Self-Diffusion of Water in Multicellular Spheroids Measured by Magnetic Resonance Microimaging

Michal Neeman, Kathryn J. Jarrett, Laurel O. Sillerud, and James P. Freyer

ABSTRACT

Nuclear magnetic resonance microimaging measurements of the self-diffusion coefficient of water in large (>2 mm) EMT-6 multicellular spheroids were performed in order to elucidate diffusion mechanisms in tumors. Pulsed gradient spin echo-imaging methods were developed for measuring diffusion in an intravoxel multicompartiment system. The self-diffusion coefficient (at 22°C) for water in the medium (Dm) consisted of only a single diffusion compartment [Dm = 1.99 ± 0.03 (SE) × 10⁻⁷ cm²/s]. Similarly, the spheroid necrotic center showed a single water diffusion compartment with a self-diffusion coefficient (D,) significantly lower than that of the medium (D. = 1.54 ± 0.05 × 10⁻⁷ cm²/s). The spheroid viable rim region showed two distinct compartments of approximately equal volume, one with a large diffusion coefficient (1.70 ± 0.12 × 10⁻⁷ cm²/s) and a second with a significantly smaller diffusion coefficient (0.25 ± 0.01 × 10⁻⁷ cm²/s). We propose that these two experimentally distinguishable compartments correspond to the extra- and intracellular regions, respectively, of the viable rim of the spheroid. Although the diffusion coefficients were significantly different in the medium, the necrotic center, and the viable rim, the activation energy for diffusion was the same in the three regions (0.20 eV). Studies of perfused spheroids at 37°C show the same dependence of the diffusion coefficients on the diffusion filter as observed for unperfused spheroids at 22°C. These results demonstrate the ability of nuclear magnetic resonance microimaging to investigate diffusion at the cellular level, which will lead to a better understanding of microenvironmental regulation in tumors.

INTRODUCTION

The NMR properties of tumor water need to be understood at a fundamental level in order to account for the contrast between tumors and surrounding tissue seen in NMR images of tumors in vivo (1–7). In addition, many kinetic and thermodynamic metabolic control mechanisms in tumors are influenced by the intra- and extracellular properties of water. Water properties also influence nutrient diffusion, which is a key control mechanism for cellular proliferation and viability (8–10).

One of the fundamental properties of water is its mobility, which is determined by the translational diffusion coefficient. PGSE NMR provides a very sensitive and direct measurement of the translational self-diffusion coefficient at steady state for a very broad spectrum of diffusion rates (11–23). In contrast to nuclear magnetic relaxation studies, in which interpretation of the data is complicated by many different relaxation and exchange mechanisms, the PGSE experiment yields pure translational motion information.

Recently, diffusion measurements have been performed in combination with NMR imaging to obtain “diffusion images” (23–31). Diffusion (along with perfusion) has proven to be an efficient generator of NMR image contrast and a very sensitive temperature marker (25–28). In fact, the resolution of the NMR microscope is thought to be limited by the diffusion of the species observed (32–36). Diffusion effects limit image resolution to between 1 and 10 μm as a result of signal loss, indirect line broadening, and movement out of the voxel (32–36). Pulse sequences that filter out signals from fast diffusing species may result in an increase in resolution, leaving signal to noise as the main practical limitation on resolution.

Multicellular spheroids (37) provide an ideal biological model system for studying water self-diffusion, as well as for studying contrast mechanisms in magnetic resonance imaging. EMT-6 spheroids can be readily cultured to >2 mm in diameter. At this size they contain a large necrotic center surrounded by a 250-μm thick layer of viable cells. Spheroids can be perfused in the NMR probe (38) to enable physiological studies to be carried out at 37°C, or they can be immersed in medium without perfusion at room temperature for 4–5 h without any significant effect on the morphology. We have previously shown (39) that significant contrast between the viable rim and the necrotic center can be detected by NMR microimaging. This contrast has been shown to be due to either T₂ or diffusion differences but not due to differences in the T₁ or spin density. In the work presented here, we show that water diffusion in spheroids is the primary contrast mechanism and that it is possible to devise a “slow diffusion filter” for NMR images which removes the signals from rapidly diffusing, extracellular water and leaves only signals from the more slowly diffusing, intracellular water. Using this filter, we were able to provide reliable measurements of the intracellular diffusion coefficient in tumor spheroids.

