental data, predicts that the ratio D/D_0 depends on the polymer volume fraction ϕ_p in the following way:

$$D/D_0 = \left(1 - \phi_p\right)^2 / \left(1 + \phi_p\right)^2 \tag{3}$$

where D is the diffusion coefficient of the cation of interest in cartilage, and D_0 the diffusion coefficient of the cation in free solution; ϕ_p is calculated from the polymer volume V_p and the water volume V_w as follows: $\phi_p = V_p/(V_p + V_w)$. The water volume fraction is correlated to the polymer volume fraction such that they add up to one. The volume fractions of the polymer are calculated from the mass fractions of the sample and their known densities (Burstein et al. 1993): 1.4 g cm^{-3} for the polymer and 1 g cm^{-3} for water. For ϕ_p the additivity of the volume of water and polymer is assumed in this study.

Materials and methods

Materials

Bovine nasal cartilage (BNC) was used instead of the physiologically more relevant articular cartilage because it does not have anisotropic properties. Cartilage was obtained from a local slaughterhouse. All chemicals were obtained from Fluka in the highest available purity, and were used as supplied. These included: poly(ethylene glycol) (PEG) with a molecular weight (MW) of about 20,000 g mol^{-1}, deuterated water (D_2O) with an isotopic purity of 99.9% (used as the solvent), tetramethylammonium chloride and tetraethylammonium chloride with chemical formulae $N(CH_3)_4Cl$, and $N(C_2H_5)_4Cl$, respectively. Both dissociate in solution, to give the TMA cation $N(CH_3)_4^+$ (with a MW of 74.1 g mol^{-1}) or the TEA cation $N(C_2H_5)_4^+$ (with a MW of 130.2 g mol^{-1}), respectively, and Cl^-. The choice of these cations was motivated by the fact that they possess longer relaxation times (for protons) than physiologically relevant cations (e.g. Na^+), and that they do not show any exchange with water. Dialysis membranes with a molecular weight cut-off of 1000 g mol^{-1} were obtained from Spectrapor.

Methods

BNC, obtained from slaughtered healthy cows, was stored at –20 °C until experiment time. The cartilage was then thawed and plugs of about 4–5 mm diameter were made from the cartilage. These were then subsequently incubated for about 12–18 h in deuterated water containing the cations TMA or TEA in defined concentrations. Because of the much larger volume of the incubation solution compared with the cartilage plugs (with a ratio of about 12:1), there is only very little dilution with the water in cartilage; thus the influence on TMA or TEA solutions was assumed to be negligible. One freezing and thawing cycle of cartilage has been shown not to affect the diffusivity relative to fresh cartilage (Burstein et al. 1993). Before each measurement, the cartilage was blotted dry to prevent (minimize) any influence on the measurement of the solution on the surface of the cartilage plugs. All measurements were performed at 298 K and after each measurement the water content of the cartilage plugs was determined by weighing, drying and re-weighing in a rapid evaporation system (Jouan, Germany) at 60 °C until a constant weight was achieved (about 4 h). D was calculated by using Eq. 3 with a fit of $\ln(\psi)$ versus g^2. At least 10 points were used for all measurements.

The compression of cartilage plugs was carried out by the osmotic stress technique (Lüsse et al. 1995; Maroudas et al. 1991). A certain amount of PEG was dissolved in a solution with the appropriate TMA or TEA concentration. The cartilage plugs, already equilibrated in a solution with the same concentration of cations, were separated from the applied PEG 20,000 solution by a dialysis membrane (with a molecular weight cut-off of 1000 g mol^{-1}) which stops PEG from penetrating into the cartilage samples (Lüsse et al. 1995). The concentrations of solutions used for incubation ranged from 10 through 20, 30 to 40 weight percent (wt%) PEG, corresponding to osmotic pressures of 0.13, 0.62, 1.77 and 4.24 MPa, respectively (Arnold et al. 1983). The incubation time was 12–18 h on average, but as a check, an incubation time of 48 h was also used for comparison. Previous experiments have shown that the equilibrium state is already reached after approximately 5 h (Lüsse et al. 1995).

