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Temperature response of $^{129}$Xe depolarization transfer and its application for ultra-sensitive NMR detection

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Trapping of exchangeable atomic xenon in functionalized cryptophane cages makes the high sensitivity of hyperpolarized (hp) $^{129}$Xe available for highly specific NMR detection of biomolecules like proteins in solution. Here, we study the signal transfer onto a reservoir of unbound hp xenon by gating the residence time of the nuclei in the cage through the temperature-dependant exchange rate. Temperature changes were detectable immediately as an altered reservoir signal and nd makes it possible to detect temperature changes as small as $\sim$0.6 K. The temperature response is adjustable with lower concentrations of caged xenon providing more sensitivity at higher temperatures, allowing ultra-sensitive detection of such molecular cages at 310 K. Functionalized cryptophane was detected at concentrations as low as 10 nM, corresponding to a $\sim$4000-fold sensitivity enhancement compared to conventional detection. This sensitivity makes hp-NMR capable of detecting such constructs in concentrations far below the detection limit by benchtop UV-visible light absorbance.

Nuclear magnetic resonance (NMR) is an extremely valuable detection tool in many fields of research because the recorded radio frequency (rf) signals are associated with almost no penetration limitations and extremely high specificity for the detected molecules. However, the method suffers from intrinsic low sensitivity. This can be overcome in some applications by use of hyperpolarized (hp) nuclei, but due to the nature of the hyperpolarization process it is difficult to make such high magnetization available for studies in biochemically relevant environments. Any detection scheme that enhances the sensitivity for biomolecular NMR applications is therefore of high interest.

Recent approaches for utilizing hp $^{129}$Xe [1] in solution NMR exploit the fact that its resonance frequency is strongly shifted when it associates with a molecular cage such as cryptophane [2, 3]. Such cages can be functionalized with a targeting unit (antibody or ligand) to form xenon biosensors [4] in order to track a specific analyte upon biochemical binding. Biotinylated cage constructs are common examples for detecting avidin and protein-linked cages have the potential to bind to specific cell membrane receptors. Changes in the NMR signal of trapped $^{129}$Xe occur upon interaction with the target and provide a powerful tool for spectroscopy and imaging. Hence, detection techniques that can identify caged Xe at low concentration ($< 10^{-10}$M) and are sensitive to the in situ physical environment will make NMR more competitive with optical and radioisotope detection.

A sensitive method of indirectly detecting exchangeable hp nuclei within cryptophanes has recently been reported (Hyper-CEST, [5]). Magnetization from encapsulated xenon can be “labeled” by a selective rf pulse that induces depolarization (i.e., saturation of the spin system). Depending on the exchange rate, hundreds to thousands of nuclei per second per cage experience this pulse, resulting in a depletion of the magnetization of free, dissolved xenon in the vicinity of the cage. This ensemble of uncaged xenon serves as a reservoir to detect the flow of saturated spins from the functionalized cage where the magnetization change is encoded.

Since chemical exchange rates are very sensitive to temperature $T$, the response of the Hyper-CEST effect upon changes in $T$ is a promising tool to achieve high-sensitivity detection of the in situ environment and of the concentration of caged $^{129}$Xe. Xenon atoms have been shown to reside in cryptophane cages in water for a few milliseconds at room temperature [2]. An increase in $T$ significantly decreases this lifetime [6] and should amplify the Hyper-CEST effect. Here, we describe the direct response of the xenon reservoir signal intensity, $I_{res}(t)$, in such a system to a temperature input function $T(t)$ in order to determine the sensitivity, $dI_{res}/dT$, of the $^{129}$Xe NMR signal to temperature changes and to push the detection limit of functionalized cages into the nM concentration range.

A gas mixture of 89% He, 10% N$_2$ and 1% xenon (natural abundance of $^{129}$Xe: 26%) was passed through a hyperpolarizer (XenoSpin$^{TM}$, Amersham Health; Durham, NC) where spin exchange with optically pumped rubidium vapor [1] generates a $^{129}$Xe nuclear spin polarization of ca. 5%. This mixture was delivered into an aqueous solution as described previously [7].

