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Study of molecular interactions with ^{13}C DNP-NMR

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ABSTRACT

NMR spectroscopy is an established, versatile technique for the detection of molecular interactions, even when these interactions are weak. Signal enhancement by several orders of magnitude through dynamic nuclear polarization alleviates several practical limitations of NMR-based interaction studies. This enhanced non-equilibrium polarization contributes sensitivity for the detection of molecular interactions in a single NMR transient. We show that direct ^{13}C NMR ligand binding studies at natural isotopic abundance of ^{13}C gets feasible in this way. Resultant screens are easy to interpret and can be performed at ^{13}C concentrations below μM. In addition to such ligand-detected studies of molecular interaction, ligand binding can be assessed and quantified with enzymatic assays that employ hyperpolarized substrates at varying enzyme inhibitor concentrations. The physical labeling of nuclear spins by hyperpolarization thus provides the opportunity to devise fast novel *in vitro* experiments with low material requirement and without the need for synthetic modifications of target or ligands.

1. Introduction

Molecular recognition between ligands and targets plays a central role in biological functionality and pharmacological activity. NMR spectroscopy has emerged as a reliable technique in the screening for biomolecule–ligand interactions, even when those interactions are weak. These NMR screening methods can be subdivided into ligand-detected and target-detected techniques [1]. Target-detected screening poses some serious restrictions on concentration, size and isotope labeling of the target. Ligand-detected techniques can circumvent these limitations, if knowledge of the binding site on the target is not required. NMR spectroscopy provides sufficient resolution to directly identify binders in complex mixtures, thus making the method amenable to high-throughput screening [1–4]. A variety of NMR parameters can be exploited for the detection of molecular interactions. These NMR parameters include changes in translational or rotational diffusion of the ligand upon binding or the direct transfer of magnetization between ligand and target [5–14].

NMR ligand screening methods have largely relied on ^{1}H NMR spectra of the ligand both due to its broad applicability and due to the high sensitivity of ^{1}H NMR [1,2,7–9,11,15]. As an alternative, ^{19}F NMR has been employed for ligands containing fluorine atoms, taking advantage of the broad chemical shift dispersion of ^{19}F compared to the more commonly employed proton [16]. In result, more complex compound mixtures can be subjected to ^{19}F NMR spectroscopy without running into chemical shift overlap [1]. The combined benefit of broad applicability to small organic ligands and large chemical shift range could be attained by using ^{13}C as a screening nucleus, which has a shift range ~20 times larger than ^{1}H. The low natural abundance of ^{13}C (1.1%) however implies poor sensitivity and excessive acquisition times and therefore impedes the direct use of 1D ^{13}C NMR screening in absence of isotope labeling (Fig. 1A) [17]. This sensitivity problem of ^{13}C NMR screening at natural abundance of ^{13}C can be easily overcompensated by hyperpolarization (Fig. 1). Polarization enhancements $>10^4$ can be achieved, if electron spin polarization is transferred to nuclear spins by dynamic nuclear polarization (DNP). After subsequent dissolution, nuclear spin polarization is temporarily retained and the sample becomes amenable to high resolution NMR spectroscopic studies [18,19]. Thus, versatile single-scan assays of ligands at ^{13}C concentrations on the order of 1 μM (e.g. ~100 μM natural abundance ligand) get feasible upon enhancing the spin polarization of the ligands.

2. Results and discussion

Hyperpolarized ^{13}C NMR ligand screening was performed as a competitive assay to characterize binding of salicylate and ascor-

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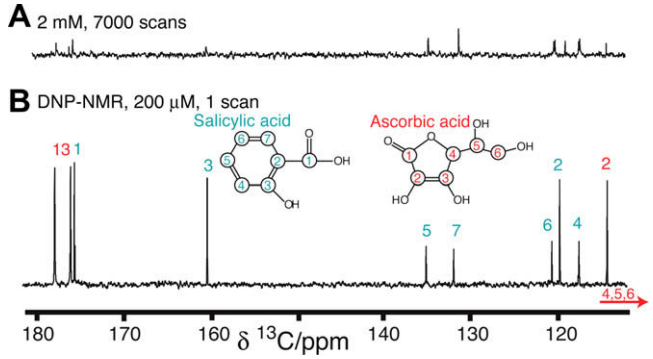