To study the dependence of the diffusion coefficient on the diffusion time (observation time) Δ, a 0.15 M TMA solution was employed. A set of experiments was performed in which g was varied for a given Δ, D was calculated and the series was repeated with a different value of Δ. These experiments were conducted

under free swelling conditions, as well as with compressed cartilage for a δ of 0.36 ms and Δ ranging from 6 to 400 ms.

The PFG NMR diffusion measurements were conducted using the home-built FEGRIS 400 spectrometer operating at a proton NMR resonance frequency of 400 MHz (Galvosas et al. 2001; Kärger et al. 1995). The field gradient g was varied between 0 and up to 34 T m^{-1} (on average), with a fixed pulse width δ of 0.36 ms. For constant diffusion time measurements, $\Delta = 15$ ms was used. The stimulated echo sequence (Knauss et al. 1999) mentioned above was utilized in all studies.

Results

Diffusion of cations and influence of cation concentration

As an example, the signal amplitudes (ψ) of 0.15 M TMA in free solution and in cartilage are shown in Fig. 1 as a function of the applied field gradient (g). Results for cation diffusion in free solution could be appropriately fitted using the model of a single D as expected from Eq. 2 (Fig. 1). The data for cation diffusion in cartilage are well described by a biexponential fit, obviously resulting from two fractions of protons. The more intense contribution with a D value of 4.8×10^{-10} m^2 s^{-1} must be assigned to the cation protons, while the less intense component with a D value of about 3.7×10^{-11} m^2 s^{-1} may be due to residual water protons moving in the interstices of the collagen molecules [since this value corresponds approximately to the one found by Knauss et al. (1996)].

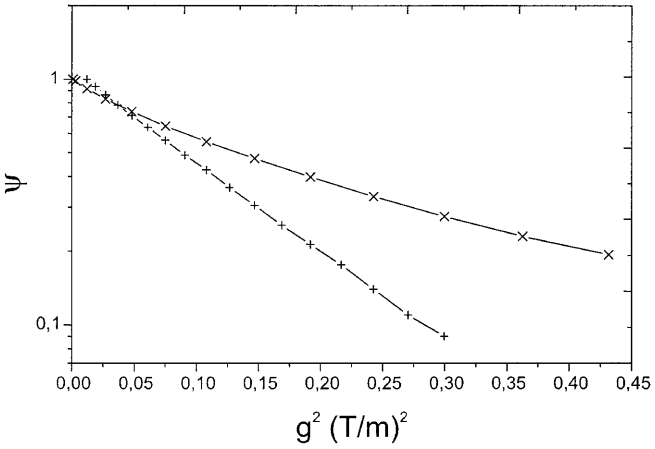

Fig. 1 Ratio (ψ) of the signal amplitude of 0.15 M TMA in free solution (*plus signs*) and in cartilage (*crosses*) obtained with a diffusion-sensitizing gradient (g) to that obtained without a gradient. Note the logarithmic scale of the y ordinate. The slope of the curve is proportional to the diffusivity as seen from Eq. 2. There is no obvious deviation from linearity for the graph of TMA diffusion in free solution, suggesting a single D. In contrast, there is a slight deviation from linearity for the diffusion behavior of the cations in cartilage, i.e. a superposition of at least two different diffusion coefficients. The more intense component is assigned to the TMA cation, while the less intense component is most probably due to residual water protons moving in the interstices of the collagen fibrils

Table 1 shows the diffusion coefficients of TMA and TEA in cartilage for different cation concentrations in the incubation solution. The diffusion coefficient relative to the free solution was found to be about 52% and 45% for 0.15 M TMA and 0.15 M TEA, respectively. It was observed that the diffusion coefficients decrease with increasing cation concentration. On checking the water content of the cartilage (determined immediately after the NMR experiments by weighing and drying as mentioned above), a corresponding decrease in the water content was noticed. This strongly indicates that the water content has a major impact on the diffusion behavior of cations.

Compression

The compression of cartilage due to the osmotic pressure of the surrounding PEG 20,000 solution causes a decrease in D values as well as in the water content of the samples. The water content (wt%) of the cartilage samples against the osmotic pressure is plotted in Fig. 2a. The diffusion coefficient of the TMA and TEA cations in BNC incubated in 0.15 M TMA/PEG 20,000 and TEA/PEG 20,000 solutions, respectively, is shown in Fig. 2b as a function of the applied osmotic pressure and, therefore, the PEG concentration.

