NOE Pumping: A Novel NMR Technique for Identification of Compounds with Binding Affinity to Macromolecules

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The drug discovery process often involves the screening of compound libraries to identify drug candidates capable of binding to target macromolecules. The recently developed affinity NMR technique, which detects complexation of a small molecule with a "receptor", promises to be a valuable tool to perform rapid screening of compounds for biological activity.[1] The basis of this technique is that the translational diffusion coefficient of a small ligand changes substantially upon binding to the target macromolecule receptor. Affinity NMR uses diffusion editing to "filter out" signals from nonbinding components, and thus, the bound ligands will be selected from the mixture.

The criterion for affinity NMR is that there are considerable differences in observed translational diffusion coefficients between binding and nonbinding ligands in order for them to be distinguished. However, this condition is not always satisfied. The free and bound ligands are in fast exchange on the diffusion time scale; thus, the observed diffusion coefficients are the weighted average of the free and bound species. Ligands in the free form should diffuse at a much faster rate than those in the bound state, so that even a small amount of the ligand in the free state causes the apparent diffusion rate of binding ligand to increase considerably. This factor can greatly narrow the gap of diffusion coefficients between binding and nonbinding ligands, leaving little room to differentiate "active" compounds from the mixture. Under such circumstances, it is difficult to draw the line between binding and nonbinding ligands by diffusion experiments alone. In addition, when the receptor is very large, the transverse relaxation time (T_2) could be too short for the bound ligands to be observed in the diffusion-edited spectrum.

Herein we propose and demonstrate a novel method, a diffusion-assisted nuclear Overhauser effect (NOE)-pumping experiment, which allows unambiguous detection of ligands that bind to a macromolecule. The concept is to distinguish from nonbinding ligands, by NOE, those molecules capable of binding to the macromolecule. Unlike the traditional affinity NMR experiment, which uses diffusion editing to eliminate signals from the unbound ligands, the NOE-pumping method relies on NOE to transfer the signal from the receptor to the bound ligands.

To prepare for the pumping, the diffusion experiment (stimulated echo (STE)[2] shown in Figure 1a or longitudinal eddy-current delay (LED)[3]) is employed to suppress all ligand signals, while preserving signals from the macromolecule receptor. We use a bipolar gradient pulse pair (BPP) to minimize spectral artifacts and chemical exchange modulation.[4] So that signals may be observed from the bound ligand, a NOE experiment (two 90° pulses separated by mixing time t_m) follows immediately as shown in Figure 1b. All of the ligand coherence is destroyed by the

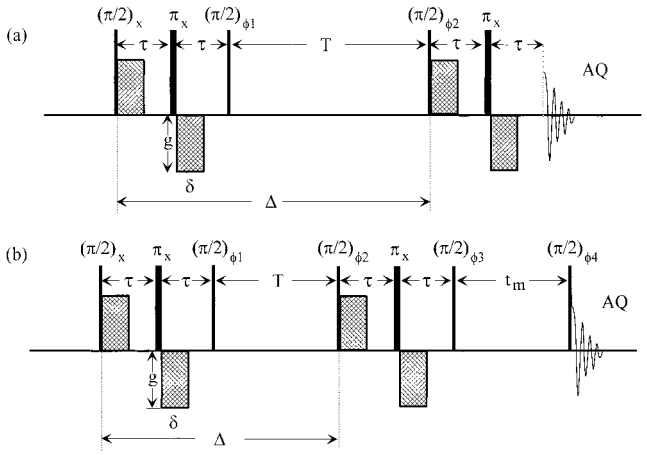

Figure 1. (a) BPP-STE pulse sequence. (b) Diffusion-assisted NOE-pumping pulse sequence. Phase cycle is as follow: $\phi1 = x, -x$; $\phi2 = 2(x)\ 2(y)\ 2(-x)\ 2(-y)$; $\phi3 = 2(x)\ 2(y)\ 2(-x)\ 2(-y)\ 2(-x)\ 2(-y)\ 2(x)\ 2(y)$; $\phi4 = 16(x)\ 16(y)\ 16(-x)\ 16(-y)$. Receiver phase for a: $\phi r = -x\ x\ y\ -y\ x\ -x\ -y\ y$. Receiver phase for b: $\phi r = 2(x\ -x\ -x\ x)\ 2(-x\ x\ x\ -x)\ 2(y\ -y\ -y\ y)\ 2(-y\ y\ y\ -y)\ 2(-x\ x\ x\ -x)\ 2(x\ -x\ -x\ x)\ 2(-y\ y\ y\ -y)\ 2(y\ -y\ -y\ y)$.

