Xenon MRI

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Supramolecular Assays for Mapping Enzyme Activity by Displacement-Triggered Change in Hyperpolarized ^{129}Xe Magnetization Transfer NMR Spectroscopy

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Abstract: *Reversibly bound Xe is a sensitive NMR and MRI reporter with its resonance frequency being influenced by the chemical environment of the host. Molecular imaging of enzyme activity presents a promising approach for disease identification, but current Xe biosensing concepts are limited since substrate conversion typically has little impact on the chemical shift of Xe inside tailored cavities. Herein, we exploit the ability of the product of the enzymatic reaction to bind itself to the macrocyclic hosts CB6 and CB7 and thereby displace Xe. We demonstrate the suitability of this method to map areas of enzyme activity through changes in magnetization transfer with hyperpolarized Xe under different saturation scenarios.*

Magnetic resonance imaging (MRI) with hyperpolarized xenon (^{129}Xe) holds promise as a minimally invasive technique for diagnosis based on disease-specific markers at low concentrations,[1] in particular in combination with contrast agents based on macrocyclic supramolecular hosts[2] and signal amplification through chemical exchange saturation transfer (Hyper-CEST).[2c] This led to highly sensitive Xe MRI applications illustrating intracellular labeling[1d,3] and the specific detection of cell surface receptors via chemically modified Xe host molecules.[1c] As a next step, following reactions with high sensitivity, for example, enzyme activity, is desirable.[4] This would make it possible to image areas of altered physiological processes associated with major diseases[5] and includes a pathway for amplifying the sensor signal.[4b]

Unfortunately, reported approaches based on substrate labeling with a Xe host are limited, because the chemical shift difference of the Xe NMR frequency before and after the enzymatic reaction is comparably small ($\Delta\delta \approx 0.5$ ppm).[4b] As a remedy, we introduce herein a carefully designed approach to MRI enzyme activity mapping by hyperpolarized Xe NMR spectroscopy, in which we 1) use cucurbit[*n*]uril (CB) hosts with $n = 6$ (CB6) and $n = 7$ (CB7) as contrast agents, 2) rationally exploit the molecular recognition properties of these hosts, and 3) for the first time apply an optimized magnetization transfer (MT) experiment for the Xe MRI of enzyme activity (Figure 1).

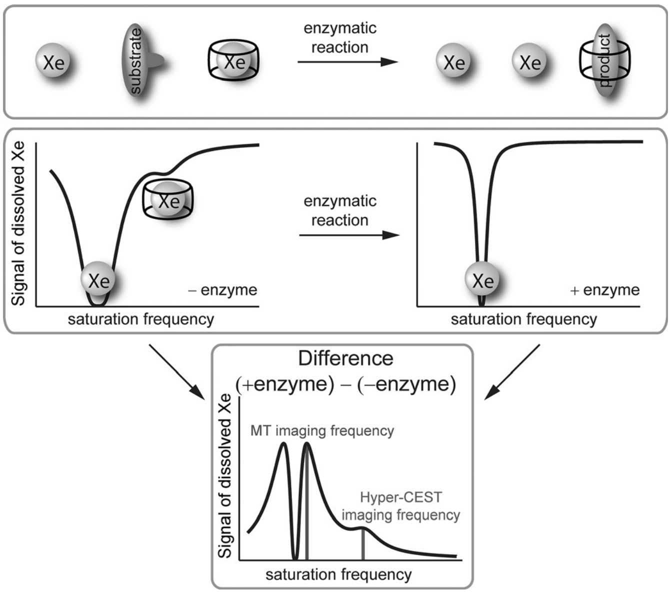

Figure 1. Displacement-based approach for ^{129}Xe biosensors (top), response in Xe saturation transfer spectra (middle), and the difference spectrum before and after displacement, which gives the optimum saturation frequency for MT imaging.

CBs are very stable and nontoxic macrocyclic hosts,[6] which have been explored in Xe NMR experiments,[2d–h] although their real potential has been underrated; for example, the utility of CB6 for Xe Hyper-CEST has been demonstrated only recently.[7] Their excellent molecular recognition properties in water have, however, led to numerous applications,[8] in particular for the detection of enzyme activity through optical sensing.[9] In this approach, the substrate and product of an enzymatic reaction bind to a host with different affinities, such that the enzyme transforms a weakly binding substrate into a strongly binding product. The concomitant displacement of a fluorescent dye changes the spectroscopic properties of the dye, which is used

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to follow the reaction. Our idea was that in a reaction mixture with Xe, its gradual displacement from the host as the enzymatic reaction progresses could be followed by overall changes of Xe saturation transfer spectra from the Xe–host complex (Figure 1).

