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Toward Nanomolar Detection by NMR Through SABRE Hyperpolarization

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Supporting Information

ABSTRACT: SABRE is a nuclear spin hyperpolarization technique based on the reversible association of a substrate molecule and *para*-hydrogen (p-H_2) to a metal complex. During the lifetime of such a complex, generally fractions of a second, the spin order of p-H_2 is transferred to the nuclear spins of the substrate molecule via a transient scalar coupling network, resulting in strongly enhanced NMR signals. This technique is generally applied at relatively high concentrations (mM), in large excess of substrate with respect to metal complex. Dilution of substrate ligands below stoichiometry results in progressive decrease of signal enhancement, which precludes the direct application of SABRE to the NMR analysis of low concentration (μM) solutions. Here, we show that the efficiency of SABRE at low substrate concentrations can be restored by addition of a suitable coordinating ligand to the solution. The proposed method allowed NMR detection below 1 μM in a single scan.

With applications in chemistry, biology and medicine, NMR is a widespread spectroscopic technique. It is often used in the analysis of complex mixtures (e.g., biofluids, food extracts, reaction mixtures, etc), mainly due to its aspecific character and straightforward, nondestructive sample preparation. However, because of NMR low sensitivity, analysis of dilute solutions is generally precluded. Therefore, we often measure (and quantitate) samples at low millimolar concentrations. One way of increasing the sensitivity of NMR is by inducing non-Boltzmann nuclear spin state populations with hyperpolarization techniques. Para-hydrogen induced polarization (PHIP) is a hyperpolarization method based on the incorporation of *para*-hydrogen (p-H_2) into a molecule by catalytic hydrogenation of an unsaturated moiety.[1,2] Signal amplification by reversible exchange (SABRE) is a variant of PHIP in which the NMR signals of small molecules in solution are enhanced without any chemical modification, such as the hydrogenation reaction which is essential in conventional PHIP.[3−16] Therefore the potential substrate scope in SABRE is much wider,[3,9−11,16] and since it is based on reversible interactions, repeated measurements of the same unmodified sample are possible.[7]

SABRE-derived hyperpolarization is based on the reversible association of both p-H_2 and a substrate molecule to a mediating metal complex. When such a complex is formed at low magnetic field, a transient scalar coupling network drives the transfer of spin order from the p-H_2-derived hydrides to the nuclear spins of the substrate molecule, resulting in strongly enhanced NMR signals.[5] Because of the reversible character of the interaction, enhanced NMR signals are also observed for substrate molecules free in solution. A schematic representation of SABRE applied to pyridine as substrate and [Ir(IMes)$(H)_2(py)_3$]Cl (**2**[Cl]) [IMes =1,3-bis(2,4,6-trimethylphenyl)imidazole-2-ylidene; py = pyridine] as metal complex is shown in Figure 1. Metal complex $\mathbf{2}^+$ is formed by a reaction of the complex precursor [Ir(COD)-(IMes)Cl] (**1**) with p-H_2 and py.[6]

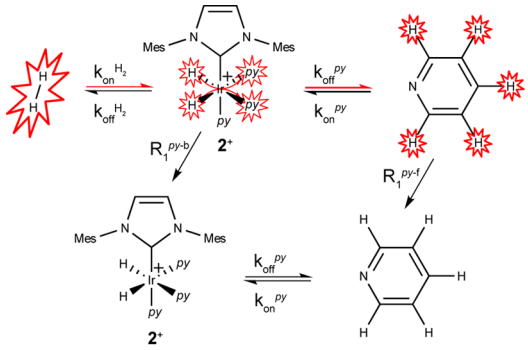

Figure 1. Schematic representation of spin order transfer from p-H_2 to py at $\mathbf{2}^+$ and resulting proton hyperpolarization of py both bound and free in solution.

