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Overhauser Dynamic Nuclear Polarization To Study Local Water Dynamics

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Abstract: Surface and internal water dynamics of molecules and soft matter are of great relevance to their structure and function, yet the experimental determination under ambient and steady-state conditions is challenging. One of the most powerful approaches to measure local water dynamics within 5 Å distances is to utilize the modulation of the nuclear spin relaxation rate of water protons through their time-dependent dipolar coupling to paramagnetic probes, here nitroxide spin labels. We recently introduced a method to obtain local water dynamics through Overhauser dynamic nuclear polarization (DNP). This has a unique advantage over other related techniques available in that a highly amplified proton nuclear magnetic resonance signal carries the information, allowing the use of minute microliter sample volumes and 100 μM sample concentrations. The outcome of our approach is the quantitative determination of the key DNP parameter known as the coupling factor, which provides local translational diffusion dynamics of the solvent within 5 Å of the spin label. In contrast to recent reports that the coupling factor for nitroxide radicals cannot be quantified due to the difficulty in determining the saturation factor for the spin label, we show the saturation factor can be accurately determined and for the first time present agreement between measurements and theory. We discuss the discrepancy between the related field cycling relaxometery technique and DNP in determining the coupling factor and present arguments in support of the DNP-determined value. DNP measurements of local hydration dynamics around nitroxides in bulk water and on the surface of proteins are presented.

Introduction

Surface and internal water dynamics play an important role in the function and structure of many biological systems. Protein folding and protein aggregation into amyloid fibers are driven or at least accompanied by the exclusion of solvent water.[1−4] The surface hydration dynamics of lipid bilayer systems are relevant for their binding to biomolecules, and the local diffusion coefficient and permeation properties of water inside lipid membranes provide direct insight into the transport mechanism of water as well as the relationship between active transport through membrane proteins and the membrane's passive water permeability.[5,6] However, the experimental analysis of local water dynamics is very difficult, especially under ambient and steady-state conditions, because the bulk and surface or internal water do not have distinct spectroscopic signatures. Recently, we have introduced Overhauser dynamic nuclear polarization (DNP) to study local water dynamics inside the hydrophobic bilayers of vesicles and micelles utilizing site-specific nitroxide spin labels, with the spin-labeled surfactants composing only 1−4% of the entire molecular assembly.[7] The Overhauser effect takes advantage of the strong, time-dependent, dipolar coupling between unpaired electrons and the solvent nuclei (here the ^{1}H nuclei of water) to enhance the nuclear magnetic resonance (NMR) signal through polarization transfer from the electron spin of a spin label to ^{1}H nuclear spins via cross-relaxation mechanisms. This dipolar coupling is short-range, with 80% of the ^{1}H spin relaxation coming within 5 Å of the unpaired electron.[8] The efficiency of polarization transfer is strongly dependent upon the magnetic field and the dynamics between the two spin-bearing molecules.[9−11] As the signal enhancement originates only from solvent molecules near the spin label, DNP has the ability to characterize water dynamics and accessibility on the surface of proteins and macromolecular assemblies when functionalized and localized spin labels are employed. However, measurements of the coupling factor (ρ), which contains the dynamic information, are complicated by the difficulty in determining the saturation factor, with a recent paper claiming

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it cannot be determined using the DNP approach.[12] For the first time, we predict the maximum possible saturation factor using a theory including Heisenberg spin exchange interactions and compare the theory to our experimental observations of the saturation factor. We conclude the saturation factor can be accurately determined and thus the coupling factor accurately measured. We use the definition of ρ, which is a function of relaxation rates involving the electron and nuclear spins, to extract the translational dynamics between the spin label and solvent molecules. We also show an application of our DNP approach to measure the hydration dynamics on the surface of an unfolded, singly labeled protein, tau187, a protein fragment truncated between residues 255 and 441 of the longest human tau isoform containing all four microtubule binding repeat regions.[13]

A related technique to study solvent dynamics near spin-labeled molecules is field cycling relaxometry (FCR). The presence of the unpaired electron enhances the T_1 relaxation rate of the solvent nuclei, again modulated by the time-dependent dipolar coupling between the two spins. Measuring T_1 over a wide range of fields gives the frequency dependence of the spectral density function, $J(\omega)$, and by applying the appropriate model for $J(\omega)$, translational dynamic information can be obtained and thus the diffusion of water (within ~5 Å) on the surface of proteins and lipid bilayers studied.[14−16] One drawback to the FCR method when employing nitroxide spin labels is that a high concentration (several millimolar) of spin-labeled molecules is required to achieve significant modulation of the ^{1}H T_1 relaxation rate. This can be difficult or impossible for many proteins due to aggregation or low solubility and can pose questions about maintaining the native structure of molecular assemblies such as vesicles when a large number of spin labels need to be employed. DNP, on the other hand, can be performed on spin label concentrations of ~100 μM and on sample sizes of only a few microliters, making it ideal for slightly soluble and expensive proteins as well as other biomolecular systems. This sensitivity comparison, however, is only valid when using site-directed spin labeling of biomolecular systems with nitroxide radicals. FCR can employ much stronger paramagnetic probes (including Gd, Fe, etc.) that require small concentrations as well as access different types of surface interactions, while DNP is only applicable to sufficiently long-lived paramagnetic probes such as nitroxide spin labels.

