NOE Pumping. 2. A High-Throughput Method To Determine Compounds with Binding Affinity to Macromolecules by NMR

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In pharmaceutical research, there is a growing need for efficient detection and reliable identification of compounds with desired binding activity. As a unique tool in probing interactions between compounds and receptors as well as providing detailed information about binding site and binding conformations in a noninvasive manner, Nuclear Magnetic Resonance (NMR) techniques have attracted broad attention as a possible front line drug screening. Several NMR techniques (transferred NOE,[1] diffusion and relaxation editing,[2,3] etc.) have been developed to identify new drug candidates capable of binding to receptor from a pool of noninteracting compounds. In the development of NMR techniques, the major focus is on shortening the experiment time and the reduction of material usage as well as automation of the data acquisition and analysis.

We have shown that a Nuclear Overhauser Effect (NOE) pumping experiment can be effectively used to distinguish those molecules that are bound to the macromolecule from nonbinding compounds.[4] The technique is very reliable and can be applied to very large biomolecules without well-resolved macromolecule signals and without the need for isotope labeling. To detect signals that are pumped from the receptor to the binding ligands in the NOE pumping experiment, a state where all the ligand signals are suppressed and the receptor signals are preserved has to be prepared first. This can be challenging because of adverse relaxation properties. Rapid relaxation (especially T_2 relaxation) generally associated with macromolecules can easily drain the receptor signals and affect the sensitivity of the experiment. To increase the sensitivity and reduce the experiment time, we propose a new experiment: reverse NOE pumping (RNP). In contrast to the original pumping experiment, this technique is designed to detect any signals transferred from the binding ligands to the receptor.

In an RNP experiment, a filter such as a relaxation (or isotope) filter is applied first to attenuate the receptor signal while preserving the ligand signals. We used a T_2 filter which was immediately followed by two 90° pulses separated by the mixing time t_m to allow signal transfer from the ligands to the receptor (Figure 1a). The ligands may lose signal intensity either through relaxation (for a nonbinding compound) or from both NOE pumping and relaxation (for a binding ligand). To detect the signal loss due to NOE pumping, a reference spectrum, which measures the signal loss by relaxation, is necessary and can be obtained by appending the same T_2 filter after the NOE experiment (Figure 1b). Because there are no unequivalent states prepared before the NOE, NOE pumping should not be detected and the spectrum

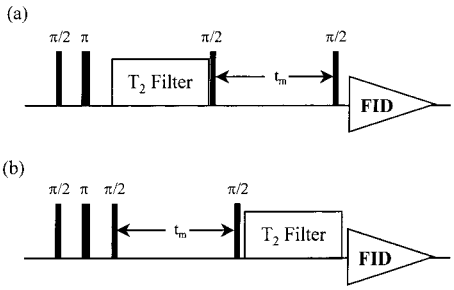

Figure 1. RNP experiment: (a) pulse sequence for RNP and (b) pulse sequence for referencing. The 180° pulse, which is optional, is used to refocus chemical shift during the preparation period of the NOE experiment. Typical NOESY phase cycle is applied.

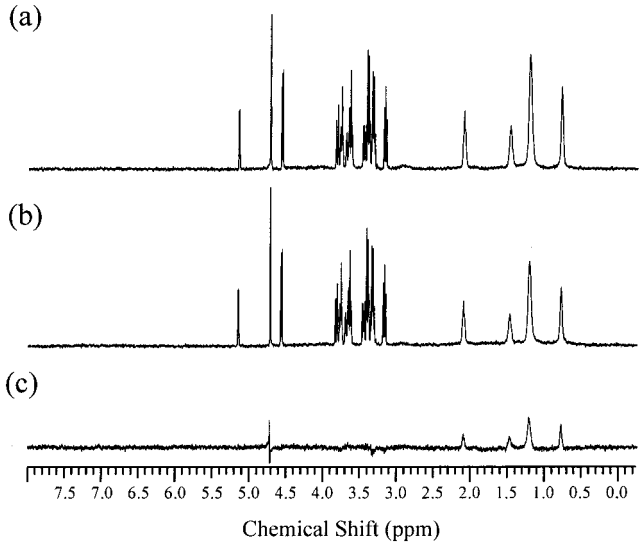

Figure 2. 1 mM octanoic acid, 1 mM glucose, and 20 μM HSA in D_2O: (a) reference spectrum obtained with Figure 1b pulse sequence; (b) RNP spectrum obtained with Figure 1a; and (c) difference spectrum of above two spectra. Here the water signal was suppressed by presaturation during recycle delay and the Carr−Purcell−Meiboom−Gill (CPMG) spin−echo pulse train was employed as a T_2 filter. The total spin−echo delay was 32ms. The NOE mixing time was 400ms. In each spectrum, 16 transients were accumulated and the experiment time was around 2 min.

records the signals after attenuation by relaxation. Comparison of spectra obtained from two experiments (Figure 1a,b) reveals the ligand that is involved in binding to the protein. Because the two spectra are taken on the same sample by interleaved acquisition, subtraction artifacts (from variation of experimental conditions, e.g. temperature fluctuation and shimming of the system) are minimized.