Fig. 1. Comparison of ^{13}C NMR spectra of a ligand mixture of salicylate and ascorbate recorded on a state-of the art NMR spectrometer (18.7 T) with cryogenic probe for signal detection (A) and recorded with DNP-NMR on a 9.4 T spectrometer (B). Ligands were 10-fold concentrated for conventional NMR (A) to allow signal detection after 7000 scans (20 h).

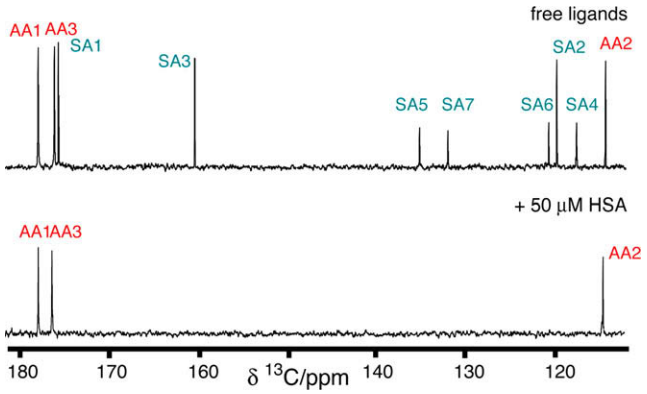

Fig. 2. Spectra of hyperpolarized salicylate and ascorbate in absence (top) and presence (bottom) of 50 µM human serum albumin. Ligands are present at 200 µM natural ^{13}C isotopic abundance. Signal was detected with a 90° detection pulse in a 9.4 T magnet. Salicylate signal is purged in presence of target due to faster polarization loss and exchange line broadening.

bate to human serum albumin (HSA). DNP-NMR provides sufficient signal to allow ^{13}C NMR ligand screening experiments that would be impossible or impracticable at thermal NMR spin polarizations. In addition, a T_1 time of 10–50 s for quaternary carbons in small to medium-sized organic ligands implies a minor signal loss of free ligands during the transfer between *ex situ* DNP polarizer and detecting NMR spectrometer. To illustrate this, Fig. 1 compares ^{13}C spectra recorded on a state-of-the-art spectrometer (18.7 T magnet, cryoprobe) by accumulation of 7000 experiments (Fig. 1A, 20 h experiment time) and single scan DNP-NMR spectra of a 10-fold diluted sample recorded on a 9.4 T spectrometer (Fig. 1B). Here, ligands were hyperpolarized to yield ^{13}C spin polarizations of 12–14% in both ligands, corresponding to signal enhancements on the order of 15,000 relative to conventional NMR experiments on a 9.4 T spectrometer. Fig. 1B shows that ample signal can be obtained in this way on 200 µM samples with natural ^{13}C isotope abundance (i.e. 2 µM ^{13}C concentration). Conventional NMR experiments on the other hand require exorbitant experiment time even for 10-fold concentrated sample due to low sensitivity and long recycle delays as result of the long ^{13}C T_1 relaxation times in small ligands. Considering the sample concentration, number of scans, pulse angle and signal-to-noise ratio, the DNP spectrum of Fig. 1A hints at an enhancement by more than three orders of magnitude relative to conventional spectra recorded with cryoprobe detection and Ernst angle conditions on a shimmed sample. The ^{13}C spectra further indicate the benefit of large chemical shift dispersion in ^{13}C NMR spectra and the possibility to use more complex ligand mixtures.

The reference spectrum of 200 µM salicylate and ascorbate in absence of the target was compared to a spectrum in the presence of 50 µM target (Fig. 2). Both spectra were obtained at a time $\Delta = 5$ s after mixing of the dissolved polarization-enhanced ligands with the target of choice. In presence of the target, signal of the stronger binder salicylate ($K_d = 5 \times 10^{-6}$ M) is quenched more strongly than the signal of the weaker binder ascorbate ($K_d = 2.8 \times 10^{-5}$ M) [20].

