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Nuclear Magnetic Resonance of Hyperpolarized Fluorine for Characterization of Protein−Ligand Interactions

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Supporting Information

ABSTRACT: Fluorine NMR spectroscopy is widely used for detection of protein−ligand interactions in drug discovery because of the simplicity of fluorine spectra combined with a relatively high likelihood for a drug molecule to include at least one fluorine atom. In general, an important limitation of NMR spectroscopy in drug discovery is its sensitivity, which results in the need for unphysiologically high protein concentrations and large ligand:protein ratios. An enhancement in the ^{19}F signal of several thousand fold by dynamic nuclear polarization allows for the detection of submicromolar concentrations of fluorinated small molecules. Techniques for exploiting this gain in signal to detect ligands in the strong-, intermediate-, and weak-binding regimes are presented. Similar to conventional NMR analysis, dissociation constants are determined. However, the ability to use a low ligand concentration permits the detection of ligands in slow exchange that are not easily amenable to drug screening by traditional NMR methods. The relative speed and additional information gained may make the hyperpolarization-based approach an interesting alternative for use in drug discovery.

High-resolution NMR spectroscopy is an important technique in drug discovery.[1−3] In an industrial setting, NMR spectroscopy is mainly used to study interactions between ligands and the target protein, either through protein observation, ligand observation, or indirect detection via a reporter ligand. Chemical shift perturbation spectroscopy of proteins in the presence of ligand can be used to demonstrate specific binding and to map the binding site, providing a high level of detail. In the context of screening large compound libraries,[4] where the primary interest lies in high-throughput determination of ligand binding, ligand-based observation is preferred. A strength of NMR spectroscopy lies in its ability to detect even weak binding, with dissociation constants of up to 1 mM,[3,5] which makes it the method of choice for the screening of fragment libraries.

Apart from work with carefully composed fragment libraries during the initial phase of a drug discovery campaign, multiple challenges remain for the general applicability of ligand-observed NMR analysis in drug discovery. Foremost, the obtainable signal-to-noise ratio is relatively low. To acquire workable ^{1}H spectra within 5−15 min, ligand concentrations of 100−200 μM are used in standard screening setups. This requirement excludes many ligands from analysis because of their often low solubility in water. Furthermore, detection of binding by ligand-observed experiments is generally limited to ligands in fast exchange, excluding the most potent, tightly binding ligands from the analysis. Most lead compounds with druglike properties fall into one of these categories (low solubility, slow exchange) and can therefore be studied only by expensive protein-observed NMR experiments[6] or, if a suitable reporter molecule is available, by reporter screening.[7,8]

Hyperpolarization of nuclear spins represents a significant opportunity to identify new leads due to a signal gain of several orders of magnitude. Dissolution dynamic nuclear polarization (DNP)[9] is well-suited for application to this problem, since most small molecules can be brought to a spin-polarized state and delivered in dilute form for use in an NMR experiment.[10] This technique has in the past most commonly been applied to ^{13}C nuclei,[11−13] which often exhibit slow spin relaxation that reduces the loss of polarization prior to the NMR experiment, among many applications also allowing the detection of ligand binding.[14] In this work, ^{19}F DNP is exploited. Experimentally, NMR detection of hyperpolarized fluorine is enabled by rapid sample injection of the polarized aliquot, which counteracts the relatively short relaxation time and reduces loss of the hyperpolarized signal prior to the NMR experiment.[11,15]

In many respects, fluorine is an ideal target nucleus for the study of protein−ligand interactions by NMR spectroscopy. The use of fluorine is motivated by its importance in pharmaceuticals, where these atoms impart specific properties pertaining to electronic structure, hydrophobicity, or metabolic stability.[16,17] In addition, 20% of marketed drugs contain fluorine.[18] For the purpose of NMR analysis, the high gyromagnetic ratio and 100% natural abundance of ^{19}F lead to a high signal intensity. With state of the art equipment in conventional NMR spectroscopy, it is possible to screen libraries of compounds containing CF_3 and CF groups at concentrations of 18 and 35 μM, respectively.[19] The chemical shift of ^{19}F is sensitive to the local environment of the nucleus and to the change that occurs upon binding. This can lead to strong exchange broadening effects, aiding detection of weakly binding compounds.[4,20] Additionally, the large chemical shift anisotropy of ^{19}F nuclei also leads to strong line broadening at slow molecular tumbling, as, for example, upon binding to a protein with a long rotational correlation time.[20] Finally, there is usually no background signal from protein or buffer components, and a pharmaceutical typically contains only one or a small number of fluorine atoms, reducing signal overlap. The resulting simplicity of an ^{19}F NMR