MATERIALS AND METHODS

Spheroid Culture. EMT-6 multicellular spheroids were cultured in a minimal essential medium supplemented with 10% bovine calf serum, as previously described (10, 40). Large spheroids of approximately 2 mm diameter were sized microscopically and placed in medium in a standard 5-mm NMR tube.

Single Spheroid Perfusion. Spheroids were perfused with medium at 37°C in a perfusion system designed to fit a 5-mm NMR tube. The spheroid was sized and inserted into a 2.4-mm internal diameter tube and allowed to settle on a porous support. Spheroids perfused under these conditions, at a rate of 2 ml/min, continued to grow at rates comparable to growth in a spinner flask.

Histology. Histological sections were obtained from all spheroids used for the NMR experiments. Spheroids were fixed in formalin immediately after measurement and imbedded in paraffin, and 5-μm thick sections were stained with eosin and hematoxylin as previously described (10).

NMR Imaging. NMR experiments were performed on a Bruker AM-400 wide bore spectrometer at 22°C and 37°C (41). Temperature was maintained using the Bruker VT1000 probe temperature controller and calibrated using a fiber optic temperature probe (Luxtron 1000B). NMR diffusion studies were performed using a spin warp-imaging
Fig. 1. The pulse gradient spin echo imaging sequence used for diffusion measurements. Details of the experimental parameters are given in the text.

version of the Stejskal-Tanner PGSE experiment (Fig. 1). The gradients were measured for all gradient values used using one-dimensional projections of a calibrated phantom. The gradient pulses were shaped with a preemphasis network to minimize the effect of eddy currents. A repetition delay of 3 s was used to avoid saturation. Selective excitation for slice selection was achieved using a sinc-shaped 90° pulse of 5 ms duration. The preemphasis unit in our system was satisfactory in minimizing the effect of eddy currents perpendicular to the main field (i.e., in the x and y directions). There was no observable effect on the free induction decay and signal line width measured 0.5 ms after a 10-ms, 12-G/cm gradient pulse. Thus, all diffusion measurements were performed using these gradients. The gradient parallel to the main field (z) was used only for slice selection. In all the measurements, the diffusion time (Δ) and the duration of the diffusion gradient (β) were held constant, at 24 and 4 ms, respectively. A variable diffusion filter was imposed by changing the duration (β) or the strength (g/β) of the frequency-encoding gradient. The echo time was constant at 68 ms, and the diffusion time for the frequency-encoding gradient pair (Ω) was

Fig. 2. Water diffusion in multicellular spheroids. Eight images (A–H) were obtained (22°C; ξ = 1.65) by incrementing the diffusion gradient in steps of 1.5 G/cm. A diffusion map (I and J) was obtained by fitting the data to Equation A ("Appendix").
Fig. 3. Diffusion images of EMT-6 spheroids showing three sets of diffusion images obtained at different slow diffusion filter (ξ) values. In each set gₓ was incremented from 0 to 8.3 G/cm in 8 steps as indicated. A, ξ = 0.25; B, ξ = 0.98; C, ξ = 6.9.

41.4 ms. The diffusion gradient (gₓ) was incremented from 0 to 9.45 G/cm in 8 steps.

All images had an in plane resolution between 20 and 50 µm, depending on the gradient strength used, and slice thickness of 0.5 mm. The width and position of the slice were determined by observing a “FLASH” (41) gradient echo image of an xy slice following presaturation of the xy slice.

Data Processing. Signal intensity was obtained from the images by averaging over a 100- x 100-µm square region of interest. Signal intensity dependence on gₓ was analyzed by a nonlinear least-square procedure using SAS software (SAS Institute Inc.) on a microVAX computer. Diffusion maps were obtained using IMAGE software (New Methods Research, Inc.).