To demonstrate the ability to identify compounds that bind to macromolecules by the RNP experiment, we analyzed the interaction of the protein receptor Human Serum Albumin (HSA) with several binding ligands (unbranched fatty acids) in the presence of a nonbinding compound glucose.[5] Here a Carr−Purcell−Meiboom−Gill (CPMG) pulse train[6] was employed as the T_2 filter. The result is illustrated using octanoic acid sample in Figure 2. Comparing the RNP spectrum in Figure 2a with reference spectrum in Figure 2b, we can see that signals from

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(5) No buffer solution other than D_2O is used to demonstrate that this method is resistant to any chemical shift change raised from chemical environment variation (e.g. pH). All the experiments are done at 300 K on a Bruker DMX at 500 MHz with a 5 mm inverse triple nuclear z gradient probe.

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Table 1. The Ratio of Each Potential Ligand Signal Integral (over the Whole Molecule) in RNP Spectrum Versus That in the Reference Spectrum and the Binding Affinity of Each Fatty Acid

sample	integral ratio		K_1 (10^4/M) of the fatty acid[7]
	fatty acid	glucose[a]	
1 mM C_8, 1 mM glucose, and 20 μM HSA	0.82	1.04	2.579 ± 0.476
1 mM C_4, 1 mM glucose, and 20 μM HSA	0.84	1.04	1.110 ± 0.161
1 mM C_2, 1 mM glucose, and 20 μM HSA	0.95	1.02	N/A

[a] The integral ratio for glucose is slightly larger than 1. This is caused by the integration of pumped protein signal that is overlapped with the glucose signal. This is verified by the observation that the ratio is approaching to 1 as the affinity of the ligand decreases (i.e. the pumped protein signal decreases).

the nonbinding compound (glucose) are similar in the two spectra. On the other hand, the signal integral of the binding ligand (octanoic acid) in Figure 2a is only about 82% of the integral in Figure 2b. The difference spectrum (Figure 2c) clearly shows the compound that has a binding interaction with the protein.

The interactions of HSA with acetic acid (C_2) and HSA with butyric acid (C_4) in the presence of glucose were also studied by RNP. The ratios of signal integral of each compound in the spectrum with RNP as compared to the reference spectrum are listed in Table 1. The RNP experiment can clearly identify the binding ligands (fatty acids with the integral ratio less than 1) as opposed to nonbinding compound (glucose with the integral ratio around 1), even in the case of acetic acid where the interaction is very weak. These short- and medium-chain fatty acids are bound to the same class of binding sites on the HSA and their affinity is known to increase with the chain length.[7] Our pumping experiment indicates that the amount of signal pumped also increases with the chain length (i.e. binding affinity). This can potentially be used to rank the affinity of the ligands involved in the same class of interactions.

The 1D RNP method is a very powerful method to rapidly pinpoint the active compounds that can bind to the target from a mixture and thus could be performed prior to using expensive labeled protein in an HSQC experiment. Most importantly, the amount of protein necessary in this experiment is drastically reduced allowing studies to be performed on a protein in which the expression is difficult or with limited solubility.

Recently, saturation transfer difference (STD) NMR spectroscopy was reported to screen compounds with binding activity in a heterogeneous system utilizing the high-resolution magic angle spinning (HR-MAS) technique.[8] This method relies on selective saturation of resonances of a receptor protein and by spin diffusion efficiently spreading over the entire protein and subsequently to the binding ligands. Therefore the cross-relaxation rate of the protein is a critical factor in the experiment. Apparently, this method is more accessible for an immobilized receptor (with much longer correlation time) in a heterogeneous system than for a mobilized target (with shorter correlation time) in a homogeneous system. Also the selective irradiation of protein signals without affecting ligand signals can be challenging in complex systems where a very crowded NMR spectrum is often observed.

The one-dimensional RNP experiment presented here offers several advantages over the HR-MAS STD experiment: (1) There is no need for HR-MAS probes. This eases both sample handling and automation. In addition, the ligand signals retain their coupling information allowing the identification of spin systems to be performed readily using 1D NMR spectroscopy. (2) The system can be homogeneous and does not need the protein to be attached to a solid support, which in principle could change the binding ability of the protein making the method less general. (3) There is no need for reference experiments using plain (no receptor bound) solid phase to rule out nonspecific binding to the solid support. (4) ^{15}N-HSQC studies can be performed on the same sample to ensure that binding of the ligand is occurring at the active site.[9]

Because it is relatively easier to suppress receptor signals while conserving small ligand signals, RNP offers higher sensitivity and shorter experiment times. The concentration required for both the receptor and the ligands is also lowered, which makes the system more tolerable by the biologic target and more manageable in the studies of compounds with limited solubility. The use of the new cryogenic NMR probe, which can significantly increase the signal-to-noise ratio in NMR spectra, could drop the sample concentration requirement even further.[10] The RNP using a T_2 filter is currently limited to the system with large differences in T_2 relaxation time between the receptor and ligands. This generally requires the binding ligands be in fast exchange between free and bound states. In the case where isotope-labeled material is available, an isotope-editing technique could be used to prepare unequivalent states without the T_2 relaxation limitation and can be applied to slow exchanging systems. This method should add to the power of the NMR screening techniques for affinity studies and can be easily incorporated into the sample automation program for high throughput.

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