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spectrum allows screening of mixtures of large numbers of compounds.

Three representative fluorinated ligands for the serine protease trypsin are shown in Figure 1. These ligands exhibit different binding kinetics in the slow and fast exchange regimes and have binding constants covering nearly 3 orders of magnitude.[21] These ligands were originally discovered by different ^{19}F-based assays [*f*luorine chemical shift *a*nisotropy and e*x*change for *s*creening (FAXS)], and their binding modes have been characterized with crystal structures.[21] Figure 2 shows hyperpolarized ^{19}F spectra of a mixture of the three fluorinated ligands together with sodium trifluoroacetate (TFA; used as an internal standard). Hyperpolarization increased the TFBC, TFMCPP, FMBC, and TFA signals by factors of 800, 450, 1500, and 3000, respectively, relative to the signals of thermal polarization in the 400 MHz NMR spectrometer used here.

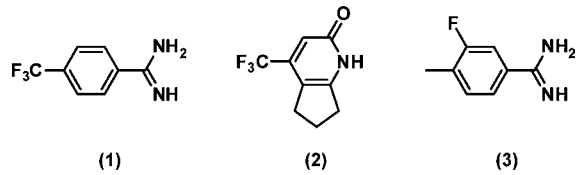

Figure 1. Structures of three fluorinated ligands binding to the serine protease bovine trypsin: (1) 4-(trifluoromethyl)benzenecarboximidamide hydrochloride (TFBC), (2) 4-(trifluoromethyl)-1,5,6,7-tetrahydro-2*H*-cyclopenta[*b*]pyridin-2-one (TFMCPP), and (3) 3-fluoro-4-methylbenzenecarboximidamide hydrochloride (FMBC).

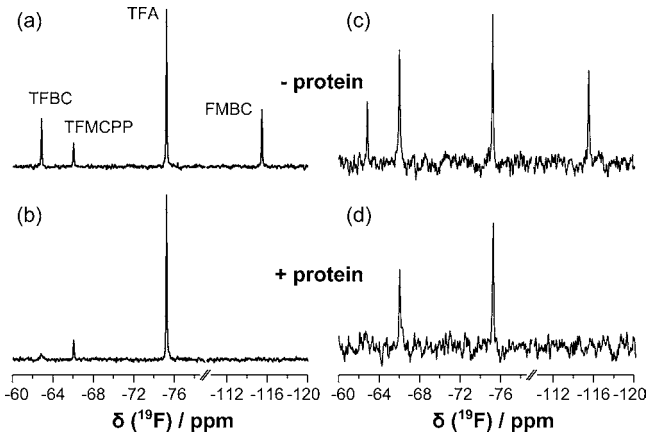

Figure 2. Hyperpolarized ^{19}F NMR spectra of TFBC (−62.8 ppm), TFMCPP (−66.0 ppm), FMBC (−115.4 ppm), and TFA (−75.2 ppm) acquired on a 400 MHz spectrometer equipped with a room-temperature broad-band probe head tuned to fluorine. Shown are spectra of (a, b) 10 μM TFBC, 10 μM TFMCPP, 10 μM TFA, and 30 μM FMBC in the (a) absence and (b) presence of 50 μM trypsin and (c, d) 1 μM TFBC, 5 μM TFMCPP, 1 μM TFA, and 3 μM FMBC in the (c) absence and (d) presence of 50 μM trypsin. Spectra (a) and (b) were acquired using a single $^{\pi}/_{2}$ excitation pulse followed by a 100 ms CPMG filter. Spectra (c) and (d) were acquired after a single $^{\pi}/_{2}$ excitation pulse.