Detection of Water Compartmentation. The imaging gradients impose a diffusion filter, whose strength can be described by a parameter, ξ, on the diffusion measurement. This parameter describes the attenuation factor for the diffusion of free water due to the imaging gradients alone. For example, a diffusion filter value of ξ = 5 implies that the free water signal is attenuated to e^−5. In a single-compartment system, this parameter would affect the signal intensity but would have no effect on the measured diffusion coefficient, and therefore the measured diffusion coefficient would be the same for all ξ values. In a multicompartment system, on the other hand, the diffusion coefficient would change as a function of ξ, starting at some weighted average at low ξ values and asymptotically approaching the lower diffusion coefficient at high ξ values. The analytical techniques used are described in the “Appendix.”

RESULTS

NMR images of spheroids immersed in medium at 22°C using frequency-encoding gradients of 9.52 G/cm (ξ = 1.97) and 8 diffusion gradient values incremented from 0 to 8.33 G/cm showed significant diffusion-dependent contrast between the rim of the spheroid and the necrotic center (Fig. 2, A–H). Analysis of the signal decay as a function of gₓ using a single-compartment model (“Appendix,” Equation A) yielded the reconstructed diffusion map (Fig. 2, I and J). The average diffusion coefficients were 1.89 ± 0.06 (SEM) for the medium, 1.41 ± 0.06 for the necrotic center, and 0.60 ± 0.04 for the spheroid rim.

The temperature dependence of the diffusion coefficients was measured in three spheroids, between 10°C and 37°C, in steps of 5°C, and was used for calculating the activation energy for diffusion. The values we obtained were 0.20 ± 0.01 eV for the medium, 0.21 ± 0.02 eV for the spheroid rim, and 0.20 ± 0.03 eV for the necrotic center. Since the activation energy was essentially identical for all regions, studies of unperfused spheroids could be performed at room temperature (22°C), at which the spheroid can survive a few hours without significant damage.

By increasing either the amplitude or duration of the frequency-encoding gradient (gₓ), which increases the ξ value, we could increase the contrast between these three zones (Fig. 3). Note that at low ξ values there was little contrast between the viable rim and the necrotic center and fits to Equation A (“Appendix”) gave similar apparent diffusion coefficients. The water line width (10–15 Hz) was in all cases at least 4-fold smaller than the frequency spread across a pixel, and the pixels were 2- to 4-fold smaller than the width of the rim. Therefore,
the loss of contrast at low $\xi$ values was not due to poor resolution. A total of 8 spheroids were measured with 4-6 different $\xi$ values for each spheroid (Fig. 4). As expected, the diffusion coefficient of the medium ($D_m$) remained the same for all $\xi$ values at $D_m = 1.99 \pm 0.03 \times 10^{-5}$ cm$^2$/s ($n = 39$). Similarly, the diffusion coefficient of water in the necrotic center [$D_c = 1.54 \pm 0.05 \times 10^{-5}$ cm$^2$/s; $n = 39$] was independent of the $\xi$ value. The diffusivity for water in the necrotic center was significantly lower than that found for the medium ($P < 0.0001$), a result which we believe may be due to the high content of cell debris in the necrotic center.

The diffusion coefficient of water in the viable rim, on the other hand, was highly dependent on the $\xi$ value (Fig. 4), implying that water was segregated into at least two compartments with different diffusion coefficients ("Appendix"). At low $\xi$ values a diffusion coefficient of $1.35 \pm 0.01 \times 10^{-5}$ cm$^2$/s was found, while at high $\xi$ the diffusion coefficient stabilized at a value of $0.24 \pm 0.02 \times 10^{-5}$ cm$^2$/s. These data were fitted to the two-compartment model ("Appendix," Equation C). The convergence for the slow (intracellular) diffusion coefficient and for the fraction of intracellular volume was good even when the data for each spheroid were analyzed separately. The diffusion coefficient for the fast (extracellular) compartment converged only when all the data were grouped together (Fig. 5; Table 1).

Based on the results from this fit, we suggest that the rim region contains two distinct compartments. In one the diffusion coefficient was approximately $0.25 \times 10^{-5}$ cm$^2$/s, while in the other the diffusion coefficient was close to that of the necrotic center, $1.70 \times 10^{-5}$ cm$^2$/s (Fig. 5; Table 1). We believe that the slowly diffusing compartment represents intracellular water, while the fast diffusing compartment is due to extracellular water. The fraction of intracellular volume, as determined by the best fit procedure, was $0.45 \pm 0.03$. A summary of the diffusion data from the rim of EMT-6 spheroids is given in Table 1.