SABRE results from the combined effect of different processes: polarization transfer via scalar couplings, metal complex dissociation and longitudinal relaxation. These processes are sketched in Figure 1. Long lifetimes of the metal complex are not favorable because of the much faster longitudinal relaxation of the substrate in the metal-bound form ($R_1^{py\text{-}b} \sim 10{-}20\ R_1^{py\text{-}f}$) and the inefficient transfer of hyperpolarized substrate molecules to the solution. On the other hand, SABRE efficiency of fast exchanging complexes is limited by the reduced contact time within the transient scalar coupling network. Therefore, an optimal dissociation rate (k_{off}^{py}) of the mediating complex appears to exist,[4,6,8] depending on the polarization transfer rate (k_{hyper}) and on the longitudinal relaxation rates ($R_1^{py\text{-}b}$, $R_1^{py\text{-}f}$). Up to now, $\mathbf{2}^+$ was found to be the most efficient mediating complex for py, with a py dissociation rate constant (k_{off}^{py}) of ~10 s^{-1}.[6,8]

So far, optimization of metal complexes, prepolarization field strength, and substrate-to-complex precursor ratio have been

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major concerns in SABRE research in an attempt to maximize nuclear spin hyperpolarization.[4,6,8,9] These studies, however, have mostly been performed at concentrations in the millimolar range and in large excess (usually ≥10:1 concentration ratio) of substrate with respect to the complex precursor. In an attempt to perform SABRE experiments on dilute solutions, we have found that at submillimolar concentrations of substrate and complex precursor, hyperpolarization is strongly attenuated and eventually disappears at low micromolar concentrations. This is illustrated in Figure 2 for a series of samples at constant (12.5:1) py-to-**1** concentration ratio, dissolved in methanol in the presence of *p*-H_2 (4 bar, 51% enrichment).

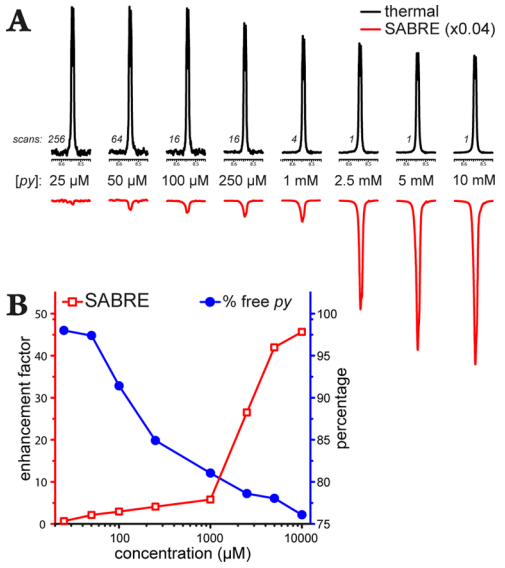

Figure 2. (A) Signals for *ortho*-protons of free py at thermal equilibrium (top, black) or following SABRE hyperpolarization (bottom, red) as a function of py concentration (indicated). Signals at thermal equilibrium are normalized with respect to the number of acquired scans and py concentration. SABRE signals are normalized with respect to py concentration. In all samples a concentration ratio of 12.5:1 of py with respect to complex precursor (**1**) was used. (B) Plot of SABRE enhancement for *ortho*-protons (red) and percentage of free py (blue) as function of py concentration. Note that in methanol the enhancement factors are reduced by ∼50% compared to methanol-d_4 due to less favorable relaxation times.[16]

Figure 2A displays the signals of the *ortho*-protons of free py at thermal equilibrium (in black) after normalization with respect to total py concentration and number of acquired scans. Such normalization allows to appreciate the effect of dilution on the relative concentrations of free and bound py. The observed increase of (normalized) signal intensity upon dilution indicates release of free py in solution due to dissociation of complex $\mathbf{2}^+$, for which a dissociation constant of 1.7 (concentrations expressed in mM) was estimated. Because of its short lifetime, all attempts to characterize the complex resulting from this dissociation process have been unsuccessful. Presumably, as a result of the dissociation, one or more py ligands are replaced by solvent molecules (see Supporting Information, SI). It should be mentioned that DFT calculations also indicate the possibility of other stable complexes, e.g. $[Ir(IMes)(H)_2(py)_2]Cl$, following the loss of a py ligand from complex $\mathbf{2}^+$.[6] Nevertheless, here it will be assumed that complex $[Ir(IMes)(H)_2(py)_2MeOH]Cl$ (**3**[Cl]) is formed upon dissociation of complex $\mathbf{2}^+$, although the exact nature of $\mathbf{3}^+$ is not relevant for the following discussion.