In this study, both DNP and FCR are employed to determine the coupling factor and translational correlation time given by[17]

$$\tau = \frac{d^2}{D_I + D_S} \tag{1}$$

where d is the distance of closest approach of the two spins, D_I is the diffusion coefficient of the solvent molecules, and D_S is the diffusion coefficient of the electron-spin-bearing molecules.

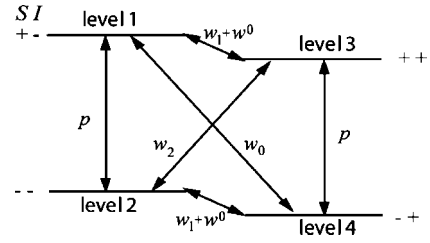

Figure 1. Four-level energy diagram appropriate for a coupled proton and electron. w_2, w_0, and w_1 are the dipolar contributions to the relaxation rate, while w^0 and p are the nuclear spin and electron spin relaxation rates in the absence of dipolar coupling and electron spin exchange.

The key parameter of interest when determining water dynamics on the surface of and inside biomolecular or other soft matter systems is the water diffusion coefficient. The details of how each technique determines ρ, τ, d, and D_I will be discussed in the Theoretical Section. The result of our data analysis, however, was that DNP and FCR experiments on small nitroxide molecules dissolved in water show large discrepancies for ρ and τ. We conclude with arguments in support of the DNP-determined values for ρ and τ.

Theoretical Section

Here we will outline how both DNP and FCR can be used to determine dynamic properties of a spin label and solvent. It will be assumed throughout this paper that the spin label is one of the commonly used nitroxide-based free radicals and the solvent is water. The theory of Overhauser-enhanced DNP is outlined in several sources, so we only mention here what is necessary for the present discussion.[9,10,18,19] The NMR signal enhancement, E, upon irradiation of the electron spin resonance (ESR) transition is given by[10]

$$E = 1 - \rho f s \frac{|\gamma_S|}{\gamma_I} \tag{2}$$

where ρ is the coupling factor describing the efficiency of the dipolar coupling between the two spins, f is the leakage factor which corrects for nuclear spin relaxation caused through other mechanisms than the time-dependent dipolar field of the unpaired electron and differences in electron spin concentration, s is the saturation factor describing the degree to which the electron spin populations have been equilibrated, and γ_S and γ_I are the magnetogyric ratios of the electron and proton, respectively. Of these, f is most easily determined; $f = 1 - T_1/T_{10}$, where T_1 is the ^{1}H relaxation time in the presence of the free radical and T_{10} the ^{1}H relaxation time in the absence of dissolved radical. Figure 1 shows the standard four-level diagram describing the two coupled spins. Here, w_0, w_1, and w_2 represent the dipolar contribution to the nuclear spin relaxation rate, while w^0 and p are the nuclear spin ($1/T_{10}$) and electron spin ($1/T_{1e}$) relaxation rates if there is no coupling between them and no electron spin exchange. The coupling factor and leakage factor defined through expressions involving relaxation rates are $\rho = (w_2 - w_0)/(w_0 + 2w_1 + w_2)$ and $f = (w_0 + 2w_1 + w_2)/(w_0 + 2w_1 + w_2 + w^0)$.

The saturation factor, s, is a function of the radiation power driving the electron spin transition, p. The hyperfine coupling

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of the unpaired electron to the ^{14}N nuclei of the nitroxide splits the electron spin transition, creating the three-line ESR spectrum. The difficulty in determining s arises because the three different transitions are not independent of one another. Therefore, creating a nonequilibrium population of one transition by (partial) saturation at the resonance frequency creates a non-equilibrium population of electron spins in the two other transitions. Bates and Drozdoski first recognized that electron spin exchange would result in a saturation factor which was concentration-dependent since the electron spin exchange rate for nitroxides increases linearly with radical concentration.[18,20] Furthermore, Armstrong and Han demonstrated that rapid ^{14}N nuclear spin relaxation would have a similar effect on DNP by mixing the three hyperfine states, which is modulated by the rotational tumbling rate of the electron-spin-bearing molecule, but is independent of the spin label concentration.[9]