An osmotic pressure of 4.24 MPa, for example, leads to about a 60% decrease in the diffusion coefficient, down to a value of about 20% compared to that in free solution for 0.15 M TMA. One clear but expected result of these measurements was that the cation diffusion coefficients in free solution as well as in the cartilage are smaller in the cartilage incubated in a TEA solution compared to that incubated in a TMA solution. This is due to the influence of the different salts on the water content of the cartilage and/or the different molecular weights of both salts.

Figure 3 shows the results from Table 1 expressed relative to the diffusion behavior in free solution for

Table 1 The diffusion coefficients of TMA and TEA in cartilage for different cation concentrations (as well as the corresponding water content of cartilage). $\phi_p = V_p/(V_p + V_w)$; V_p and V_w are the polymer volume and the water volume, respectively (Eq. 3)

Cation	Concentration	Water content of cartilage (wt%)	Polymer volume fraction, ϕ_p	D (10^{-10} m^2 s^{-1})
TMA	0.15 M	79.8	0.153	5.2 ± 0.1
TMA	0.3 M	77.8	0.169	4.8 ± 0.1
TMA	1 M	76	0.184	4.6 ± 0.1
TMA	3 M	61.8	0.306	3.2 ± 0.2
TEA	0.15 M	77	0.234	3.0 ± 0.2
TEA	1 M	36	0.559	2.0 ± 0.1

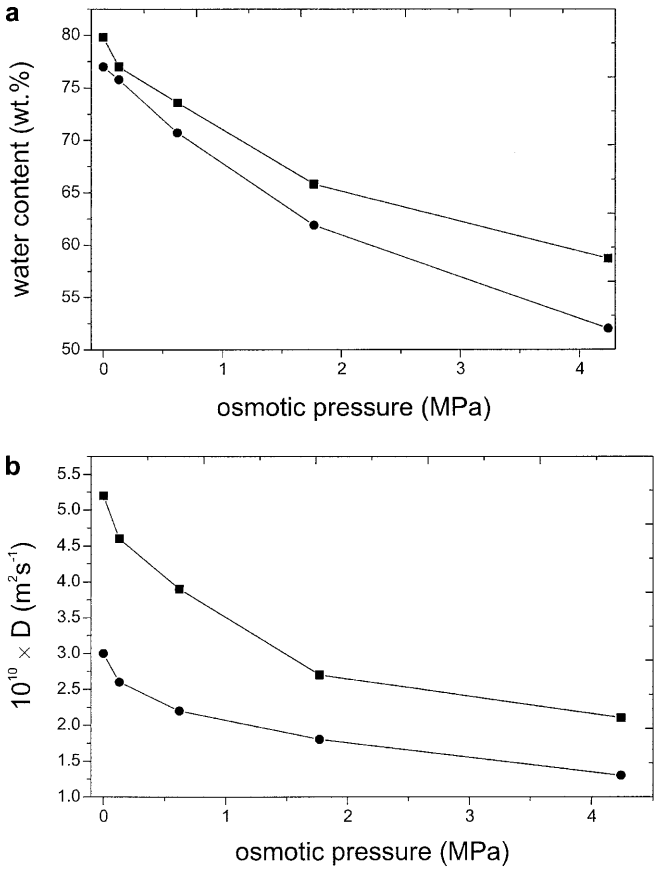

Fig. 2 **a** Water content of cartilage incubated in 0.15 M TMA (*squares*), and 0.15 M TEA (*circles*) as a function of the osmotic pressure from the surrounding PEG 20,000 solution. Different osmotic pressures were achieved by incubation of the cartilage plugs in different concentrations of 0.15 M TMA/PEG 20,000 solutions (see text). The cartilage samples were separated from the solution by a dialysis membrane to prevent the PEG from entering the cartilage plugs. **b** Diffusion coefficient of TMA (*squares*) and TEA (*circles*) cations (both as 0.15 M solutions) in cartilage as a function of the osmotic pressure at 298 K. Different osmotic pressures were realized by incubating the cartilage samples in differently concentrated PEG 20,000 solutions. The diffusion coefficients decrease with decreasing water content

the different concentrations of the TMA cation. The solid line in this figure shows the curve obtained by the use of the equation derived by Mackie and Meares (Eq. 3). Analogous calculations yield a D/D_0 for 0.15 M TEA of about 46%. This compares favorably with the 49% obtained according to Mackie and Meares with a D_0 of $6.6 \pm 0.2 \times 10^{-10}$ m^2 s^{-1}. For the very high TMA concentration of 3 M, there is a deviation from the curve according to Mackie and Meares, possibly due to electrostatic shielding of the matrix-fixed charges.