diffusion experiment at the beginning of NOE experiment; therefore, any ligand signals detected at the end of the NOE experiment arise from polarization transferred from the receptor which is preserved after the diffusion filter. Here, the macromolecule signals are serving as a reservoir to deliver polarization only to ligands that are bound to the receptor. As the ligands disassociate from the target macromolecule, the transferred magnetization is carried over and conserved by the relatively long T_1 of the ligands in the free state. The ligand signals observed arise not only from the signals transferred to the ligand currently in the bound state but also from the signals pumped to the ligand in the free state. As a result, binding ligand signals can still be detected even if T_2 is too short for the direct observation of bound-state ligand signals. It can be envisioned that this method works efficiently where excess free ligands are present and the free and bound ligands are in fast exchange during diffusion and mixing times. This situation is generally of great interest in the pharmaceutical industry.

The concept of this experiment, which focuses on the efficient identification of binding ligand signals by a 1D experiment, is fundamentally different from other multidimensional diffusion NOESY experiments.[5,6] In those experiments, diffusion NMR is used to resolve or select chemical shift overlapped 2D NOE cross-peaks (from intra- or intermolecule) rather than to prepare for NOE pumping. In fact, any experiment (besides diffusion NMR) that can suppress ligand signals while conserving macromolecule signals can be used before the NOE pumping in order to identify binding ligands.

To demonstrate the ability to identify compounds that bind to macromolecules by the diffusion-assisted NOE-pumping experiment, the interactions of the protein receptor of human serum albumin (HSA) with a binding ligand (salicylic acid[7]) and nonbinding ligands (L-ascorbic acid and glucose) in D_2O were studied.[8] The normal proton NMR spectrum of the mixture is shown in Figure 2a.

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(8) No buffer solution is used to demonstrate that this method is resistant to any chemical shift change raised from chemical environment variation (e.g., pH). All of the experiments are done at 300 K on a Bruker DMX 500 with a 5-mm inverse triple nuclear *z* gradient probe.

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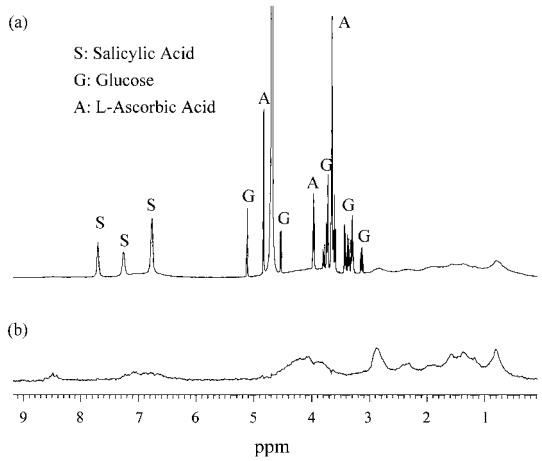

Figure 2. 10 mM salicylic acid (S), 10 mM L-ascorbic acid (A), 10 mM glucose (G), and 100 μM HSA in D_2O. (a) Normal 1D ^{1}H NMR spectrum. (b) 1D ^{1}H NMR spectrum with BPP-STE, where $g = 34.8$ g/cm, $\delta = 3$ ms, $T = 50$ ms, $\tau = 3.5$ ms, and 512 scans.

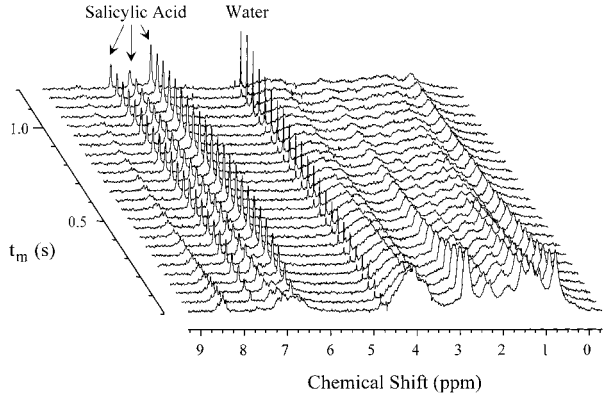

Figure 3. Stack plot of 1D ^{1}H spectra of HSA with three ligands as the mixing time t_m increases (from 5 ms to 1.2 s in intervals of 0.05 s) at a temperature of 300 K, where $g = 34.8$ g/cm, $\delta = 3$ ms, $T = 50$ ms, $\tau = 3.5$ ms, 512 scans were collected, and the experiment time for each spectrum is 30 to 40 min. Signals from bound ligand salicylic acid and water are indicated.