An additional zone was observed (Fig. 6) between the viable rim and the center of the necrotic portion at low and intermediate $\xi$ values ($\xi = 0.95-3.14$). This intermediate zone appeared to have two diffusion compartments. A fit of data from the intermediate zone of all 8 spheroids suggested that this zone contained a compartment in which water diffusion resembles that found in the necrotic center, with $D = 1.7 \times 10^{-5}$ cm$^2$/s, and an additional compartment with a lower diffusion coefficient. Setting this lower diffusion coefficient to the intracellular diffusion coefficient found in the rim ($0.25 \times 10^{-5}$ cm$^2$/s) enabled us to estimate that 12% of the volume in this region had restricted diffusion. Comparison of NMR images and histological sections of the same spheroid, for 7 spheroids, shows that there was also a morphologically distinct intermediate zone in the spheroid which corresponded closely with that...
Table 1  Two-compartment diffusion data for the viable rim of EMT-6 multicellular spheroids (22°C)

<table>
<thead>
<tr>
<th>Experiment</th>
<th>$D_m$</th>
<th>$D_w$</th>
<th>$A_m$</th>
<th>$A_w$</th>
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</thead>
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<td>2.2*</td>
<td>0.50 ± 0.08</td>
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</tr>
<tr>
<td>2</td>
<td>0.43 ± 0.06</td>
<td>2.2*</td>
<td>0.62 ± 0.10</td>
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<tr>
<td>3</td>
<td>0.27 ± 0.03</td>
<td>1.9 ± 0.09</td>
<td>0.33 ± 0.03</td>
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<tr>
<td>4</td>
<td>0.36 ± 0.02</td>
<td>2.2*</td>
<td>0.58 ± 0.02</td>
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</tr>
<tr>
<td>5</td>
<td>0.17 ± 0.04</td>
<td>2.2*</td>
<td>0.37 ± 0.16</td>
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</tr>
<tr>
<td>6</td>
<td>0.47 ± 0.05</td>
<td>2.2*</td>
<td>0.62 ± 0.06</td>
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</tr>
<tr>
<td>7</td>
<td>0.41 ± 0.06</td>
<td>1.9 ± 0.10</td>
<td>0.69 ± 0.17</td>
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</tr>
<tr>
<td>8</td>
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<td>1.85 ± 0.06</td>
<td>0.37 ± 0.10</td>
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</tr>
<tr>
<td>Average</td>
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<td>1.9 ± 0.10</td>
<td>0.51 ± 0.12</td>
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<tr>
<td>All</td>
<td>0.25 ± 0.01</td>
<td>1.70 ± 0.12</td>
<td>0.45 ± 0.03</td>
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</tr>
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</table>

* Estimated value (± the asymptotic SE) for the intracellular ($D_m$) and extracellular ($D_w$) diffusion coefficients $10^{-5} \text{cm}^2/\text{s}$ and for the apparent fraction of intracellular volume (not corrected for $T_2$ effects). Each experiment represents diffusion data obtained from a single sphere at 4–6 different diffusion filter (ξ) values.
* Upper limit imposed on the fit.
* Average of the results obtained from experiments 1–8 (±SD).
* Estimated values obtained from fitting all the data, combined from experiments 1–8, to the two-compartment model (± the asymptotic SE).

![Fig. 6. NMR image and histological section showing the intermediate zone in an EMT-6 multicellular spheroid. The NMR image (left) was obtained at 22°C, with ξ = 3.14 and no diffusion gradient. Histological section was taken through the center of the same spheroid (right).](image)

observed in the NMR images. Histological sections show no intact cells in that region but do show pyknotic nuclei, dispersed in dense cytoplasmic cell fragments, that could account for the 12% restricted diffusion.

The results presented up to this point were obtained at a temperature of 22°C, at which the spheroids survived without damage, and were obtained over times up to 4 h without flowing perfusate. In order to obtain data for the spheroids at physiological temperature (37°C), we repeated the experiments using a perfusion system which held a single spheroid stationary during the data acquisition.