Figure 3 also shows a plot of D/D_0 (for 0.15 M TMA and 0.15 M TEA) against the polymer volume fraction of cartilage obtained from the simulation of the mechanical compression. Obviously, the good agreement with the model of Mackie and Meares suggests that the water content of the sample and not the charges have the most pronounced effect on the diffusion behavior of monovalent cations in cartilage.

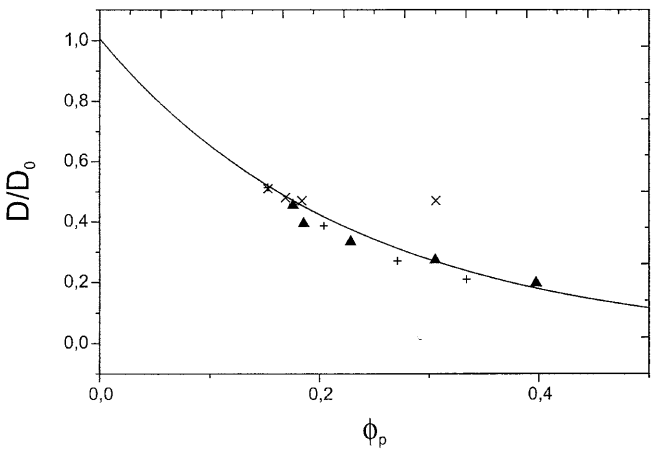

Fig. 3 Relative self-diffusion coefficient D/D_0 for different concentrations of TMA (Table 1) in cartilage (*crosses*) as a function of the volume fraction ϕ_p of the solid component. The D/D_0 ratios of 0.15 M TMA (*plus signs*) and 0.15 M TEA (*triangles*) in cartilage as a function of the volume fraction ϕ_p of the solid component (calculated from the compression experiment) are also shown. D_0 and D are the diffusion coefficients of the cations in free solution and cartilage, respectively. The *solid line* represents the curve calculated according to the model of Mackie and Meares (Eq. 3)

The apparent diffusion coefficient as a function of diffusion time

As expected, the measured apparent diffusion coefficient of the cations in cartilage was dependent on the time over which the diffusion was observed. As the diffusion time Δ was increased (from 6 to 400 ms for cartilage with a water content of about 77 wt%, and from 6 to 200 ms for cartilage with a water content of about 64 wt% due to the decreasing signal amplitude), the apparent diffusion coefficient of the TMA cation (0.15 M TMA solution) in cartilage decreased (Fig. 4). For means of comparison, the apparent diffusion coefficient for 0.15 M TEA in cartilage with a water content of 70 wt% is also shown for different observation times. A decrease of D for increasing observation times underscores restricted diffusion for the cations, but is less expressed than in the case of TMA.

For TMA, the ratio of the apparent diffusion coefficient at a diffusion time Δ of 15 ms to that at 200 ms, for example, was found to be about 1.4 for the cartilage with a water content of 77 wt%. When measurements were repeated with cartilage compressed by an osmotic pressure of 1.77 MPa (64 wt% water content), a downward shift of the D-Δ curve was observed (Fig. 4). In this case, the ratio of D at 15 ms to that at 200 ms was about 1.6. Interestingly, this latter ratio is about the same as in the case of a water content of about 77 wt%. Therefore, we assume that the compression affects the magnitude but not the general shape of the dependence of D on the diffusion time. This is an observation already made by