To account for discrepancies in the applied radiation power exciting the electron spin transition, E can be measured at various powers and the enhancement factor extrapolated to infinite power:[9]

$$E_{\max} = E(P \rightarrow \infty) = 1 - \frac{\omega_S}{\omega_I}\rho f s_{\max} \tag{3}$$

with

$$s_{\max} = \frac{1}{3}\left[\frac{(2 + w_N/p + 6\kappa' C/p)(2 + 3w_N/p + 6\kappa' C/p)}{4 + (w_N/p + 2\kappa' C/p)(w_N/p + 6\kappa' C/p) + 2(3w_N/p + 8\kappa' C/p)}\right] \tag{4}$$

where w_N is the ^{14}N nuclear spin relaxation rate, κ' is the electron spin Heisenberg exchange rate, C is the nitroxide concentration, and ω_S and ω_I are the frequencies at which the electron and ^{1}H nuclear spins are being irradiated, respectively. While $s_{\max}$ is a complicated function, it can be seen that at high radical concentrations $s_{\max}(C \rightarrow \infty) = 1$ regardless of the value for w_N/p. Also, f will also approach 1 at high concentrations. Thus, we have

$$E_{\max}(C \rightarrow \infty) = 1 - \rho\frac{\omega_S}{\omega_I} \tag{5}$$

This implies if $E_{\max}$ is measured as a function of concentration, the results can be extrapolated to infinite concentration to quantitatively determine ρ. In the simple case of a nitroxide free in solution, we have previously found that f approaches 1 at concentrations around 15−20 mM.[9] We also demonstrate in the Results that $s_{\max}$ nears 1 at 15 mM.

For spin-labeled biomolecules, it may not be possible to achieve the large concentrations needed for $s_{\max}$ to approach 1; however, the term w_N/p in eq 4 is large for a wide range of rotational correlation times corresponding to tumbling of species that are larger than the small nitroxide molecules, e.g., proteins, micelles, or vesicles, making $s_{\max}$ approximately 1 for many biological systems.[7,21] In such a dilute electron spin system, f will not approach 1, which will reduce the extrapolated enhancement factor, but one can quantitatively determine ρ upon measurement of f and determination of s.

Once the coupling factor has been determined from a DNP experiment, one has to employ the appropriate model governing the dynamic parameters of the solvent molecule interacting with the spin labels to obtain τ. In the case of nitroxide radicals dissolved in water, the coupling between the ^{1}H spin and electron spin has been shown to be almost exclusively dipolar.[19,22] Also, the electron spin relaxation times, T_{1e} and T_{2e}, are long compared to τ (eq 1) and thus can be ignored. If τ is the only relevant time scale, a single spectral density function is sufficient in describing the interaction between the two spins.[14,17,23] FCR data from other studies[12,14,23] including the data presented in this paper (see the Results) show little evidence for a rotational component to the relaxation, implying that translational diffusion predominantly modulates the dipolar coupling between the two spins. With these assumptions, ρ is given by[10]

$$\rho = \frac{6J(\omega_S + \omega_I, \tau) - J(\omega_S - \omega_I, \tau)}{6J(\omega_I + \omega_S, \tau) + 3J(\omega_I, \tau) + J(\omega_S - \omega_I, \tau)} \tag{6}$$

Once ρ has been measured via the DNP technique, τ can be determined if the form of the spectral density function is known. In general, this may not be a trivial task, although FCR measurements can provide the shape of the spectral density function. For the case of nitroxide spin labels and water, the hard-sphere, force-free model developed by Hwang and Freed[17,24] has been shown to give a good fit to the FCR data.[12,14,16] In this case the spectral density function is given by

$$J(\omega, \tau) = \frac{1 + \frac{5\sqrt{2}}{8}(\omega\tau)^{1/2} + \frac{\omega\tau}{4}}{1 + (2\omega\tau)^{1/2} + (2\omega\tau) + \frac{\sqrt{2}}{3}(\omega\tau)^{3/2} + \frac{16}{81}(\omega\tau)^2 + \frac{4\sqrt{2}}{81}(\omega\tau)^{5/2} + \frac{(\omega\tau)^3}{81}} \tag{7}$$

In this manner τ can be found, and if d in eq 1 can be estimated or determined in combination with another experiment, local solvent diffusion coefficients can be determined with our DNP approach.

Utilizing the same basic principle, an FCR experiment can also find ρ and τ, the validity of which also relies on the model used for the spectral density function. Again, employing the often used[25] hard-sphere, force-free model, the ^{1}H relaxation rate is given by[14]

$$\frac{1}{T_1} = \frac{1}{T_{10}(\omega)} + \frac{32\pi}{405}\gamma_I^2\gamma_S^2\hbar^2 S(S+1)\frac{N_A}{1000}C\frac{\tau}{d^3}(6J(\omega_S + \omega_I, \tau) + 3J(\omega_I, \tau) + J(\omega_S - \omega_I, \tau)) \tag{8}$$

where S is the spin of the electron (1/2 for nitroxide radicals), N_A is Avogadro's number, and all other variables have been previously defined. The parameters τ and d are the fit parameters in an FCR experiment, so the sum of local diffusion coefficients, D, can be determined using eq 1. The fit value for τ can also be inserted into eq 6 to determine ρ.