Studies of water diffusion at 37°C in perfused stationary spheroids showed that the dependence of the diffusion coefficient at the rim on the diffusion filter and the dependence of the contrast on the applied gradients were similar at both temperatures. However, because of the faster diffusion at 37°C, the same contrast patterns appear at lower gradient values. At ξ = 0.65 the diffusion coefficient of the water in the spheroid rim was $0.67 \pm 0.06 \times 10^{-5} \text{cm}^2/\text{s}$, while at a higher diffusion filter value (ξ = 4.08), the diffusion coefficient of water in the rim was found to be $0.27 \pm 0.03 \times 10^{-5} \text{cm}^2/\text{s}$. As observed for the unperfused spheroids, diffusion of water in the necrotic center was not dependent on the diffusion filter ($1.73 \pm 0.10 \times 10^{-5} \text{cm}^2/\text{s}$ at ξ = 0.65 and $1.79 \pm 0.07 \times 10^{-5} \text{cm}^2/\text{s}$ at ξ = 4.08). The diffusion coefficient for the medium could not be measured accurately because of the bulk medium flow.

**DISCUSSION**

NMR microimages obtained from EMT-6 multicellular spheroids showed a diffusion-dependent contrast between the viable rim and the necrotic center. Diffusion measurements, using an imaging variation of the PGSE experiment, were used to obtain the activation energy for water diffusion in the medium and in different regions in the spheroid. We found no significant differences among the activation energies for water diffusion in the different regions, and the values we obtained agree with literature values for the energy of breaking two hydrogen bonds (26, 43). As previously reported, this enables diffusion measurements to be used reliably for temperature mapping (26). The similar temperature dependence implies that studies performed here at 22°C could be applied also at 37°C. Indeed, studies of perfused spheroids at 37°C show a behavior of the diffusion coefficients very similar to that seen at 22°C.

We have previously shown that the high imaging gradients used in NMR microscopy impose a “diffusion filter” on the images (41). We have used this effect for differentiating between diffusion in a single compartment and in a two-compartment region. In the static spheroid system the medium surrounding the spheroid served as an internal reference for single-compartment diffusion. Water diffusion in the necrotic center of the spheroid also showed single-compartment diffusion, but the diffusion rate was significantly reduced relative to that in the medium. Since histological sections show no intact cells in this zone, and the diffusion measurements showed no compartmentation, we assume that the lower diffusivity for water is due to a high content of proteins and membrane fragments. The composition of the necrotic region is not known: histological sections show that this is not an empty hole but rather contains dense, uniform material that stains pale red with eosin (39), indicating that it consists primarily of proteins rather than nucleic acids. The presence of proteins and membrane fragments may cause partial structuring and binding of water in the necrotic center and thus reduce the average diffusion rate, as reported previously for the diffusion of water in gelatin and in protein solutions (23). The exact diffusion rate in necrotic regions would depend on the structure and density of these extracellular matrix proteins and may differ between different tumors.

Water diffusion in the viable rim region of the spheroid showed a distinctive two-compartment behavior. Analysis of the diffusion measurements performed at different diffusion filter (ξ) values revealed the existence of one compartment with a diffusion coefficient close to that of the necrotic center, probably corresponding to interstitial extracellular water, and another compartment with a diffusion coefficient of $0.25 \times 10^{-5} \text{cm}^2/\text{s}$, probably corresponding to intracellular water.

Susceptibility and background gradients could affect the accuracy of the diffusion measurements. Analysis of the susceptibility effects in concentric spheroids predicts large field gradients at the outer layers of the spheroid and at the water outside the spheroid (44). Similar to the effect of the imaging gradients, such background gradients would have cross-terms with the diffusion gradient, leading to enhanced coherence loss.
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and to overestimated diffusion coefficients (41). The fact that the lowest diffusion rate was found at the spheroid rim implies that susceptibility effects were not significant in our case.