Pronounced signal loss of the stronger binding salicylate follows from the larger fraction of time in the faster-relaxing target-bound state and from exchange line broadening. Dynamic nuclear polarization creates a non-equilibrium state and the enhanced spin polarization returns to its equilibrium with the relaxation time T_1. At low magnetic field, $1/T_1$ is proportional to the rotational diffusion time and thus to a good approximation inversely proportional to the molecular weight. As a result, relaxation of enhanced polarization can be used to distinguish free and bound states, as enhanced polarization is lost faster in macromolecule-bound ligands than in free ligands. Notably, DNP-NMR obviates the need to use ligand in large excess relative to the target so that even strong ligand binding can be detected in form of signal loss upon complexation.

In the case of weak ligand binding, the observed relaxation rate $1/T_1^{obs}$ of the ligand is given by the weighted average of relaxation rates in free and bound state according to Eq. (1) (see Ref. [12]).

$$\frac{1}{T_1^{obs}} = f_L^{bound}\frac{1}{T_1^{bound}} + (1 - f_L^{bound})\frac{1}{T_1^{free}} \quad (1)$$

where f_L^{bound} is the fraction of bound ligand in dependence on target and ligand concentration as well as dissociation constant K_d

$$f_L^{bound} = \frac{((K_d + [T] + [L]) - \sqrt{(K_d + [T] + [L])^2 - 4[T]\cdot[L]})}{2\cdot[L]} \quad (2)$$

with ligand and target concentrations $[L]$ and $[T]$, respectively.

Under the conditions applied in Fig. 2, i.e. 200 µM ligands and 50 µM target, the published dissociation constants of HSA with ascorbate and salicylate translate into concentrations [HSA] = 1.3 µM, [HSA·SA] = 40.2 µM and [HSA·AA] = 8.6 µM [21]. Due to sizeable differences of T_1^{bound} and T_1^{free}, $1/T_1^{obs}$ contains large contributions from ligand binding to a macromolecule, if only few percent of the ligand are target-bound (Eq. (1)). Accordingly, binding of only 4.3% ascorbate (8.6 µM/200 µM) translates into a detectable polarization loss of 40% (Fig. 2) of the ascorbate carbonyl signal during 5 s transfer in presence of enzyme. According to Eq. (1), this polarization loss translates into an estimate for $1/T_1^{bound} - 1/T_1^{free} = -\ln(0.6)/(0.043\cdot 5\text{ s})$ and yields a T_1^{bound} of 430 ms in agreement with low-field carbonyl ^{13}C T_1 values in proteins at natural ^{13}C isotopic abundance [22]. Overall, binding of ligand even for only a small fraction of time gets detectable in an altered T_1^{obs}. Weak interactions with K_d up to ~mM can then be detected for ligand and target concentrations on the order of 100 µM (Fig. 3).

The signal-to-noise ratio >50 for 200 µM hyperpolarized ligand at natural isotope enrichment (Fig. 2) indicates that binding studies can be performed under conditions that do not require excess of ligand relative to commonly employed target concentrations. Under these conditions (Fig 3, black curve), strong binders should lead to a strong signal attenuation due to small fractions of free ligand. The current sensitivity can be additionally increased by using cryogenic NMR probes to reduce thermal noise ~4-fold in the NMR signal detection [17] or by employing high field polarizers (4.6 T) to roughly double the attainable polarization [23]. In consequence,