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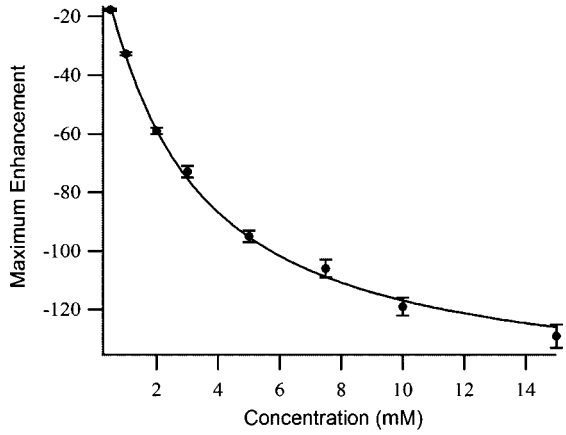

Figure 2. Measured maximum ^{1}H NMR enhancement versus nitroxide concentration. The solid curve is the fit to eq 3 with s_{max} given by eq 10.

Alternatively, since an FCR experiment typically samples very low magnetic fields where the extreme narrowing condition holds (w_0:2w_1:w_2 = 1:3:6), the dipolar ^{1}H nuclear transition rate, w_1, can be determined from the low-field limit of the FCR curve. The coupling factor can then be calculated with the following equation:[10]

$$\rho \cong \frac{5}{7}\left[1 - \frac{2w_1}{1/T_1 - 1/T_{10}}\right] \tag{9}$$

where it is assumed that w_1 is field-independent in the range being studied, $\omega_S \gg \omega_I$, and the coupling is purely dipolar. Even though the spectral density function does not appear explicitly in eq 9, the same assumptions used in eq 6 must also hold for eq 9 to be valid.

Both DNP and FCR techniques rely on the dipolar coupling between the unpaired electrons and solvent protons, which is modulated by the dynamics between the two. Therefore, one should be able to employ either technique to determine parameters such as ρ or τ with the same result.

Results

DNP and FCR experiments were performed with the radical 4-oxo-2,2,6,6-tetramethyl-1-piperidinyloxy (4-oxo-TEMPO) dissolved in water at room temperature. In DNP experiments, the applied power driving the electron spin transition was varied and the ^{1}H NMR signal enhancements were measured for several different radical concentrations. The magnetic field was approximately 0.35 T (~9.8 GHz electron frequency, 14.85 MHz NMR frequency). For each sample, the maximum possible enhancement was determined using eq 11 (see the Materials and Methods) and the T_1 time measured to determine f. A plot of E_{max} vs C is shown in Figure 2 along with a fit to eq 3, assuming w_N/p to be 0, where eq 4 reduces to

$$s_{max} = \frac{1}{3}\left(\frac{1 + 3\kappa' C/p}{1 + \kappa' C/p}\right) \tag{10}$$

This assumption is shown to be valid in Table 1 and will be further discussed below. Extrapolation of the E_{max} vs C curve to infinite concentration gives a coupling factor of ρ = 0.22 (eq 5).

Table 1 shows the largest signal enhancement measured for each sample (E_{measd}), the maximum predicted signal enhancement determined from eq 11 (E_{max}), the actually measured saturation factor (s_{measd}) determined from eq 2 with ρ = 0.22, the extrapolated maximum saturation factor from DNP determined from eq 3 (s_{max}(DNP)), the saturation reached (%), and the theoretically predicted maximum saturation factor using eq 10 (s_{max}(eq 10)). The ratio κ'/p was determined in a previous study to be 1.1 mM^{-1} by independent measurements of the electron spin exchange rate, κ', with ESR line width measurements and relaxation time, T_{1e}, with pulsed ESR.[9] Hence, the calculated values of s_{max} from eq 10 shown in Table 1 are determined independently from DNP and then compared to saturation factors measured through DNP. There is excellent agreement between the measured (DNP) and predicted (eq 10) values of $s_{max,}$ validating both the assumption of w_N/p = 0 and the prediction that s_{max} approaches 1 at large concentrations (~15 mM) for nitroxide radicals. This finding is important as it allows us to confidently determine ρ and extract the dynamic information it contains. For small concentrations of radicals we were able to experimentally reach ~100% saturation. This was further confirmed by measuring no decrease in the observed signal enhancement while decreasing the power by nearly 50%. Note that 100% saturation at 0.5 mM radical concentration still leads to an s_{max} of only 0.58 due to incomplete mixing of the hyperfine states. Therefore, s_{max} approaching 1 should not be confused with a 100% saturation level.