An estimate of the self-diffusion coefficient of intracellular water can be obtained directly from the high £ diffusion experiments. The value we found of 0.24 x 10^{-9} cm^2/s (Table 1) corresponds to a mean square displacement of 3.5 ¡m. This value is substantially lower than previously reported for tissues and cell suspensions (11-17) but agrees well with a recent spectroscopic study of water self-diffusion in perfused single MCF-7 cells embedded in agarose filaments (19). The diffusion time in our measurements was 24 ms, which is substantially shorter than most previous studies (11-17). Thus, the lower diffusion coefficient is not due to diffusion restriction. Averaging of intra- and extracellular signals was probably a main cause for the higher diffusion coefficients reported earlier. The ability to selectively observe the slowly diffusing, intracellular compartment could possibly improve the achievable resolution of NMR microscopes, since diffusion is thought to limit resolution in NMR microscopy (32-36).

The extracellular water in the rim region diffuses at a rate comparable to that found for the necrotic center and significantly slower than free diffusion of water in the medium. Since it is the viscosity, tortuosity, and extracellular matrix proteins of the extracellular space that determine nutrient diffusion into the spheroids and tumors, it would be interesting to study nutrient diffusion relative to consumption and determine the limiting factors associated with the onset of necrosis. Unfortunately, our results for the extracellular diffusion coefficient were not as accurate as for the other compartments because of problems in convergence for this parameter in the fitting procedure.

The fraction of extracellular space in the viable rim region, estimated from the two-compartment model, was 45% (Table 1). This fraction agrees well with previous, non-NMR measurements of the extracellular volume (9). Calculations of the fraction of extracellular space were done assuming the same $T_2$ relaxation time for intra- and extracellular water. The similarity between the NMR and non-NMR results suggests that the $T_2$ differences between extra- and intracellular water in the rim region may not be very large.

For intermediate diffusion filter values, we could clearly observe an intermediate zone between the viable rim and the necrotic center of the spheroids. Similar intermediate zones were observed previously in studies of the response of spheroids to radiation (45) and to Adriamycin (46) treatments. We currently do not know whether the intermediate zones reported in those studies correlate with one another and with our results. Our diffusion measurements suggest that this region contains a small fraction (12%) of intact diffusion barriers. Histological sections performed on the spheroids used for microimaging show, in the intermediate region, pyknotic nuclei dispersed in dense cytoplasmic cell fragments. The slow diffusion component in this region may represent water confined within these nuclei.

The unique sensitivity of NMR to kinetic properties such as diffusion, perfusion, and chemical exchange, combined with the nondestructive nature of NMR, make it a very appealing tool for biological studies. In fact, these are the areas in which NMR microscopy may be most useful, in spite of its inherently low resolution and sensitivity relative to other microscopy techniques. In this work we have shown that self-diffusion measurements are sensitive to cellular integrity and packing. Using NMR microscopy we were able to probe the self-diffusion coefficients of water in the necrotic center, the cells in the viable rim, and the extracellular space in the rim of EMT-6 spheroids. An additional zone has been observed between the viable rim and the center of the spheroid which contains a small fraction of diffusion-restricted water. With improvements in sensitivity and by combining these measurements with specific chemical selection (50), it will be possible to map diffusion and compartmentation of specific metabolites and catabolites.

APPENDIX

The spin echo pulse sequence used for diffusion studies (Fig. 1) includes a pair of diffusion gradients ($g_0$), applied along the direction of frequency encoding, of duration $\delta$ and delay $\Delta$ between their leading edges. Slice selection was performed using a 5-ms sinc-shaped 90° excitation pulse. The dependence of the signal intensity on $g_0$ is given by Equation A.

$$S(g_0) = \frac{S(0)}{S(0) e^{D_D \Delta}} e^{D_D \Delta}$$

Where

$$a = \gamma^2 g_0^2 \left[ \Omega - \delta \left( \frac{\delta}{3} \right) 2 g_0 g_0 g_0 \right]$$
$$b = \gamma^2 g_0^2 \left[ \Omega - \delta \left( \frac{\delta}{3} \right) \gamma^2 g_0^2 \alpha^3 \right]$$

Here $g_0$ is the slice selection gradient applied for duration $\alpha$, $g_0$ is the frequency-encoding gradient, applied for duration $\beta$ with a delay of $\Omega$ between the leading edges, and $\gamma$ is the gyromagnetic ratio of the proton.

