Nuclear Overhauser Effect on Diffusion Measurements

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Diffusion NMR has proven to be a powerful tool in mixture analysis.[1] The close relationship with molecular size also makes the diffusion coefficient an effective indicator of molecular interactions and a useful measure for binding constant. Due to its convenient and noninvasive nature, diffusion NMR has found its widespread application in various fields. In biological systems, it becomes one of the few techniques available to characterize molecular association and measure partition ratio in intact cells.[2] In the pharmaceutical industry, a great deal of effort has been made to incorporate diffusion-based NMR techniques into drug screening as well as binding affinity evaluation.[3] Because diffusion experiments are gaining in popularity for the investigation of intermolecular interactions and determination of binding ratios, it has been decided to examine this methodology in detail.

The basis for binding ratio determination is that the diffusion coefficient changes upon binding to a target. Under the condition when fast chemical exchange takes place during diffusion experiment, the signal intensity (I) decays single exponentially according to eq 1.[4]

$$I(K) = I(K=0)\exp[-K^2 D(\Delta - \delta/3)] \qquad (1)$$

where $K = \gamma g \delta$, γ is the gyromagnetic ratio, g is the gradient strength, δ is gradient pulse width, D is the diffusion coefficient, and Δ is the diffusion time during which the diffusion is being monitored. The observed D is the averaged coefficient of free (D_{free}) and bound (D_{bound}) states weighted by the fraction of free molecule (x_{free}) and bound molecule (x_{bound}):

$$D = x_{free} D_{free} + x_{bound} D_{bound} \qquad (\text{where } x_{free} + x_{bound} = 1) \quad (2)$$

As long as D_{free} and D_{bound} can be determined, the binding percentage ($x_{bound} \times 100\%$) can be readily calculated. The binding ratio[5] (or dissociation constant), even the number of binding sites,[6] can be further estimated.

The effect of chemical exchange on diffusion NMR has been extensively examined theoretically and experimentally.[7] However, another important effect that can also have severe impact on diffusion coefficient measurements has been ignored in low-viscous liquids,[8] that is nuclear Overhauser effect (NOE). During the diffusion time with the magnetization stored in the longitudinal direction in the stimulated echo (STE) type of experiments, not only chemical exchange but also intermolecular NOE (cross-relaxation) will affect the signal decay. Under the condition of slow motion limit, strong NOE can rapidly build up within the diffusion time window generally used in diffusion experiments. As a result, the diffusion experiment can be severely compromised, and the outcome of the experiment will depend on experimental conditions employed.

Suppose during the gradient encode period, a molecule A at position $\mathbf{r}_A$ obtains a spatial label $\Phi_A = \gamma(\mathbf{g}\cdot\mathbf{r}_A)\delta$ and a molecule B at position $\mathbf{r}_B$ gets a label $\Phi_B = \gamma(\mathbf{g}\cdot\mathbf{r}_B)\delta$. During the diffusion time of STE and longitudinal Eddy current delay (LED) experiments, magnetization is stored in the z direction for signal conservation. If there is no NOE between molecules, then each molecule will still carry its own label when it is detected. However, the spatial label scheme could be disrupted if there is longitudinal magnetization transfer between the two molecules through cross-relaxation. As a result, molecule A obtains the spatial label Φ_B from molecule B and molecule B is now labeled by Φ_A. When the signal is detected, molecule A will appear to move from position B rather than from position A. Because the spatial label exchanges between different molecules, the signal decay may deviate from eq 1 since it no longer directly reflects the displacement of the molecule.

To demonstrate this effect, we performed a series of diffusion experiments[9] on a mixture of 150 μM human serum albumin (HSA) (from Sigma), 10 mM benzoic acid (carboxyl-^{13}C, 99% from Cambridge), and 10 mM glucose (1-^{13}C, 99% from Cambridge) in D_2O. The interaction between binding ligand benzoic acid and HSA provides us a way to monitor intermolecular NOE effect on the diffusion measurement. Glucose does not bind to HSA and is present as a comparison.

The interaction between benzoic acid and HSA has a strong effect on the diffusion measurements (Figure 1a). We can see clearly that when the diffusion time is short enough for NOEs to be ignored (e.g., $\Delta = 20$ ms), the benzoic acid signal does show a single-exponential decay as expected from eq 1. The diffusion coefficient determined (5.9×10^{-10} m^2/s) is much slower than that measured without protein (7.7×10^{-10} m^2/s), which indicates that the benzoic acid is interacting with protein and the on- and off-rates are fast on the diffusion time scale. However, as the diffusion time increases, the signal for the benzoic acid decays more slowly while HSA signal decays faster. The signals also show increased deviation from a single-exponential decay. Although diffusion experiments can always be forced to yield a result according to eq 1, the diffusion coefficient determined can be distant from the real value. By forcing a single-exponential fit to the decay, the resulting D at $\Delta = 1$ s is less than half of the value determined at $\Delta = 20$ ms. Apparently eq 1 no longer holds when there are intermolecular NOE and chemical exchange during the measurement. Under these conditions, the result from the diffusion experiment depends on the diffusion time Δ employed. On the other hand, signals from noninteracting ligand glucose show single-exponential decay (Figure 1b), and they decay at the same rate (within experimental error) regardless of diffusion time Δ employed in the experiment (Table 1).