Not surprisingly, the dissociation process described above has a strong impact on the hyperpolarization by SABRE. This is evidenced by the signals displayed in Figure 2A (in red) after normalization with respect to py concentration. At concentrations close to 25 μM all py is virtually free in solution, and no SABRE effect is observable, as summarized in Figure 2B. The rapid decrease of SABRE hyperpolarization between 2.5 and 1 mM is concomitant with the dissociation of mediating complex $\mathbf{2}^+$. Note that in this concentration range, almost 20% of py in solution is still bound to the metal center (see Figure 2B). The drop in SABRE efficiency suggests therefore that metal center $\mathbf{3}^+$ resulting from the dissociation of complex $\mathbf{2}^+$ is not effective in polarizing the nuclear spins of bound py. This is probably related to its short lifetime, as indicated by the line width of the proton NMR signals (see SI).

Loss of SABRE hyperpolarization was also observed at higher (mM) concentrations, for py-to-**1** concentration ratios below stochiometry (3:1). This is summarized in Figure 3, where the

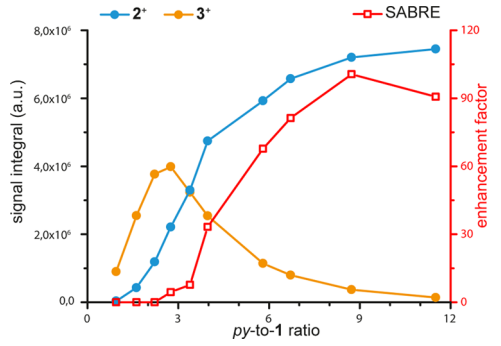

Figure 3. Signal integrals for IMes protons in complex $\mathbf{2}^+$ (blue) and complex $\mathbf{3}^+$ (orange) and signal enhancements for the *ortho*-protons of free py (red), as a function of py-to-**1** ratio. SABRE enhancements are determined with respect to NMR signals measured at thermal equilibrium at 600 MHz.

SABRE enhancement factor is plotted as a function of py concentration for a series of samples containing 2 mM **1** dissolved in methanol-d_4 together with *p*-H_2 (4 bar, 51% enrichment). At large excess of py (py-to-**1** ratio of 9:1) the signal enhancement factor for the *ortho*-protons of free py is 101 (0.48% polarization). As the ratio is lowered, a progressive decrease in signal enhancement is observed. Again, this negative trend follows the decrease in concentration of mediating complex $\mathbf{2}^+$ and the concomitant formation of metal complex $\mathbf{3}^+$ that is ineffective in polarizing the nuclear spins of py, as noted above. Complete loss of hyperpolarization is observed below the stochiometric ratio, due to the disappearance of the active complex $\mathbf{2}^+$.

The reported behavior at low substrate-to-complex precursor ratios appears to be general, as we have observed the formation of such SABRE-inactive complexes parallel to hyperpolarization loss for all substrates and complex precursors tested so far (see SI).

These results indicate that formation of inactive complex $\mathbf{3}^+$ must be prevented in order to obtain SABRE hyperpolarization. The approach presented here shows that this can be achieved even at low substrate concentration, provided a second ligand, referred to as co-substrate in the following, is added to the solution.

The co-substrate should fulfill the following requirements: (a) It should bind to the metal complex with much higher affinity

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than the solvent. Its binding affinity, however, should be lower or comparable to the affinity of the substrate under investigation; (b) it should be present in excess with respect to the concentration of the complex precursor; (c) the asymmetric complex $[Ir(IMes)(H)_2(co\text{-}substrate)_2(substrate)]Cl$ (**4**[Cl]) should display a lifetime that is favorable for SABRE; and (d) its ^1H signals should not overlap with the resonances of the substrate of interest.

If the concentrations of substrate (S), co-substrate (cS), and complex precursor (M) satisfy the condition:

$$[S] \ll [M] < [cS]$$

the distribution of substrate between free and bound forms is determined by the chemical equilibrium (see SI):

$$[Ir(IMes)(H)_2(S)(cS)_2]Cl + cS \overset{K_{eq}}{\rightleftarrows} [Ir(IMes)(H)_2(cS)_3]Cl + S$$

from which eq 1 can be derived:

$$\frac{[S]_{free}}{[S]_{bound}} = 3 \times K_{eq} \frac{[cS]_{free}}{[cS]_{bound}} \approx 3 \times K_{eq} \frac{C_{cS} - 3 \times C_M}{3 \times C_M} \quad (1)$$

Here, K_{eq} indicates the relative affinity of substrate and co-substrate for the metal center. The symbols C_{cS} and C_M denote the analytical concentrations in solution of co-substrate and complex precursor, respectively. The numerical factor 3 in eq 1 derives from the number of co-substrate molecules bound to the metal center. Equation 1 indicates that the distribution between the free and bound forms of a dilute substrate is independent of its total concentration and is essentially determined by the amount of co-substrate and complex precursor present in solution.