Table 1. Summary of DNP Enhancements and Saturation Factors with Comparison to the Theoretical Value Given by Eq 10 with κ'/p = 1.1 mM^{-1} [a]

concn (mM)	E_{measd}	E_{max}	s_{measd}	s_{max}(DNP)	saturation (%)	s_{max}(eq 10)
0.2	−5.3	−5.3	0.44	0.44	100	0.45
0.5	−17.7	−17.7	0.58	0.58	100	0.57
1	−32.6	−32.6	0.64	0.64	100	0.68
2	−56	−59	0.74	0.78	95	0.79
3	−68	−73	0.76	0.81	94	0.84
5	−87	−95	0.82	0.90	91	0.90
7.5	−91	−106	0.79	0.91	87	0.93
10	−97	−119	0.80	0.98	82	0.94
15	−101	−129	0.79	1.00	79	0.96

[a] The measured value of s assumes ρ = 0.22.

We note that our previous study of 4-oxo-TEMPO reported a coupling factor of only 0.176[9] instead of 0.22 as reported here. Since that first study, we have developed a high-power X-band amplifier capable of delivering several watts to the sample instead of the 200 mW maximum power used previously.[26] In this study we are reaching 80% saturation at 15 mM compared with only 30% saturation in our earlier study (see Table 1) and believe that this low value for the saturation factor caused erroneous extrapolations to infinite power. We now have an order of magnitude larger power, which allows a more accurate extrapolation to infinite power.

Using the measured value of ρ = 0.22 with eq 6 and employing the spectral density function of eq 7, τ was found to be 76 ps. A pulsed field gradient diffusion ordered spectroscopy (DOSY) NMR experiment was used to determine the diffusion coefficient of 4-oxo-TEMPO in water (data not shown) to be 4.1×10^{-10} m^2 s^{-1}. Using the literature value of 2.3×10^{-9} m^2 s^{-1} for the self-diffusion of water at 24 °C,[27] the distance of closest approach was found through eq 1 to be 4.5 Å.

DNP enhancements of water were measured using the singly spin-labeled human protein isoform tau187, truncated between

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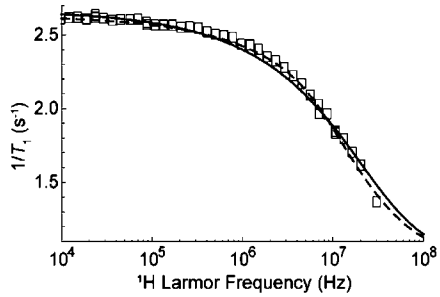

Figure 3. Water ^{1}H relaxation rate with 5 mM 4-oxo-TEMPO dissolved. The solid curve is the fit of the data to eq 8, while the dashed line includes a 9% rotational component.

Table 2. Comparison of DNP Results with 4-oxo-TEMPO Free in Solution to Those with a Nitroxide Attached to Solution-State Tau

sample	E_{max}	ρs_{max}
200 μM tau	−8	0.14
1 mM tau	−27.5	0.13
200 μM TEMPO	−5.3	0.10
1 mM TEMPO	−32.6	0.15

residues 255 and 441, (with further details described in ref 13) as the source of unpaired electrons. The protein is largely unfolded in its native state, where the spin label at residue 322 is expected to be exposed to solvent as well as mobile. As the NMR signal enhancement comes from an electron on the protein's surface, the DNP magnitude is determined by the translational dynamics of the hydration water near the surface of the protein. In Table 2, the quantity ρs_{max} is compared for two different concentrations of 4-oxo-TEMPO free in solution and the spin-labeled protein tau187. The fact that ρs_{max} is equal within error for both 200 μM and 1 mM concentrations of tau187 shows that Heisenberg spin exchange is not effective, but also that the other mechanisms affecting s_{max} must be insensitive to the spin label concentration, as ρ is already known to be concentration-independent. Ultimately, the observation that ρs_{max} is 0.14 for 200 μM spin-labeled protein is larger than that of 200 μM free spin label dissolved in solution provides evidence that rapid nitrogen nuclear spin relaxation (w_N) increases s_{max} according to eq 4, since ρ is not expected to increase when the radical is attached to a protein compared to its freely dissolved state. If w_N/p is large enough due to slow protein tumbling (>28 from eq 4) so that s_{max} approximates 1, then $\rho \approx 0.14$ for water interacting with the nitroxide spin label near the surface of the unfolded tau187 protein. Putting $\rho = 0.14$ into eq 6 gives $\tau = 130$ ps. Assuming the distance of closest approach between water and the unpaired electron of the spin label does not change as the surface of an unfolded protein is hydrated, the solvent diffusion coefficient of the hydration layer of the protein is found to be $D_I \approx 1.6 \times 10^{-9}$ m^2 s^{-1}.