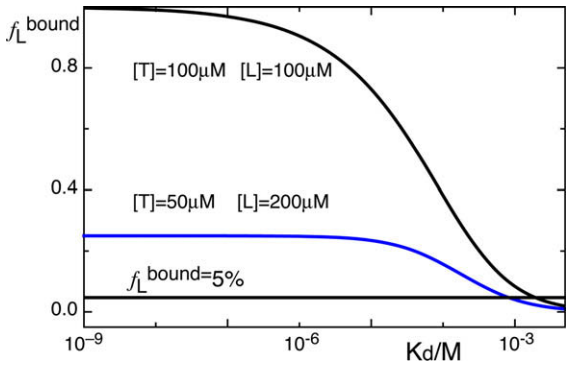

Fig. 3. Fraction of bound ligand as function of K_d for various ligand and enzyme concentrations. Strong effects of ligand binding on T_1 relaxation imply the capacity to detect ligand binding to macromolecular targets, if the target-bound fraction of ligand is more than few %. Horizontal lines indicate a population of 5% bound ligand.

relaxation screening with hyperpolarized signal is feasible with low ligand concentrations over a broad range of K_d values.

Depending on exchange kinetics and relaxation behavior, binding may also get detectable as a resonance frequency shift for binders in fast chemical exchange in initial lead screens. The detection of signals from a binding ligand in presence of its target is facilitated by injecting hyperpolarized ligand into a solution of the target equilibrated to 37 °C inside the NMR spectrometer. Hilty and coworker have recently described a high pressure injection system with a reaction dead time of 300 ms [24]. Even for manual injections of hyperpolarized sample into assays of a binding reaction, short times Δ before signal detection and reduced low-field relaxation of the complex result in detectable signal of binders (Fig. 4) that show most pronounced polarization loss, line broadening and shift changes for the stronger binding salicylate.

Notably, DNP-NMR permits that ligand binding to enzymes can be assessed not only as a molecular interaction, but also via real-time activity assays [24–28] of enzyme in presence and absence of an inhibitor. As an example, the inhibition of jack bean urease by acetohydroxamate was monitored with real-time initial rate assays of urease activity *in vitro*. Inhibitors of urease activity have recently attracted attention as prospective anti-ulcer drugs [29]. Urease is produced by a number of pathogens and thus has additional diagnostic value for the detection of pathogens.

Functional ^{13}C DNP-NMR assays were conducted by monitoring the decomposition of 90 μM hyperpolarized ^{13}C-urea (δ^{13}C = 163.1 ppm) to carbon dioxide (125.1 ppm) and ^{13}C-bicarbonate (161.3 ppm) by urease in 10 mM phosphate buffer of pH 7.6 (Fig. 5). For assays of inhibitor binding, the reaction was assayed after urease (4 U/ml) had been pre-incubated with the slowly binding inhibitor acetohydroxamate at varying concentrations for 30 min. Enzymatic activity in dependence of inhibitor concentration is displayed in Fig. 5. An IC_{50} value of 5×10^{-5} M of acetohydroxamate against jack bean urease was determined in this way.

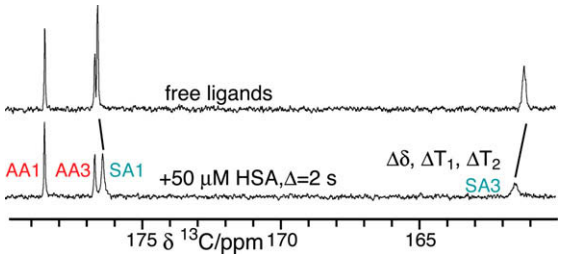

Fig. 4. Spectra of 200 μM hyperpolarized salicylate and ascorbate at natural ^{13}C isotopic abundance injected into buffer (top) and into solutions of 50 μM human serum albumin. Spectra are recorded 2 s after injections. ^{13}C signals of the salicylate are detected in the binding reaction and show attenuated signal amplitude, line broadening and shift changes.