Figure 1 illustrates the $^{129}$Xe NMR spectrum in D$_2$O
with a functionalized cryptophane-A cage designed as a universal precursor for biosensors that are prepared by attaching a targeting unit like biotin to the terminal amine group [9]. Data were recorded on an NMR spectrometer (Unity Inova; Varian Inc., Palo Alto, CA) at 7.05 Tesla with a 10 mm $^{129}$Xe NMR probe. The resonance of the dissolved, uncaged xenon at $\delta_1 = 193.8$ ppm can be detected with a signal-to-noise ratio of $S/N = 185$ after 8 acquisitions at 295 K, whereas the diluted encapsulated xenon signal (33 $\mu$M cage concentration) at $\delta_2 = 62.7$ ppm gives only a $S/N = 11$.

For this experiment, Hyper-CEST detection used a frequency-selective saturation pulse of 500 ms (amplitude $B_1 = 6.48 \mu$T, bandwidth $\Omega = 0.93$ ppm) centered at $\delta_2$, followed by observation of the subsequent change in the reservoir signal. Previous studies showed that the resonance frequency of the caged xenon shifts linearly with increasing temperatures and vice versa. To calibrate the temperature dependence can be modeled with an empirical fit to a sigmoidal Boltzmann function (correlation coefficient $R^2 = 0.98$)

$$I_{\text{res}} = A_1 + \frac{A_2 - A_1}{1 + e^{(T - T_0)/dT}}$$

with $A_1 = 0.04 \pm 0.04$, $A_2 = 1.00 \pm 0.05$, $T_0 = (297.5 \pm 0.5)$ K, and $dT = (3.9 \pm 0.6)$ K$^{-1}$. The derivative of this function yields the differential temperature sensitivity $dI_{\text{res}}/dT$ plotted in Fig. 3b (solid line), showing a maximum sensitivity of ca. 6% decrease per K around 297.5 K. Since the systematic noise is about 3.6% (see below), this corresponds to temperature resolution of ca. 0.6 K.

The differential sensitivity is adjustable to some ex-
FIG. 2: (a) Input function $T(t)$ (black solid line) for Hyper-CEST signal transfer using the construct shown in Fig. 1. The saturation frequency offset $\omega_{sat}$ (grey dashed line) is adjusted according to the resonance shift of 26.6 Hz/K. Bars indicate the bandwidth of the saturation pulse of $\Omega = 76.7$ Hz (0.925 ppm). (b) Response $I_{res}(t)$ to $T(t)$. Varying the temperature changes $^{129}$Xe signal transfer through the cage that is present at 27.2 $\mu$M. The reference signal for no saturation transfer is illustrated by the dashed line.

tent by adjusting certain system parameters. For example, lower cage concentrations, shorter saturation pulses, or lower saturation power would all yield less efficient saturation transfer at a given $T$, thus shifting the Boltzmann function to the right. This was demonstrated with a solution of 11.2 $\mu$M cage concentration (only one temperature sweep from 293K to 313K in this case). As seen in Fig. 3a, the overall saturation is decreased and the Boltzmann transition stretched over a wider temperature range ($A_1 = 0.11 \pm 0.03$, $A_2 = 1.00 \pm 0.03$, $T_0 = (303.7 \pm 0.3)$ K, $dT = (5.7 \pm 0.5)$ K$^{-1}$, $R^2 = 0.99$). In addition, the range of maximum sensitivity is shifted by 6.2 K to give a high-$T$ sensitivity (Fig. 3b, dashed line). Similarly, a low-$T$ sensitivity can be achieved by opposite modifications.

The significant increase in saturation transfer upon increasing $T$ shown in Fig. 3a can be used to detect very low concentrations of caged xenon. To determine the detection threshold for this construct at body temperature (ca. 310K), a solution of 10 nM concentration was prepared. Figure 4 illustrates that Hyper-CEST induces a signal decrease of $\sim$16% with this concentration after 20s saturation with a pulse amplitude of $B_1 = 25.8 \mu$T ($\Omega = 544$ Hz). The standard deviation (SD) of the signals detected in control experiments with off-resonance saturation was 3.6%.