From a comparison of the spectra recorded in the absence (Figure 2a) and presence of protein (Figure 2b), binding of the three ligands can readily be observed from several NMR parameters, including reduction in peak height, increase in line broadening, and chemical shift change. To increase the effects of spin−spin relaxation of the ligand in bound form, a Carr−Purcell−Meiboom−Gill (CPMG)[22,23] filter was applied. In the presence of trypsin, significant signal loss was observed for the strong binder FMBC, line broadening for the intermediate binder TFBC, and only a small signal loss for the weak binder TFMCPP. The difference in peak height for this molecule was further accentuated by increasing the CPMG time (Figure S1 in the Supporting Information). The signal intensity and chemical shift of the control molecule, TFA, was used as a reference, since there was no interaction with the protein. Traces (c) and (d) in Figure 2 are experiments carried out near the detection limits of the instrumentation used, with 1 μM TFBC, 5 μM TFMCPP, and 3 μM FMBC. It would be straightforward to reduce the detection limit further by using a cryogenically cooled NMR probe or higher NMR field strength.

Binding of the strong binder FMBC, which exhibits binding kinetics in the slow exchange regime, can clearly be detected through total signal loss, which occurs because of an excess of protein in the DNP experiment. In contrast, conventional ligand-based NMR experiments often use a 10- to 20-fold excess of ligand. For ligands in the slow exchange regime, conventional experiments give rise to only a small reduction in the overall signal, resulting in unreliable detection of binding.[24] Furthermore, multiple ligands are often tested simultaneously in one sample for drug screening. If more than one ligand binds to the protein competitively in conventional NMR experiments, the weaker binder may not be detected because the stronger binders occupy most of the binding sites of the protein.[20] This problem is also eliminated by using ligand concentrations smaller than the protein concentration in DNP experiments with multiple ligands.

As an alternative to 1D spectroscopy, weakly binding ligands can readily be identified by measuring the transverse relaxation rate (R_2). Single-scan CPMG experiments are applicable to DNP-polarized samples under the condition that only one ^{19}F signal is present. Signal decays from such experiments, involving 174 μM TFMCPP in the presence or absence of 88 μM trypsin, are shown in Figure 3. Fits of the two traces to single exponentials indicated relaxation rates of 2.2 and 0.59 s^{-1} for the samples with and without protein, respectively. These rates are in agreement with values obtained from conventional CPMG experiments (2.0 and 0.54 s^{-1}; Figure S2). The

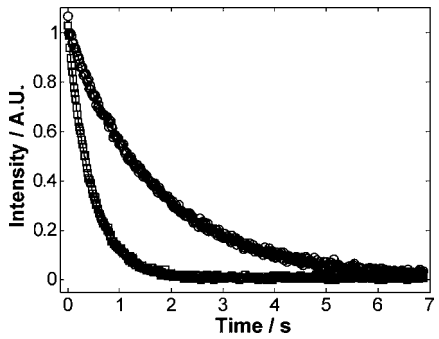

Figure 3. Magnitudes of the time-domain signals of 174 μM DNP-polarized TFMCPP in the presence (□) and absence (○) of 88.0 μM trypsin. One averaged data point is shown for each time interval between π pulses in a CPMG experiment, and the delay between adjacent π pulses was 420 μs. Background signals from the NMR probe were removed by subtracting the signal acquired using the same pulse sequence without a sample in the magnet. Data were independently normalized to unit intensity at t = 0.

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difference in R_2 values clearly indicates that TFMCPP interacts with the protein. In contrast, R_2 values of the control molecule TFA, which were measured from separate samples, did not show a significant change in the presence (0.53 s^{-1}) and absence (0.50 s^{-1}) of the same concentration of trypsin (Figure S3).