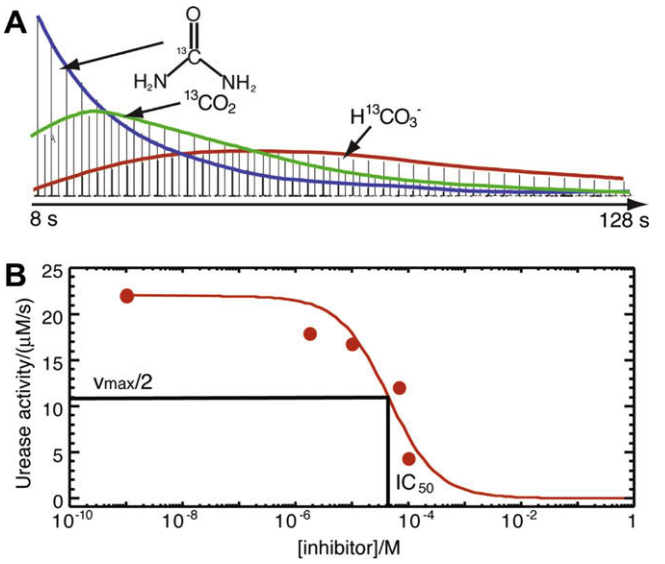

Fig. 5. Functional screen of jack bean urease activity. Hyperpolarized ligand, here ^{13}C-urea, provides sufficient signal for real time reaction assays *in vitro*. Diverse chemical species can be detected via their chemical shifts and quantified via their signal areas. NMR spectra were recorded every 3 s with 15° flip angle pulses (a). Enzymatic activity assays at varying concentrations of acetohydroxamate yield the IC_{50} of acetohydroxamate against urease (b).

Use of functional ^{13}C DNP-NMR allows to conduct inhibition experiments at low substrate concentrations relative to the Michaelis–Menten constant K_M (K_M = 3.8 mM for jack bean urease in phosphate buffer of pH 7.6) [30] and the dissociation constant is very well approximated by IC_{50} both in the case of competitive and noncompetitive inhibition [31]. A comparable value of 1.3×10^{-5} M for IC_{50} of acetohydroxamate against jack bean urease had previously been obtained using an assay based on an ammonium sensitive electrode [32]. Thus, DNP-NMR assays can be used in a variety of implementations for the detection of molecular interactions using either hyperpolarized ligands or hyperpolarized enzyme substrates. The use of hyperpolarized enzyme substrates has the benefit not only to detect binding, but at the same time to yield K_d values and identify functional effects of binders on enzyme activity.

3. Conclusions and outlook

In summary, we show that hyperpolarized NMR bears great promises for studies of molecular interactions. Binders can be identified either in ligand-detected assays of molecular interaction or in functional assays detecting the impact of effector binding on reaction rates. Hyperpolarized NMR yields unambiguous and easy to interpret results, which should be amenable to automation. NMR is by now an established and invaluable technique in ligand screening, but a central focus on the reduction of material usage and NMR experiment time has remained [6]. Technological advances such as those provided by polarization-enhanced NMR bear a great potential for alleviating the limitations of conventional NMR screening on sample amount, labeling and solubility as well as the detectable affinity range. Hyperpolarized single scan ^{13}C NMR should be particularly useful for interaction studies, where the precise identification of binding epitopes is not vital, and where ^{1}H NMR ligand spectra are crowded or require acquisition

times that are considered too long. It is further noted that signal enhancement by more than four orders of magnitude may imply the possibility to use ligands at natural ^{13}C isotopic abundance in order to collect structural information of the bound state by cross correlated relaxation experiments that else rely on ^{13}C labeling [33,34]. Due to the specific polarization enhancement of ligands of choice, the detection of ligand signal without interfering signals from the non-hyperpolarized target or other background may eventually even prove feasible for the direct detection of molecular targets in complex backgrounds such as blood serum or cell homogenates. The methodology is currently limited in throughput by the DNP polarization time (1 h in our case), which can be reduced by parallel polarization and separate dissolution of samples in polarizers with several separate sample spaces [35] and by optimized DNP protocols.

4. Experimental

4.1. Materials

Salicylic acid, ascorbic acid, human serum albumin, urease and ^{13}C-labelled urea were purchased from Sigma–Aldrich. Trityl radical OX063 was obtained from GE Healthcare, UK.