To test the hypothesis that this phenomenon is caused by NOE, we measured the diffusion coefficients on a part of the molecule with little or no NOE interactions: the carboxyl carbon of benzoic acid. Because of the relatively small value of the gyromagnetic

(1) (a) Morris, K. F. Johnson, C. S., Jr. *J. Am. Chem. Soc.* **1993**, *115*, 4291−4299. (b) Stilbs, P. *Prog. Nucl. Magn. Reson. Spectrosc.* **1987**, *19*, 1−45.
(2) (a) Lennon, A. J.; Scott, N. R.; Chapman, B. E.; Kuchel, P. W. *Biophys. J.* **1994**, *67*, 2096−2109. (b) Andreasson, B.; Nordenskiold, L.; Schultz, J. *Biophys. J.* **1996**, 70, 2847−2856. (c) Price, W. S.; Barzykin, A. V.; Hayamizu, K.; Tachiya, M. *Biophys. J.* **1998**, *74*, 2259−2271.
(3) (a) Lin, M.; Shapiro, M. J.; Wareing, J. R. *J. Am. Chem. Soc.* **1997**, *119*, 5249−5250. (b) Hajduk, P. J.; Olejniczak, E. T.; Fesik, S. W. *J. Am. Chem. Soc.* **1997**, *119*, 12257−12261. (c) Xu, F.; Detlefsen, D. J.; Marshall, E. A.; Rourick, R.; Whitney, J. L.; Lee, M. S. *Book of Abstracts*, 39th ENC, March 22−27, **1998**, 220.
(4) Stejskal, E. O.; Tanner, J. E. *J. Chem. Phys.* **1965**, *42*, 288−292.
(5) Chen, A.; Wu, D.; Johnson, C. S., Jr. *J. Phys. Chem.* **1995**, *99*, 828−834.
(6) Liu, M.; Nicholson, J. K.; Lindon, J. C. *Anal. Commun.* **1997**, *34*, 225−228.
(7) (a) Johnson, C. S., Jr. *J. Magn. Reson.* **1993**, 102, 214−218. (b) Chen, A.; Johnson, C. S., Jr.; Lin, M.; Shapiro, M. J. *J. Am. Chem. Soc.* **1998**, *120*, 9094−9095. (c) Moonen, C. T. W.; van Gelderen, P.; Vuister, G. W.; van Zijl, P. C. M. *J. Magn. Reson.* **1992**, 97, 419−425.
(8) Fischer, E.; Kimmich, R. *J. Chem. Phys.* **1997**, *106*, 9883−9888.
(9) All of the data were collected on a Bruker DRX500 spectrometer with 5 mm QNP probe at temperature of 300 K.

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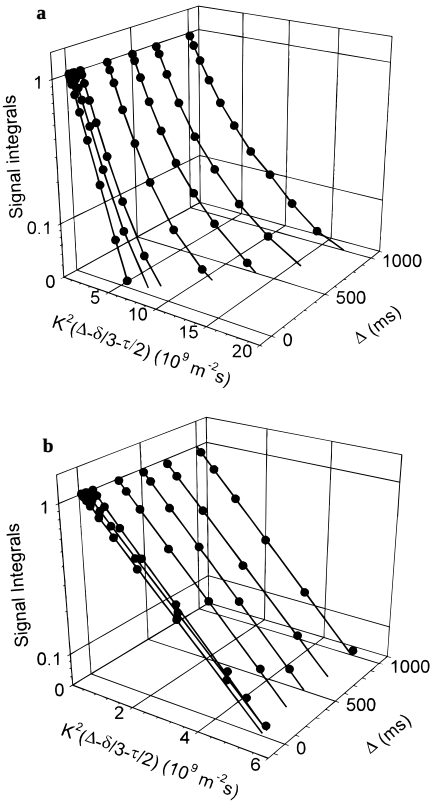

Figure 1. Diffusion measurement with bipolar LED pulse sequence* on proton signals. (a) Normalized signal integrals of benzoic acid ($H_{2,6}$) vs $K^2(\Delta - \delta/3 - \tau/2)$ at different Δ values. (b) Normalized signal integrals of glucose (H_1) vs $K^2(\Delta - \delta/3 - \tau/2)$ at different Δ values. * In the bipolar LED pulse sequence,[10] τ is the delay between the bipolar gradient pulse pair, and the signal decays according to $I(K) = I(K = 0) \exp[-K^2D(\Delta - \delta/3 - \tau/2)]$.