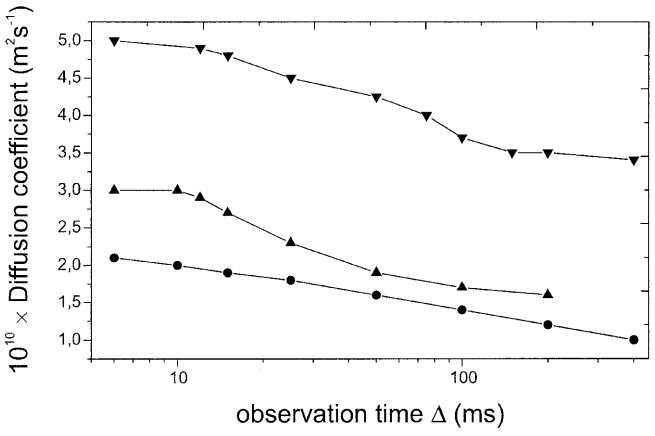

Fig. 4 The apparent diffusion coefficients of 0.15 M TMA in cartilage with a water content of 77 wt% (*down triangles*) and 64 wt% (*up triangles*) as a function of observation time. For comparison, the diffusion coefficient of 0.15 M TEA (*circles*) in cartilage with a water content of 70 wt% as a function of observation time is also shown. Bovine nasal cartilage (BNC) was investigated by the stimulated-echo sequence

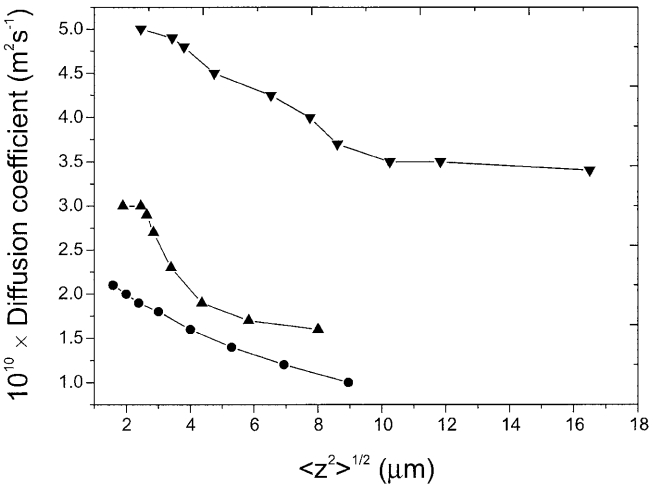

Fig. 5 Diffusion coefficient of 0.15 M TMA in cartilage with a water content of 77 wt% (*down triangles*) and 64 wt% (*up triangles*) as a function of the mean displacement of the cations calculated from Eq. 1. The diffusion coefficient as a function of the mean displacement of TEA cations (*circles*) in cartilage with a water content of 70 wt% is also shown. Apparently, the movement of the cations exhibits a relatively high sensitivity to the cartilage internal structures as they encounter restrictions

Burstein et al. (1993) for the diffusion measurements of water in cartilage.

Figure 5 shows the results obtained in terms of the mean square displacement of the diffusing cations. This is very illustrative as it shows the distances covered for different observation times. It is evident that, for a lower water content of cartilage (about 64 wt%), restrictions encountered by the diffusing TMA cations are more pronounced than in the case of a higher water content (77 wt%). This is seen from the rather drastic decrease after a mean distance of about 2.5 μm. After just about 4 μm, most of the restrictions have been met by the TMA cation, while for a higher water content a distance of about 9 μm is needed for restriction. This is expected, since compressing cartilage increases the solid volume fraction, hence providing a higher extent of restrictions within a shorter distance. The result is also shown for TEA cations. Apparently their higher molecular weight permits these cations only to diffuse over shorter distances during the same observation time as compared to TMA cations. Other factors like the shape of the cation and different salt as well as solubility properties may also be responsible for this observation. Compared to water diffusion (Knauss et al. 1999), the cations show a higher sensitivity to the restrictions encountered.