4.2. DNP preparations

A stock solution of salicylic acid and ascorbic acid was prepared by dissolving 23.9 μmol of both ligands in 150 μl DMSO to yield a ligand concentration of 136 μmol/g. Trityl radical OX063 (1.22 mg) was dissolved in 57 μl of this solution and 2 μl of a 14.5 mM 3-Gd stock solution were added, resulting in a radical concentration of 15 mM and a 3-Gd concentration of 0.5 mM. The sample was sonicated and vortexed and 5.5 mg of the preparation (0.7 μmol of each ligand) were frozen at liquid helium temperature in the prototype polarizer described in Ref. [19]. After a DNP period of one hour, ascorbic acid (0.7 μmol) and salicylic acid (0.7 μmol) were solubilized to 0.2 mM final concentration in 3.5 ml phosphate buffer (10 mM) of pH 7.6 containing 100 mg/l EDTA. In the reference experiment, the dissolved ligand sample was collected directly into a 10 mm NMR tube. In the target-binding experiment, the dissolved sample was transferred into a 10 mm NMR tube containing human serum albumin at a final concentration of 50 μM. Resultant ligand concentrations for reference and binding experiment are 200 μM.

For the functional assays, ^{13}C-labelled Urea was prepared in a water/glycerol 40:60 mixture with 15 mM trityl radical OX063 and frozen in the prototype polarizer [19] at a temperature of 1.2 K. Addition of a glass forming agent such as glycerol to the sample can prevent crystallization and ensure a homogenous distribution of the radical and target molecule. Microwave irradiation was performed at ~94 GHz in a field of 3.35 T. This irradiation was performed for 1 h. The polarized sample was dissolved in 3 ml of a heated phosphate buffer (pH 7.6, 10 mM), resulting in a sample temperature of approximately 37 °C. Spin polarizations were calculated from signal enhancements in hyperpolarized samples relative to ^{13}C isotope labeled standard at thermal equilibrium at 37 °C and 9.4 T.

4.3. NMR experiments

Hyperpolarized samples were mixed with 10 mM phosphate buffer or human serum albumin solutions in phosphate buffer prior to signal detection in a 9.4 T Varian spectrometer equipped with a room temperature probe to obtain the data of Figs. 1B and 2. Sample transfer was timed to 5 ± 1 s in this setup. Alternatively, samples were injected into buffer or protein solution equilibrated to 37 °C inside a 14.1 T Bruker DRX spectrometer equipped with a room temperature probe (Fig. 4). DNP spectra were recorded on 3 ml samples in 10 mm sample tubes. The thermal equilibrium spectrum of Fig. 1 was recorded with a 30° flip angle on a Bruker Avance 18.7 T spectrometer equipped with a TCI cryoprobe. All spectra were recorded with a spectral width of 240 ppm and acquisition time of 0.68 s. Spectra were processed with an exponential apodization function with 3 Hz line broadening.

4.4. Urease activity assays

About 16.4 μmol of ^{13}C-urea were hyperpolarized and solubilized in 3 ml of a 10 mM phosphate buffer of pH 7.6. Five hundred μl sample out of a dissolution volume of 3 ml were transferred to a 10 mm NMR tube containing 2.5 ml phosphate buffer and 12 U urease. A series of 1D spectra was collected as a function of time with 15° flip angle pulses and an acquisition time of 0.68 s and on a Varian 9.4 T spectrometer. Spectra were processed with an exponential apodization function with 3 Hz line broadening. Urease activity was assessed from the initial rate of $^{13}CO_2$ formation. In order to measure inhibition of the urease reaction, the competitive urease inhibitor acetohydroxamic acid was added at varying concentrations from 1 to 100 μM. The NMR signal intensities of urea in the individual experiments were used to determine enzymatic activity. Measurements of activity in dependence of inhibitor concentration provide an estimate for the inhibitor concentration needed to inhibit the urease reaction to half activity (IC_{50} value).

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We acknowledge GE for funding this work. The high-field spectrum of Fig. 1 was recorded on the Bruker 800 MHz spectrometer equipped with a TCI cryoprobe at the Danish Instrument Center for NMR Spectroscopy of Biological Macromolecules.

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