Figure 3 shows the results of an FCR experiment for a sample of 5 mM 4-oxo-TEMPO dissolved in water. Although water has little frequency dependence over the field range studied here, $T_{10}(\omega)$ was also measured for inclusion in eq 8. The relaxation data were fit to eq 8, with $\tau = 24$ ps and $d = 2.4$ Å giving the best fit. Putting the value of $\tau = 24$ ps into eq 6 at 0.35 T gives $\rho = 0.36$. Alternatively, subtracting off $1/T_{10}$ (T_{10} was found to be 2.6 s) in the low-field limit and taking advantage of the extreme narrowing condition gives $2w_1 = 0.665$ s^{-1}. This value was put into eq 9 with $1/T_1 = 1.74$ s^{-1} and $T_{10} = 2.60$ s at 0.35 T, again giving $\rho = 0.36$ in agreement with eq 6. Putting the values of $\tau = 24$ ps and $d = 2.4$ Å back into eq 1 gives $D = 2.4 \times 10^{-9}$ m^2 s^{-1}. Our FCR results also agree with a recently published study by Höfer and colleagues utilizing FCR for nitroxide radicals dissolved in water where they also determined τ to be between 15 and 20 ps and ρ, through eq 10, to be 0.36.[12]

Discussion

In a DNP experiment, as long as the leakage factor and saturation factor can be quantified, ρ can be directly extracted from the DNP E_{max} values. It is important to point out that the measurement of ρ through DNP does not depend on the form of the spectral density function nor the type of coupling (dipolar or scalar) between the electron and proton, which is not the case when determining ρ from FCR data. Therefore, the DNP approach should be more reliable in determining ρ. The determination of ρ for nitroxide radicals is however made difficult because of the mixing of the three hyperfine electron spin states. For nitroxides dissolved in water, this mixing is caused primarily through Heisenberg spin exchange. Bates and Drozdoski predicted that, for high concentrations, a nitroxide radical would behave as a radical with a single electron spin transition; i.e., $s_{max} = 1$ instead of 1/3, if the hyperfine states are completely independent.[18] The calculated values of s_{max} from eq 10 shown in Table 1 come from measurements of the electron spin exchange rate and electron spin relaxation rate and are thus completely independent of DNP measurements. This is the first time that predicted values for the maximum saturation factor of nitroxide radicals are presented and also compared to measured values from DNP studies. The good fit of the E_{max} vs C curve in Figure 2 to eq 3 with the predicted s_{max} given by eq 10 provides further strong evidence that the saturation factor can indeed be quantified for nitroxide radicals. This finding is important for measuring ρ and thus determining dynamic parameters.

The DNP-determined value of $\rho = 0.22$ implies $\tau = 76$ ps according to eq 6. Using the known diffusion coefficient of water and our measured diffusion coefficient for the dissolved nitroxide implies a distance of closest approach between the unpaired electron and solvent proton of 4.5 Å. This value is in good agreement with the predicted van der Waals contact distance.[15] When the nitroxide is attached to a well-hydrated biomolecule, this distance is not expected to change.[14] D_S of tethered spin labels will also be significantly smaller than D_I in eq 1 and can be ignored. Thus, by measuring ρ and extracting τ, the local diffusion coefficient of water can be determined as shown for the unfolded protein tau187 above. The value of $D_I = 1.6 \times 10^{-9}$ m^2 s^{-1} represents an average diffusion coefficient for water that is heavily weighted toward water molecules very close to the radical, with 80% of the relaxation coming within 5 Å (the distance of closest approach already being 4.5 Å) and 90% within 10 Å.[8] Our measured value of 1.6×10^{-9} m^2 s^{-1} for the hydration layer water on the surface of an unfolded protein is in good agreement with the value of 1.65×10^{-9} m^2 s^{-1} for the hydration layer water on the surface of a model protein, as determined by quasi-elastic neutron scattering (QENS).[28] QENS is one of the few experimental techniques able to measure diffusion in the hydration layer, but requires highly concentrated samples, e.g., 500 mM peptide solution in the above example, where every water molecule can be assumed to be in the hydration layer. This requirement, however, makes QENS not viable for characterizing most biomolecular systems.