Discussion

The results of our study indicate a significant change in D when the concentration of the cations is changed. This corresponds, however, to a change in water content and, consequently, a changed water or a changed polymer volume fraction. One should notice that the D/D_0 values (Fig. 3) are approximately 50% for both cations and for the different concentrations of TMA. Furthermore, as depicted by Fig. 3, the changes in D/D_0 with the solid volume fraction ϕ_p are in good agreement with the prediction by the model of Mackie and Meares (Eq. 3). Both facts support the view that charge has little or no effect on the diffusion of monovalent cations, as previously suggested by Burstein et al. (1993). Additionally, these observations lend credit to the findings of Knauss et al. (1999). The latter authors observed that D depends mainly on the water content at short diffusion times ($\Delta = 13$ ms). Since the water content of cartilage plays a significant role in pathological processes (Mankin and Thrasher 1975), cation diffusion studies also provide valuable information with respect to this.

As seen from Fig. 3, a higher concentration of up to 3 M for TMA leads to a slight deviation from the curve calculated according to Mackie and Meares' equation. The D/D_0 is the same as that for 1 M TMA (47%), different from the D/D_0 calculated according to the model of Mackie and Meares (28.2). This must be due to complex polyion/mobile ion interactions like electrostatic shielding of the matrix-fixed charges. The shielding of matrix-fixed charges is followed by a lower repulsion, leading to a corresponding approach of individual glycosaminoglycan chains, resulting in more pronounced obstruction of diffusion (Lüsse et al. 1995). This explains the deviation in the case of a 3 M TMA concentration. The apparently insignificant influence of charges on the diffusion of monovalent cations may, however, not be readily extended to the diffusion of divalent cations. Potter et al. (1997) concluded from their cation studies with Cu^{2+} that matrix-fixed charges influence divalent cation diffusion in cartilage in a significant way. In another study, Lüsse et al. (1995) concluded that the concentration of Na^+ ions neither influences the water content nor the relaxation times in cartilage, while Ca^{2+}

(a divalent cation) causes a small reduction in these parameters.

The diffusion coefficient D depends on the MW in the following manner: $D \approx (\mathrm{MW})^{-1/2}$ for molecules with a MW less than 1000 g mol^{-1} (from the Stokes-Einstein relation). The molecular weights of the TEA and TMA cations are 130.2 g mol^{-1} and 74.1 g mol^{-1}, respectively. Within the limit of experimental errors and due to the asymmetric structure of the cations, these obey roughly this relationship between D and the MW. For example, for approximately the same water content of 77 wt% in cartilage, we expect that $D = 4.6\times10^{-10}$ m^2 s^{-1} for 0.15 M TMA (Fig. 2b), would correspond to $D = 3.4\times10^{-10}$ m^2 s^{-1} for 0.15 M TEA. This agrees favorably with the measurements which give $D = 3.0\times10^{-10}$ m^2 s^{-1}.

Compression, an intervention mimicking an event that occurs under physiological conditions, resulted in detectable changes in the diffusion coefficient of the cations. The diffusivity decreased with compression of the tissue, a condition in which only the interstitial fluid is lost, leading to an increased solid volume fraction. In general, the shape of the curves (Fig. 2a and b) tends to be exponential and compares favorably with graphs obtained in previous studies on compression effects on the diffusivity of water in cartilage. The change in solid volume fraction due to compression of the tissue is also in good agreement with the model of Mackie and Meares (Fig. 3), and one might imply the same as already done above in the discussion of concentration effects. The D values tend to agree with the fact that the steric hindrance of the extracellular matrix is an important determinant of the diffusivity of small solutes in cartilage (Maroudas 1970).

The water content of cartilage is reduced with an increase in the osmotic pressure. One therefore expects the D values of the TMA cation to be reduced with an increase in osmotic pressure, as the water content decreases. This observation holds for both TMA and TEA, as seen in Fig. 2a. This basic dependence of D on the water content of cartilage could also be of great relevance in medical and biological applications, considering that degenerated cartilage due to, for example, osteoarthritis, is characterized by an increased water content of cartilage (Burstein et al. 1993).

Strong evidence exists that the structure of the collagenous network in cartilage is connected with the pattern of restricted diffusion. The data found on the variation of diffusivity with the diffusion time suggest that for diffusion times of less than 10 ms the cartilage tissue is almost homogeneous with respect to the diffusion behavior of TMA cations (Fig. 4). This is not apparent for TEA cations, probably due to factors like shape or structure of the TEA action. Although changes in matrix density by compression led to a decrease in the observed D, the functional change in the measured diffusivity remained essentially unchanged. To obtain a very rough estimation of the diffusion distance corresponding to diffusion times (Fig. 5), Eq. 1 was used. In cartilage the potential barriers of diffusion include the glycosaminoglycan chains (spacing 3–4 nm) and the collagen fibrils (40–400 nm) (Byers et al. 1983). Therefore, using a diffusion time of 10 ms, these barriers are being encountered.