We have found that 1-methyl-1,2,3-triazole (mtz) satisfies the requirements (a–d) above and therefore can be used as a co-substrate for several substrates that are suitable for SABRE.[3,9,11] The binding affinity of mtz for the metal center is sufficiently high to prevent solvent binding at a mtz-to-**1** ratio of at least 5:1, for millimolar complex precursor concentrations. Furthermore, the value of K_{eq} in the case of py as (dilute) substrate and mtz as co-substrate is ~0.09; this corresponds to a fraction of bound py up to 80% (mtz-to-**1** ratio of 5:1). As previously mentioned, the fraction of bound py can be reduced by increasing the total concentration of mtz (see eq 1).

The effect of co-substrate (mtz) addition is shown in Figure 4A for a series of samples containing 2 mM **1** dissolved in methanol-d_4 together with p-H_2 (4 bar, 51% enrichment) and a fixed py-to-**1** ratio of 2:1. As previously shown in Figure 3, at this py-to-**1** ratio most of the bound py is found in the inactive $\mathbf{3}^+$ complex, and no hyperpolarization is observed. However, by titrating mtz to the solution, a progressive intensity decrease for signals from metal complex $\mathbf{3}^+$ is observed, while new signals for the complexes $\mathbf{4}^+$ and $\mathbf{5}^+$ ($[Ir(IMes)(H)_2(co\text{-}substrate)(substrate)_2]^+$) appear in the ^1H NMR spectrum. Concomitantly, restoration of py hyperpolarization is observed in the SABRE spectrum, as shown in Figure 4A. A maximal signal enhancement factor of 87 (0.42% polarization) on the *ortho*-protons of free py was found, which is comparable to what is shown in Figure 3. However, the dissociation of mediating complexes $\mathbf{4}^+$ and $\mathbf{5}^+$ is a much slower process than for $\mathbf{2}^+$, as reflected in the low free-to-bound py ratio (60% bound py, from Figure 4A) for which maximum signal enhancement is observed. Note that the free-to-bound ratio of py in this series is not correctly described by eq 1 since the complex precursor is not present in large excess (py:**1** = 2:1). In Figure 4B, the signals of *ortho*-protons of free py at thermal equilibrium (top) and the corresponding SABRE signals (bottom) at different mtz-to-**1** ratio are shown. Note that the conditions for highest SABRE signal intensity and highest enhancement factor do not coincide.

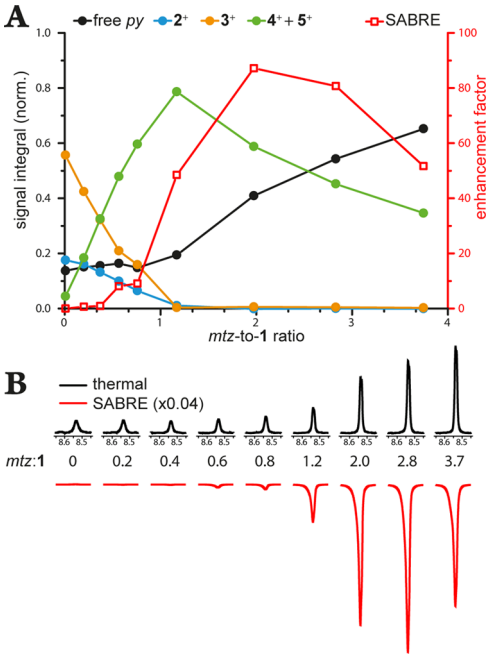

Figure 4. (A) Signal integrals and SABRE enhancement factors for the *ortho*-protons of py as a function of mtz-to-**1** ratio (py:**1** = 2:1). The following color coding is used: free (black), complex $\mathbf{3}^+$ (orange), complex $\mathbf{2}^+$ (blue), combined co-substrate complexes $\mathbf{4}^+$ and $\mathbf{5}^+$ (green). Signals are normalized with respect to the cumulative ortho py integral. Signal enhancements are reported as red squares. (B) Signals of *ortho*-protons of free py at thermal equilibrium (top, in black) and in SABRE experiments (bottom, in red) at different mtz-to-**1** ratios.