To demonstrate the transferability of supramolecular enzyme assays to MRI, we first chose the strongly Xe-binding homologue CB6 as the contrast agent and lysine decarboxylase (LDC) as the enzyme. LDC converts the weakly binding substrate L-lysine (Lys) into the strongly binding product cadaverine (Cad) and has a key role in tumor growth and inflammatory processes.[10]

As an initial control and for comparison with the Xe NMR studies, we developed a fluorescent assay with CB6 and LDC using a putrescine derivative of 1-aminonaphthalene-5-sulfonic acid as fluorescent dye, which responds to CB6 binding with an increase in fluorescence.[11] The binding constant was determined by fluorescence titration as $(4\pm1)\times10^{4}\,\text{M}^{-1}$ in the NH_4OAc buffer (Figure SI-1), which lies between $4.3\times10^{7}\,\text{M}^{-1}$ in 1 mM HCl (pH 3.0) and $2.5\times10^{3}\,\text{M}^{-1}$ in 50 mM NaOAc (pH 5.5).[11] The higher binding constant in 1 mM HCl results most likely from an increased protonation of the dye in the more acidic solution and binding in 50 mM NaOAc is lower because of the competitive binding of Na^+ to CB6.

The binding constant of Cad to CB6 (10^{9} to $10^{10}\,\text{M}^{-1}$)[9e] is clearly higher, and the binding of Lys is too weak to efficiently compete with the dye at relevant concentrations (Figure SI-2). Consequently, dye displacement and fluorescence decrease were expected upon progress of the enzymatic reaction, which was in fact observed (Figure SI-3). The fluorescence traces gave a turnover number of 74 nmol $\text{min}^{-1}\,\text{mg}^{-1}$ at 25 °C and 300 μM Lys. These fluorescence results 1) demonstrate the principle feasibility of the enzyme assay under these conditions, 2) exclude potential undesirable interactions between CB6 and the enzyme, and 3) ensure that the enzymatic reaction completes within an acceptable time to be detected by Xe NMR spectroscopy.

Next, we explored the reporter system with hyperpolarized Xe NMR spectroscopy and MRI. The Hyper-CEST spectrum of CB6 (Figure 2) displayed resonances at $\delta=193$ ppm ascribed to direct saturation of free dissolved Xe, and at $\delta=105$ ppm originating from saturation transfer via Xe encapsulated in CB6,[12] demonstrating that the exchange kinetics of Xe are compatible with Hyper-CEST.[7] After addition of LDC to the mixture containing only CB6, the Xe@CB6 resonance remained unchanged, indicating no undesirable interaction of CB6 or Xe with the enzyme in line with the fluorescence results. The Xe@CB6 resonance vanished completely after addition of 100 μM Lys and incubation for 30 min. This is due to decarboxylation of Lys by LDC producing strongly binding Cad, which efficiently suppresses the saturation transfer by occupying the CB6 cavity. This was also observed when Cad was added to a solution containing CB6 only. Another control, in which Lys was added to a CB6-containing solution, showed a small response, in which the CEST signal became slightly smaller and shifted to lower ppm values. This is likely due to weak binding of Lys as also observed by fluorescence (Figure SI-2). These results clearly show the applicability of Hyper-CEST to detect enzyme activity through competitive macrocycle occupation and allow Hyper-CEST MRI in solution, where only the area not containing LDC displayed a high Hyper-CEST effect (Figures 2d and SI-4).