The first term of $a$ is the Stejskal-Tanner relation for a normal PGSE experiment. The second term is a cross-term (41) between the diffusion gradient ($g_0$) and the imaging gradient applied in the same direction ($g_0$). The first and second terms in $b$ are the signal attenuation caused by the frequency-encoding and slice selection gradients, respectively. Signal attenuation due to the phase-encoding gradient was neglected in this treatment because this gradient is small and of short duration relative to the others (31). As can be seen in Equation A, the imaging gradients ($g_0$ and $g_0$) impose a diffusion filter on the experiment even without applying the additional diffusion gradient, $g_0$. A slow diffusion filter parameter ($\xi$) was defined as the expected attenuation in signal intensity of free water, due to the imaging gradients alone:

$$\xi = D_0 b$$

where $D_0$ is the self-diffusion coefficient of water at 22°C (2.2 x 10^{-5} cm^2/s). The slow diffusion filter represents the reduction (due to diffusion) of the free water signal to $e^{-\xi}$. The effect of $\xi$ is to filter out signals from rapidly diffusing species, such as free water, while retaining signals from slowly diffusing species.

In a single-compartment system, signal decay is independent of this diffusion filter, and of $T_1$ relaxation; therefore, the derived diffusion coefficient should not depend on the strength and duration of the imaging gradients or on the echo time (TE). The influence of the diffusion filter becomes significant in complex systems in which a number of water compartments exist within a voxel. Signal intensity in a simple two-compartment system is expected to depend on $g_0$ according to the following relation:
The apparent relative fraction of each compartment ($R'$) would be weighted by the T2 relaxation (Equation A). As long as all diffusion measurements are performed with the same value of TE, different $T_2$ values for the two compartments would not affect the determination of the diffusion coefficients for the two compartments. The apparent relative fraction of each compartment ($R'$) would be weighted by the $T_2$ relaxation (Equation D), and separate $T_2$ measurements are necessary in order to obtain the true ratio ($R$).

$$S(g_d) = \frac{A_0 e^{-TE/T_2\sigma^2}}{S(0)}$$

where $a$ and $b$ are as defined in Equation A, and $A$ and $B$ label the two separate compartments.

The signal attenuation $S(g_d)/S(0)$ as a function of $g_d$ and $\xi$ differs between single- and two-compartment systems. Simulation of a two-compartment model predicts a significant dependence of the diffusion coefficient on $\xi$ in such a system when analyzed using a single-compartment model (Equation A), while in a true single-compartment system the diffusion coefficient should be independent of $\xi$. In the case of a two-compartment system, the data can be analyzed using Equation C to obtain the two diffusion coefficients and the relative fraction ($R$) of each compartment (Equation D). As long as all diffusion measurements are performed with the same value of TE, different $T_2$ values for the two compartments would not affect the determination of the diffusion coefficients for the two compartments. The apparent relative fraction of each compartment ($R'$) would be weighted by the $T_2$ relaxation (Equation D), and separate $T_2$ measurements are necessary in order to obtain the true ratio ($R$).

$$R' = \frac{A_0 e^{-TE/T_2a}}{A_0 e^{-TE/T_2a} + B_0 e^{-TE/T_2b}}$$

$$R = \frac{A_0}{A_0 + B_0}$$

where $A_0$ and $B_0$ are the spin densities for the two compartments and $T_{2a}$ and $T_{2b}$ are their transverse relaxation times.

The problems of quantitative $T_2$ measurements from NMR images are well documented (47-49). Experimental separation and measurement of $T_2$ from two different intravoxel diffusion compartments is even more complex. The effect of $T_2$ can be reduced by using higher gradients and shorter echo times. In this work the fractional volumes reported were not corrected for $T_2$ differences.

Water exchange between the intra- and extracellular compartments during the diffusion time may also contribute to an error in the analysis. Diffusion restriction and exchange across diffusion barriers can be measured by observing the dependence of the apparent diffusion coefficient on the diffusion time $A$ (11, 12, 14–18, 20). In this work the diffusion times $A$ and $\Omega$ were kept constant and relatively small so as to minimize the effects of permeability and water exchange between the compartments (11, 12, 14, 16) and the effect of non-Brownian diffusion (17).

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