As summarized in Figure 4, addition of co-substrate allows to preserve SABRE hyperpolarization by preventing the formation of inactive complex $\mathbf{3}^+$. We have tested the validity of this method by acquiring SABRE spectra on samples containing trace amounts of substrate. The detection of 2 μM py (S/N ratio = 8:1) was achieved with 2 mM complex precursor **1** and 13 mM co-substrate mtz (data not shown). In an attempt to further improve SABRE efficiency, we have employed a different mediating complex than the slow exchanging $\mathbf{4}^+$ (k_{off}^{py} = 0.23 s^{-1}). For substrate concentrations down to 0.5 μM, complex precursor [Ir(SIMes)(COD)Cl] (SIMes = 1,3-bis(2,4,6-trimethylphenyl)imidazolin-2-ylidene) (**6**) was used. The p-H_2 and py exchange processes in co-substrate complex $[Ir(SIMes)(H)_2(mtz)_2(py)]Cl$ (**7**[Cl]) were approximately three times faster than for $\mathbf{4}^+$. Accordingly, a ~3-fold increase in signal intensity was observed in the SABRE spectra obtained with $\mathbf{7}^+$.

The SABRE spectra of py at micromolar concentrations in samples containing 1 mM **6** dissolved in methanol-d_4 together with p-H_2 (4 bar, 51% enrichment) and 18 mM mtz as co-substrate are shown in Figure 5A. The thermal equilibrium spectrum (top trace, black) was acquired with 512 scans for the 5 μM sample. The colored spectra were acquired at concentrations between 0.5 and 5 μM with a single scan following SABRE hyperpolarization. By comparing the signal integrals at thermal

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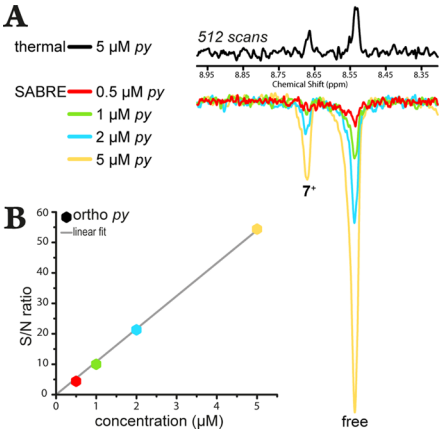

Figure 5. (A) ^{1}H NMR signals acquired at 600 MHz, at thermal equilibrium (black) or following SABRE hyperpolarization (colored) of samples containing trace amounts of py together with **6** (1 mM), mtz (18 mM) and 4 bar *p*-H_2 in methanol-d_4. The displayed signals originate from the *ortho*-protons of py in the free and bound form. (B) Plot displaying the signal-to-noise ratio of the free py signals in (A) as a function of py concentration.

equilibrium and after SABRE, an enhancement factor of 121 is obtained for the 5 μM py sample.

A detection limit <1 μM can be estimated based on the series of SABRE spectra in Figure 5A. The plot in Figure 5B clearly shows a linear dependence of the signal intensity on py concentration. This stems from the fact that at such low concentrations the free-to-bound substrate ratio is essentially determined by the concentration of co-substrate, and it is, therefore, constant for all the spectra of Figure 5A (see eq 1). This linear dependence suggests a possible use of the proposed approach for quantitative applications of SABRE at low substrate concentration.

This approach to extend SABRE applicability is obviously not restricted to the model substrate py: comparable detection limits were found for other substrates using co-substrate complex 7^+ (see SI).

In conclusion, we demonstrate that SABRE can be successfully applied at concentrations of only a few micromolars. Employing SABRE at low substrate concentrations requires the addition of a more concentrated co-substrate molecule to the solution. In the present study 1-methyl-1,2,3-triazole was used as co-substrate, resulting in stable hyperpolarization transfer complexes (4^+, 7^+) which gave reproducible signal enhancements for diluted substrates, even a week after sample preparation by simply refreshing the *p*-H_2 in the sample. As illustrated by the plot in Figure 5B, a linear dependence exists between SABRE signal intensity and substrate concentration in the low micromolar regime. This result is important as it indicates that hyperpolarization does not *a priori* preclude quantitative NMR applications. With the current experimental setting, a detection limit <1 μM was obtained for py as a substrate. A further reduction down to ~100 nM should already be feasible in a single scan via the proposed co-substrate approach, by using the automated polarization setup previously described[6] (gain: 7×) and fully enriched *p*-H_2 (gain: 3×).

■ ASSOCIATED CONTENT

Supporting Information

Experimental procedures and NMR data. 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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