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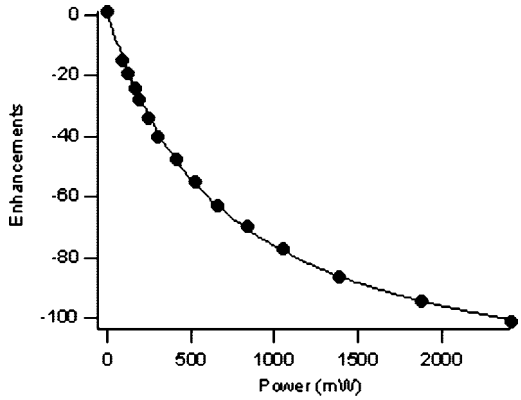

Figure 4. Measured enhancement versus applied microwave power to the sample for 15 mM 4-oxo-TEMPO. The solid curve is the fit of the data to eq 11, giving $E_{max} = -129$.

The key advantage of DNP over QENS and FCR is its capability to use dilute samples of minute quantities in bulk water and under physiological conditions.

It is surprising that DNP and FCR experiments can give such different results for τ and ρ, as they both rely on the dipolar coupling between the unpaired electron spins of the free radicals and the ^{1}H nuclear spins of the solvent, in this case water. If the spectral density function of eq 7 is inappropriate for this system, the value of τ with each technique would be incorrect, unless it varied by only a constant parameter in front of the frequency-dependent terms. There is no obvious explanation for the discrepancies in ρ and τ from the two techniques, so here we will discuss how plausible each of the parameters are starting with ρ.

We have shown quantitatively for the first time that electron spin exchange does increase s_{max} to 1 at large radical concentration. Thus, if ρ were 0.36 as FCR suggests (from the fit value of $\tau = 24$ ps), we would expect possible enhancement factors nearing -240 at 0.35 T according to eqs 3 and 5. We have seen no evidence of enhancements coming close to this value in our own experiments under optimized conditions (e.g., a critically coupled microwave cavity with a high quality factor, high power, and small sample volumes for complete microwave penetration) nor in any published results. In Figure 4 a plot of measured enhancement against applied microwave power is shown, with the solid line being the fit of the data to eq 11. The fit gives $E_{max} = -129$, and the largest enhancement actually measured is -101. This is far below the FCR-suggested value of -240-fold, even though s_{max} and f are both very close to 1. For this reason we believe the DNP-measured value of $\rho = 0.22$ to be valid and not the FCR value of 0.36. Höfer et al. report DNP enhancements of -140-fold and state to be power limited, but do not mention additional adjustments to avoid sample heating, which is notoriously problematic at high microwave powers and high frequencies.[29] Higher temperatures cause much more rapid diffusion, thus decreasing τ greatly according to eq 1 and increasing the coupling factor. This increase in the coupling factor can easily more than offset the loss in thermal polarization, also caused by sample heating. Our own experiments at the X-band have shown a 15% increase in signal enhancement when the cooling air is turned off.

The FCR fit value of 2.4 Å for d is only half the value predicted for the proton of water and the electron spin of nitroxide molecules through van der Waals contact.[15] Equation 8 could underestimate the value of d if a constant term in front of the spectral density function is missing. Steric factors and order parameters have been employed in FCR experiments on other systems to account for nonspherical shapes and internal motions of the molecules.[30,31] Also, it has been reported that the local concentration of paramagnetic oxygen near the radical may be at least twice that of the bulk oxygen concentration,[32] which could manifest itself as a ^{1}H relaxation rate larger than predicted by eq 8 due to the higher local concentrations of paramagnetic species. This last effect likely does not play a role as our samples were not degassed, but those of Höfer and colleagues were, while for both sample preparations the FCR results are in good agreement. If steric factors or order parameters were significant, FCR would give a value of d which was too small, and also overestimate w_1 since these would add other modes of increased relaxation other than through dipolar coupling to the electron. This effect however would need to be field-independent as Höfer et al. measured the ^{1}H relaxation rates over a large field range up to 900 MHz, with the value for w_1 at high field agreeing with the value at low field.[12] It is important to note that if the error lies in a constant term in front of the spectral density function, it will fall out of eq 6, thus leaving the determination of $\tau = 76$ ps from the DNP-measured ρ unaffected, while providing higher d values for the FCR analysis. Another relevant observation in this context is a systematic FCR study in the literature on lipid bilayers using localized spin labels, reporting d values of 4.3−4.7 Å between water and the electron spin. The authors also note that the van der Waals distance between water and spin labels within these well-hydrated volumes is not expected to change from that in bulk water.[14] Another FCR study on bovine serum albumen, however, reported $d = 1.9$ Å.[16] This suggests there exists a discrepancy within the FCR methodology, possibly depending on the size or shape of the molecule onto which spin labels are attached.