To estimate the sensitivity gain compared to direct detection shown in Fig. 1, parameters summarized in Tab. II were used. The xenon occupancy of the cages calculated from the binding constant, $K = 6000$ M$^{-1}$ [7], is 55% [11]. This information must be considered when designing cages with different affinity for the noble gas [3]. The saturation transfer observed in Fig. 4 is caused by a concentration of caged $^{129}$Xe that is only 1.4 nM.

FIG. 3: (a) Calibration for temperature sensitivity of $I_{res}$ shown in Fig. 2b at 27.2 $\mu$M cage concentration (×) and for 11.2 $\mu$M cage concentration (+). The signal intensity is normalized to a control experiment without saturation pulse. Data are fitted to Eq. 1. and shown with a 95% confidence band (dashed lines). (b) The differential sensitivity of $^{129}$Xe signal, intensity change per Kelvin, is given by the first derivative of the fit results in a). Reducing the concentration of the cage construct shifts the maximum sensitivity to higher temperatures. Since the experimental standard deviation in determining of $I_{res}$ is $\sim$3.6% (see below), the temperature resolution is limited to ca. 0.6 K for the 27.2 $\mu$M solution.
TABLE II: Experimental parameters for comparison of direct and indirect detection. The amount of detectable, encapsulated xenon, i.e. \([^{129}\text{Xe}]_\text{cage}\), is determined by the concentration of the cryptophane cage, the abundance of the isotope \([^{129}\text{Xe}]\) and the xenon partial pressure of the gas above the solution. The final acquisition time is determined by the # of scans and the repetition time \(TR\).

<table>
<thead>
<tr>
<th></th>
<th>([^{129}\text{Xe}]_\text{cage}) ([\text{nM}])</th>
<th>([^{129}\text{Xe}]_\text{cage}) @ 58.4 mbar ([\text{nM}])</th>
<th># scans</th>
<th>(TR) ([s])</th>
<th>S/N</th>
<th>(T) ([K])</th>
</tr>
</thead>
<tbody>
<tr>
<td>direct detection</td>
<td>33,300</td>
<td>8.710</td>
<td>4.616</td>
<td>8</td>
<td>33</td>
<td>3.6</td>
</tr>
<tr>
<td>Hyper-CEST detection</td>
<td>10</td>
<td>2.6</td>
<td>1.38</td>
<td>2</td>
<td>53</td>
<td>4.6</td>
</tr>
</tbody>
</table>

Even though the S/N of direct detection at 310 K will be slightly higher than at 313 K (ca. 6 instead of 5.1 [12]), this comparison still yields a ∼4000-fold sensitivity enhancement with respect to direct detection; the direct measurement time would be ∼55 years to achieve the same S/N rather than 106 seconds.

Under conditions described here the exchangeable, hyperpolarized xenon detection makes NMR much more sensitive than optical methods in this specific case. Conventional, benchtop UV-visible absorbance detection of the cryptophane-A cage (\(\epsilon_{282} = 8000 \text{M}^{-1}\text{cm}^{-1}\), [8]) requires a minimum concentration of ∼1 \(\mu\text{M}\) [13]. Thus the temperature-controlled depolarization transfer detection is very promising for new applications of high-sensitivity NMR with functionalized biosensors.

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9. In contrast to previously used constructs, this one has a negatively charged side chain to minimize interactions with the glass wall and remain detectable at low concentrations.
10. For sufficient S/N, resonance shifts of ca. 10% of the line width can be detected, thus 4 Hz is the resolution limit at 295 K, yielding ca. 0.15 K thermal resolution.
11. Xenon dissolves in water to a concentration of 190 \(\text{L}\) [9].
12. Based on data in [6], the exchange rate increases by a factor of ∼14 for \(T = 22^\circ\text{C} \rightarrow 40^\circ\text{C}\) but only by ∼9 for \(T = 22^\circ\text{C} \rightarrow 37^\circ\text{C}\); the corresponding line broadening would then reduce S/N from 11 to 6.
13. Extinction coefficients can be as high as \(100,000 \text{M}^{-1}\text{cm}^{-1}\) for many macromolecules, thus being ca. 12 times more sensitive than for cryptophane. However, this would still yield a minimum concentration of ca. 400 nM for detection with UV-vis absorbance.