In addition to simple determination of binding, it can be of interest to quantify the strength of the protein−ligand interaction, expressed in the form of the dissociation constant, K_D. The fraction of bound ligand, p_b, is given by

$$p_b = \frac{c_P + c_L + K_D - \sqrt{(c_P + c_L + K_D)^2 - 4c_P c_L}}{2c_L} \quad (1)$$

where c_P and c_L are the total protein and ligand concentrations, respectively.[25] In traditional NMR experiments for the determination of K_D, fast exchange between the bound and free forms of the ligand is often assumed. In this case, two readily observable spectral parameters, the line width at half-maximum, $\nu_{1/2}$, and the change in chemical shift, $\Delta\delta$, are proportional to p_b.[19] A fit of one of these experimentally determined quantities to eq 1 can then be used to estimate K_D. In the hyperpolarized experiments used here, the same approach is in principle viable. Since this method relies on comparison of spectra obtained from different stopped-flow sample injections,[15] the precision of the measurement can be greatly improved by comparing the chemical shift or line width of the ligand under study to that of a nonbinding reference in order to remove the effect of variations between experiments. Practically, we prefer the measurement of the line width, which is easier to determine than the chemical shift of the typically broad lines. The parameter of interest derived from the hyperpolarized experiment is then the change in line width as a function of ligand concentration,

$$\Delta\Delta\nu_{1/2}(c_P, c_L) = [\nu^L_{1/2}(c_P, c_L) - \nu^{ref}_{1/2}(c_P, c_L)] - [\nu^L_{1/2}(0, c_L) - \nu^{ref}_{1/2}(0, c_L)] \quad (2)$$

Carrying out a titration of protein or ligand concentration requires a new hyperpolarized sample for each data point, so the number of data points should be chosen judiciously. Titrations using five points are shown in Figure 4. The change in line width can then readily be fit to the proportionality relation

$$\Delta\Delta\nu_{1/2}(c_P, c_L) = p_b \Delta\Delta\nu_{1/2,max} \quad (3)$$

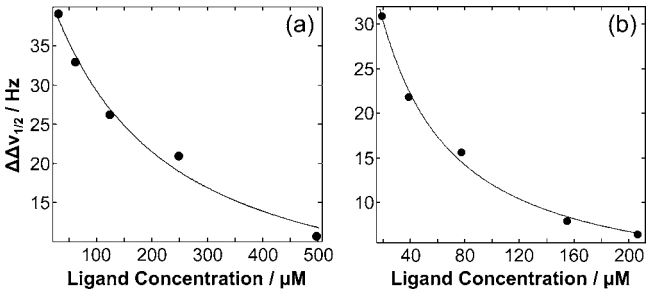

Figure 4. Titration of trypsin with the DNP-polarized ligands (a) TFBC and (b) FMBC. Each data point represents $\Delta\Delta\nu_{1/2}$ calculated according to eq 2 using TFA as the reference. The fits to eq 3 are indicated by the solid lines. K_D values of 148 (66−230) and 24 (12−36) μM were obtained for TFBC and FMBC, respectively (the 95% confidence ranges obtained from the individual fits are indicated in parentheses). The resulting $\Delta\Delta\nu_{1/2,max}$ values were 299 (191−407) and 167 (130−230) Hz, respectively. The titrations used (a) 26 and (b) 9 μM trypsin.

The fit determines two independent parameters, K_D and the apparent maximum change in line width, $\Delta\Delta\nu_{1/2,max}$. For comparison, titrations using conventional NMR spectroscopy and a K_D determination by isothermal titration calorimetry (ITC) are shown in Figures S4 and S5. The results obtained from the various experimental measurements are summarized in Table 1.

Table 1. Summary of K_D Determination

ligand	method	K_D (μM)[a]	$\Delta\Delta\nu_{1/2,max}$ (Hz)	no. of expts
TFBC	DNP NMR titration	142 ± 6	284 ± 13	5
	thermal NMR titration	122, 133	301, 280	2
	ITC titration	86	N/A	1
FMBC	DNP NMR titration	15, 24	167, 217	2
	DNP NMR peak fitting	35 ± 18	N/A	10

[a]Standard deviations of values obtained from more than two experiments are indicated; otherwise, the individual values are given. The 95% confidence interval obtained from each individual fit was larger than the spread of values; for typical ranges, see Figure 4.