Table 1. Diffusion Coefficients of Glucose ($T = 300$ K)

^{1}H diffusion measurements ($D = (5.44 \pm 0.13) \times 10^{-10}$ m^2/s)							
Δ (ms)	20	54.2	104.2	304.2	504.2	704.2	1004.2
D($\times 10^{-10}$ m^2/s)	5.30	5.28	5.63	5.48	5.55	5.39	5.48

^{13}C diffusion measurements ($D = (5.5 \pm 0.2) \times 10^{-10}$ m^2/s)			
Δ (ms)	56.4	504.2	1004.2
D($\times 10^{-10}$ m^2/s)	5.4	5.7	5.4

ratio for ^{13}C, the NOEs between ^{13}C and other nuclei are generally insignificant. Also, this (carboxyl) carbon of benzoic acid is shielded from the protein by NMR inactive nuclei.

The diffusion measurements with various diffusion times were carried out as before, now monitoring the ^{13}C of the sample (Figure 2). Although benzoic acid is interacting with HSA, the signal of carboxyl carbon shows single-exponential decay even at a diffusion time around 1 s. The resulting diffusion coefficients measured at different diffusion times (Table 2) now give the same value within experimental error. The average diffusion coefficient measured on ^{13}C signal for benzoic acid is $(5.7 \pm 0.2) \times 10^{-10}$ m^2/s, which is consistent with the value $(5.9 \times 10^{-10}$ m^2/s) measured on proton at $\Delta = 20$ ms. The diffusion coefficients of glucose determined at different diffusion times are also very similar and agree with the results obtained from proton measurements (Table 1). It is clear that, in the absence of NOE, there is no deviation from eq 1 observed and the diffusion coefficient can be measured consistently and accurately under different experimental conditions. This strongly suggests that the difference of proton experiment results obtained at different diffusion times is caused by NOE built up during diffusion experiments.

It is interesting to note that if there is only physical exchange (between free and bound states), then the spatial labels stay on the same molecule, and the movement of each molecule can be recorded with no problem. Only when intermolecular NOE is present, does it become difficult to track the molecules individually.

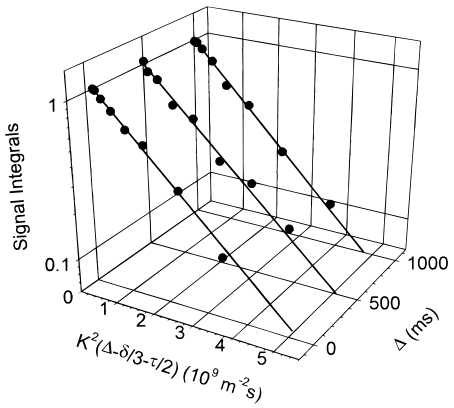

Figure 2. Diffusion measurement with bipolar LED pulse sequence on acid ^{13}C signal (normalized).

Table 2. Diffusion Coefficients of Benzoic Acid from ^{13}C Measurements ($T = 300$ K)

Δ (ms)	56.4	504.2	1004.2
D($\times 10^{-10}$ m^2/s)	5.7	5.6	5.9

A possible benefit from this effect is that it may be used to discriminate binding ligands against nonbinding compounds. Unlike nonbinding species whose decay rate does not depend on diffusion time Δ, binding ligands decay at different rates as diffusion time Δ varies. Therefore by adjusting the diffusion time, a NMR spectrum can be obtained with only binding ligand and receptor signals. It should be noted that in our case benzoic acid can be "separated" from glucose by intermolecular NOE rather than by a simple diffusion filter, given the fact that the observed diffusion coefficients of the two compounds are very similar. Because of signal attenuation by diffusion, this method is less sensitive than our NOE pumping experiment.[11]

In summary, intermolecular NOE can interfere with diffusion measurement when: (a) there is intermolecular NOE between molecules, (b) the interacting species have different diffusion rates, and (c) chemical exchange takes place during the experiment. The consequence is more significant when a strong NOE is expected, which is generally the case when the binding target under investigation is a macromolecule. Strong NOE can build up rapidly in the exact time window generally used in the diffusion experiment and cause serious disruption in the measurement. The implications are extremely important, given the surging interest in the diffusion NMR study of interactions and association constants involving macromolecules. Deviation from a single-exponential decay in the fast exchange limit is a symptom of NOE interference. Variation of signal decay rate with diffusion time (Δ) is another strong sign of NOE perturbation. Lack of consideration of this effect can lead to large errors in the determined diffusion coefficient and the binding affinities. Currently we cannot separate this NOE effect from the diffusion measurement experimentally. Because of strong spin diffusion, selective diffusion measurements with selective excitation cannot completely negate the problem. To minimize this effect, it is suggested that signals with little or no intermolecular NOE be chosen to determine the diffusion rate (if possible) and use diffusion time as short as possible.

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(10) Wu, D.; Chen, A.; Johnson, C. S., Jr. *J. Magn. Reson. A* **1995**, *115*, 260−264.
(11) Chen, A.; Shapiro, M. J. *J. Am. Chem. Soc.* **1998**, *120*, 10258−10259.