Using a standard BPP-STE diffusion-filtered experiment (Figure 1a), it is very difficult to identify a binding ligand from the mixture. As the gradient is increased, all three ligand signals decay at a similar rate. Although salicylic acid is bound to HSA, the apparent diffusion coefficients of the three ligands are not much different: 4.8×10^{-10} m^2/s for salicylic acid, 5.6×10^{-10} m^2/s for glucose, and 5.8×10^{-10} m^2/s for ascorbic acid.[9] This is because bound and free salicylic acid are involved in fast exchange on the diffusion time scale, and the weighted average diffusion coefficient is closer to the diffusion rate of the free state than to that of bound state. Under the condition where a large enough gradient is applied to suppress the nonbinding ligand signals, the binding ligand signals disappear as well (Figure 2b).

In this case, where the affinity NMR method fails to distinguish a binding ligand from nonbinding ones, the diffusion-assisted NOE-pumping experiment can clearly identify the binding ligands, as demonstrated in Figure 3. The bound ligand signals including the macromolecule associated water signal, which can be hardly seen before NOE transfer (Figure 2b), grow substantially as the mixing time t_m increases. On the other hand, all of the other signals are decreased by relaxation. Because the ligand signals that reappear after the NOE experiment are transferred from protein polarization, it is analogous to observing 2D NOESY cross-peaks (from protein to ligand) in a 1D fashion. The salicylic acid signal intensity starts to build up in the early phases of the NOE experiment and reaches its maximum at $t_m \approx 600$ ms before the magnetization begins to decay by T_1 relaxation. This is consistent with the large-molecule-mass protein tumbling at a slow rate (long correlation time τ_c in slow motion limit) which gives a strong negative NOE (positive cross-peaks and positive magnetization transfer). As a result of this negative NOE, an increase in the bound ligand signal intensity is observed.

As shown in Figure 3, signal loss with an increase of the mixing time is much more severe for the protein than for the binding ligand. This results not only from the longer T_1 of the small ligand favoring conservation of the signal but also from constant signal pumping from the protein to the ligands. As a consequence, a longer mixing time can be used to suppress the protein signal, giving a cleaner 1D NMR spectrum consisting of mainly the binding ligand signals. A T_2 relaxation filter could also be appended at the end of the experiment to further suppress protein signals. This 1D NOE-pumping experiment, which provides a relatively clean spectrum with signals from the bound species, permits direct observation of the molecular interaction in a very time efficient manner.

The 1D NOE-pumping method can be a very powerful method to rapidly pinpoint the active compounds that can bind to the target from a mixture. However, detailed binding site information could also be obtained by extending the experiment into multidimensions. For example, inserting t_1 before the fourth 90° pulse in Figure 1b should result in a 2D NOESY with protein signals in the first dimension and binding ligand signals in the second dimension. As a result, the diagonal peaks will be suppressed. Binding site information can be read out directly from cross-peaks, assuming peak assignment of the receptor is available.

The 1D NOE-pumping experiment offers several advantages. Because the detected signals of "binding" ligand are transferred from the receptor, false positives can be greatly eliminated. Binding activities are observed by directly detecting binding ligand signals; therefore, any chemical shift changes in the spectrum due to any chemical environment variation will not affect the result. Unlike other drug screening schemes which monitor ^{15}N or ^{1}H NMR chemical shift changes in the protein,[10] the NOE-pumping experiment can be applied to very large biomolecules without well-resolved macromolecule signals and without the need for isotope labeling. Actually, this method favors larger receptors because the NOE cross relaxation rates increase with the correlation time (i.e., receptor size) in slow motion limit and the broader macromolecule signals will be less likely to obscure bound ligand signals. There is also no need to perform extra experiments to subtract unwanted signals.[11] Compared to the simple diffusion-edited affinity NMR experiment, this method does not require that the apparent diffusion coefficients of binding ligands be substantially different from the nonbinding ligands. Furthermore, this method could be applied to the study of weakly bound ligands such as solvents.

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(9) The diffusion coefficients were measured at $T = 300$ K. To avoid NOE effect during diffusion measurement, the diffusion time was chosen to be as short as possible (16.2 ms in this case).

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