Equation 3 is true only in the case of fast exchange; in other cases, significant nonlinearities can be introduced as a result of exchange broadening. Consideration of the exchange contribution is not specific to the hyperpolarized experiment. However, in the hyperpolarized experiment, because of the enhanced sensitivity, a larger range of values for p_b becomes accessible, and the exchange effects can become more important. The evolution of the spin system under the influence of chemical exchange was examined on the basis of the Bloch equations (Figures S6 and S7).[26,27] From the simulations it became evident that under the experimental conditions examined, the line broadening depends linearly on p_b for small values of p_b ($p_b \lesssim 0.2$) even when there is a significant exchange broadening. In this case, the parameter $\Delta\Delta\nu_{1/2,max}$ obtained from eq 3 loses its significance. However, the accuracy of the K_D values obtained is not affected.

For the strongly binding ligand FMBC, the signals for the free and bound forms can be detected separately since the ligand is in slow exchange. In this case, K_D can also be calculated from a single one-dimensional spectrum (Figure 5 and eq 1).

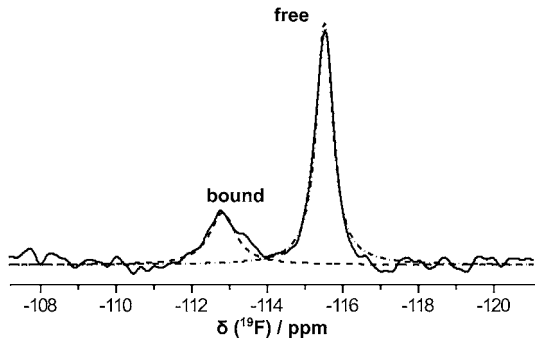

Figure 5. Hyperpolarized ^{19}F spectrum of 50 μM FMBC in the presence of 32 μM trypsin. The fraction of bound ligand, p_b, was directly determined from the ratio of the integrals obtained by peak fitting for the free (dot-dashed trace) and bound (dashed trace) forms, yielding a K_D value of 34.3 μM.

The obtained K_D values (Table 1) are on the same order as the value from the ligand titration and that from previous research, where a value of <20 μM was determined.[21] Since

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the determination of K_D in this way relies on the observation of broad signals, which concomitantly lowers the signal-to-noise ratio, the use of hyperpolarization is particularly beneficial for high-affinity ligands with nanomolar to low-micromolar K_D values.

In summary, NMR experiments based on hyperpolarized fluorine that permit the identification and quantification of ligand binding over a wide range of K_D values and with different binding kinetics have been shown. Hyperpolarization allows a wide range of protein to ligand ratios spanning up to 6 orders of magnitude to be accessed without the need for very long measurement times. The concentration of the ligand can be varied over a larger range (typically 1−200 μM) than in conventional NMR experiments, as can the concentration of the protein (0.1−100 μM). The ability to record spectra at low ligand concentration with a relatively high signal-to-noise ratio is an important advantage that (i) enables direct detection of binding, especially for stronger binders in the slow exchange regime; (ii) allows the study of ligands with low solubility in aqueous buffer; and (iii) represents an advantage for K_D determination experiments, since a ligand:protein ratio of <1:1 can be reached even when using typically low protein concentrations. Using ligand concentrations that are equal to or smaller than the protein concentration permits the robust detection of binding, including that of strong binders with slow off rates, which are easily missed in conventional ligand observation experiments. Furthermore, the single-scan CPMG experiments would offer a way for robust, automated T_2 determination. An obvious limitation of the above experiments is that they require fluorine-containing compounds. However, using a well-characterized fluorinated ligand as a reporter molecule could expand the applicability to other types of ligands.[7,8] On this basis, hyperpolarized ^{19}F NMR spectroscopy may be a viable alternative for inclusion in current workflows for validating protein−ligand interactions.

■ ASSOCIATED CONTENT

Supporting Information

Experimental section; hyperpolarized ^{19}F spectra of TFMCPP; conventional 2D CPMG experiments; single-scan DNP CPMG experiments; and K_D determination by conventional NMR analysis, ITC, and simulations. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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