It is worth remarking that, just as is the case in this study, on the basis of direct obstruction effects, cartilage will retard the rate of movement of small molecules up to 50–60% of their migration rate in water (Burstein et al. 1993; Maroudas 1968; Maroudas and Evans 1974). Similar values have been obtained for the transport of Na^+, Cl^- and SO_4^{2-} across articular cartilage slices (Maroudas 1968; Maroudas and Evans 1974), where the fixed-charge concentration varies from 0.05 to 0.2 M. This indicates that the interaction of mobile ions with the proteoglycans of cartilage has little or no effect on their transport.

Several technical issues worthy of consideration should be briefly mentioned in the interpretation of the results of the NMR diffusion measurements. The measured data were fit to a model consisting of a single D for free solution data, with a linear relation (Eq. 2) between $\ln(\psi)$ and g^2. Two populations (compartments) of protons were noted for D in cartilage: a more intense one, associated with the protons of the cations, and a less intense one, which is most probably due to residual water protons moving in the interstices of the collagen fibrils (Knauss et al. 1996). From our compression experiments we also realized that the higher the compression (lower water content), the greater the tendency towards mono-exponential behavior. The minor component becomes increasingly less significant. This supports the conclusion that the minor component is due to water. Experiments conducted by some authors of this paper (Knauss et al. 1996, 1999) in the absence of cations also demonstrated multiple component behavior by diffusing water in cartilage. A biexponential fit was therefore appropriate. Otherwise, if two or more different populations of nuclei exist within the sample with different T_1 and T_2, the calculated D will be an average of the associated diffusion coefficients, weighted by the relative populations and their relaxation times.

We also have noticed that the NMR signal amplitude is dependent on inherent NMR relaxation phenomena described by the relaxation times T_1 and T_2. Although the loss of magnetization due to inherent NMR relaxation processes during the same period the diffusion is taking place does not directly influence the estimation of D (as the calculation is based on the ratio of the signal with and without the diffusion-sensitizing gradients), the relaxation times may affect which nuclei actually contribute to the estimation of D. In particular, by measuring the dependence of the apparent diffusion coefficient on the diffusion time with the stimulated-echo sequence, the time in which T_1 relaxation of the observed spins takes place is possibly varied. Measurements using different echo sequences to account for this, however, showed no variation of D with T_1 for the diffusion times used in this study.

In perspective, the possibility of applying very short field gradient pulses of large magnitude in PFG NMR enables measurements with very short diffusion times. The diffusion coefficients in cartilage could be measured down to values as low as $\Delta = 6$ ms. However, the exclusive use of the stimulated-echo sequence limits measurements at much lower diffusion times.

Conclusions

Different cation concentrations in cartilage result in different diffusion coefficients of the cations. Apart from possible binding or shielding effects of these cations respectively with or by the matrix-fixed charges, cation diffusion in cartilage shows essentially the same behavior in comparison to water diffusion in cartilage. The main difference is a change of the magnitude of the diffusion coefficients and the varying degrees at which the occurrence of these effects becomes evident. As in previous studies on water diffusion, the dependence of the diffusion coefficient of cations on the water content at short diffusion times ($\Delta \approx 15$ ms) may (together with D values obtained for water diffusion) be used for an estimation of the water content of an arbitrary sample. The PFG NMR results are in that case independent of structure and composition. This is valuable information, since the water content in cartilage plays an important role in many pathological processes.

With increasing compression the diffusion coefficient of the cation decreases, a result consistent with D being dependent on the composition and density of the solid tissue matrix or the water content. The results also lend support to the fact that the collagenous network of cartilage is mainly responsible for the observed restriction.

Finally, the main result obtained by using TEA in comparison to TMA is that there is a shift in magnitude to lower values for the diffusion coefficient. This is possibly due to the higher molecular weight of TEA. Apart from that, conclusions made for TMA essentially also hold for TEA.

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