While a missing constant could explain a difference in d, it cannot explain the large difference seen in τ. In the spectral density function of eq 7, τ is always found as a product with ω and thus is determined by the frequency dependence of the T_1 relaxation data, which is unaffected by constants in front of the spectral density function. This is important as both d and τ are needed to determine a local diffusion coefficient with these methods. If the form of the spectral density function in eq 7 is inappropriate for this system, the determination of τ from each experiment will be incorrect, perhaps bringing them into agreement once the correct model is used. Although the fit of the FCR data to eq 8 shown as a solid line in Figure 2 is not perfect, it does show that the spectral density function of eq 7 is a good approximation to describe the frequency dependence of the data. We were able to obtain an improved fit when we added a rotational component of less than 9% to the relaxation, shown as a dashed line in Figure 2. This modification changed the fits for the key parameters, τ and d, by less than 5%, but added three new fit parameters. To date, we have found no literature that applies a different form of the spectral density function to the nitroxide radical and water system. More

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complicated spectral densities are obtained if one removes the force-free assumption, but this adds significant complications and requires numerical methods and/or simulations to determine the radial distribution function describing the forces between the molecules. Reports in the literature so far suggest that taking forces into account requires custom design of the spectral density function for every different molecular system.[24,25,33] Also, the fact that eq 7 already well approximates the FCR data does not make it obvious whether the problem lies in the general form of the spectral density function and whether this can explain the factor of ~3 difference in τ between the two techniques.

Conclusion

In this study, we have for the first time predicted and quantified the DNP saturation factor for nitroxide radicals. This is a key step to employ DNP to determine the local water dynamics on spin-labeled molecules and macromolecular assemblies because quantifying the saturation factor is a necessary step to determining the coupling factor. The significance of being able to experimentally quantify local water diffusion coefficients through the ^{1}H DNP method using nitroxide-based spin labels is two-fold: (1) The orders of magnitude sensitivity gain of the DNP methodology compared to other techniques allows for the use of very small sample volumes (3−4 μL) and concentrations (~100 μM), making biological applications readily feasible. (2) The use of nitroxide spin labels allows the utilization of the site-directed spin-labeling technology for DNP analysis for a wide range of biological applications. This allows for the quantification of local hydration dynamics at the surface of and inside the same biological systems studied by ESR, thus of proteins, protein complexes, or lipid membrane complexes with residue- and site-specific resolution. These systems are the focus of our current studies. Here, we have shown one example application of how DNP can measure the diffusion of water in the hydration layer of the unfolded protein tau187.

Although the FCR technique employing nitroxide spin labels is impractical due to the low solubility of most proteins, it can determine both τ and ρ at the same time. However, for the simple system of a nitroxide dissolved in water, DNP and FCR give very different results. As the measurement of ρ via DNP does not depend on the type of coupling or model for the spectral density functions, we believe the coupling factor of $\rho = 0.22$ measured through DNP is the correct value. Besides, we have seen no reported DNP enhancements suggesting the FCR-measured value of $\rho = 0.36$ to be physical. Additionally, FCR gives a fit for the distance of closest approach, which varies greatly depending on the molecule to which it is attached. Because these techniques rely on the same dipolar coupling between the unpaired electrons and solvent protons and the same dynamics model was applied, the origin for these discrepancies remains an unresolved question and needs to be further investigated. The correct evaluation of local solvent diffusion measurements with each technique requires the development of better models describing the spin dynamics interactions. DNP is a very promising tool to study local water dynamics in and on biological systems, given the lack of alternative techniques to characterize samples under biologically relevant conditions.

Materials and Methods

4-oxo-TEMPO was purchased from Sigma-Aldrich and used without further purification. Samples were initially dissolved in DMSO at a high concentration and then diluted in deionized water, with all samples containing less than 5% DMSO by volume. Samples used for all experiments were not degassed. For DNP experiments, samples were loaded into a 0.7 mm i.d. silica capillary and sealed with beeswax. The capillary was loaded into a home-built NMR probe and placed inside a Bruker TE_{102} X-band cavity. ESR spectra were taken to determine the electron spin resonances with a Bruker EMX spectrometer. NMR measurements were made with a Bruker Avance 300 NMR spectrometer. The magnetic field for DNP experiments was ~0.35 T.

For irradiation of the electron transition during a DNP experiment, a home-built 8−10 GHz microwave amplifier was used. This system was described in detail previously.[26] The sample was continuously irradiated at the electron spin resonance while the NMR experiment was carried out. Cooling air was flowed over the sample, and microwave powers were kept lower than maximum to prevent sample heating.

To determine the maximum enhancement, the enhancement vs power data were fit to

$$E = I_0 - \frac{AP}{1 + BP} \tag{11}$$

where I_0, A, and B are the fit parameters and contain all the constants in eq 2. The enhancement at infinite power is given by $E_{max} = I_0 - A/B$,[9] and Igor Pro 5.05 data analysis software was used to provide error estimates to the fits. Field cycling experiments were performed at the Technical University of Illmenau on a Stelar FFC 2000-1T field cycling relaxometer, and the data were fit in Mathematica 6.0.

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