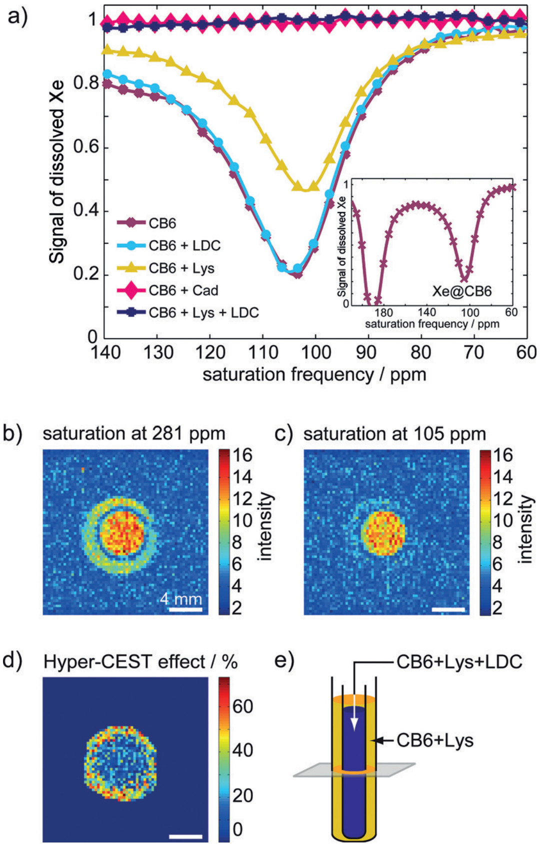

Figure 2. a) Details and overview (inset) of Hyper-CEST spectra (Xe gas as $\delta=0$ ppm reference). Details: only CB6 (dark red), with 15 $\mu\text{g}\,\text{mL}^{-1}$ LDC (light blue), with LDC and 100 μM Lys (dark blue), with 50 μM Cad (pink), and with 100 μM Lys (yellow). Saturation parameters: $B_1=16\,\mu\text{T}$, $t_{\text{sat}}=4$ s. b)–e) Hyper-CEST MRI (on-resonant (b) and off-resonant saturation (c), Hyper-CEST effect (d), and two-compartment setup (e)). In addition to Lys, 15 $\mu\text{g}\,\text{mL}^{-1}$ LDC was present only in the inner compartment, which reduced its Hyper-CEST effect (d) significantly. Conditions: 20 μM CB6, 10 mM NH_4OAc, 25 °C, $[\text{Xe}]\approx975$ μM. No. of averages: 6. Total acquisition time: ca. 4.6 min.

When we attempted to transfer this approach to cell lysates, no clearly resolved Xe@CB6 resonance was observed at 20 μM CB6, but the CEST response at $\delta=193$ ppm was significantly broadened as previously observed in blood plasma (Figure SI-6).[7a] This is attributed to two factors: 1) CB6 is partially occupied by more competitive guests and 2) Xe now additionally interacts with other potential binding partners, for example, proteins and lipids, with a mutual influence on the exchange kinetics causing line broadening per se,[4i] such that it becomes impractical to detect the presence of CB6 by saturating at $\delta=105$ ppm (Figure SI-6).

As a modified strategy, application of saturation pulses slightly off-resonant from an exchange-broadened bulk signal is, however, a well-established method in ^{1}H NMR spectroscopy called magnetization transfer (MT)[13] and has been used, for example, to follow myelination of neuronal cells in vivo.[14] We therefore reasoned that a similar approach may be applied to Xe NMR spectroscopy. To further reinforce the exchange-mediated line broadening, we considered the yet unexplored CB7 with its larger cavity, which should facilitate fast exchange. Surprisingly, CB7 gave clear Hyper-CEST spectra in pure H_2O and a well-defined Xe@CB7 resonance at $\delta = 100$ ppm (Figure SI-7), which can be used for Hyper-CEST MRI (Figure SI-8) and vanished completely upon addition of Cad. Most importantly, the dissolved Xe resonance at $\delta \approx 190$ ppm was further broadened compared to the CB6 spectra confirming the anticipated faster Xe exchange with CB7.

Fluorescence-based LDC assays were originally established with CB7,[9a] such that no control was required and imaging of LDC activity using Hyper-CEST MRI was directly pursued. As for CB6, Hyper-CEST spectral resolution with CB7 is expected to decrease in cell lysates rendering the contrast of Figure SI-8 unachievable under such conditions. To identify the ideal frequency of the saturation pulse for MT, CB7 in a lysate of macrophage cells before and after addition of LDC was measured (Figure 3 a). Therein, the original CEST response of CB7 at $\delta \approx 108$ ppm is indeed hard to identify due to the fast exchange, but the change in the direct saturation response of the bulk Xe is well pronounced. This is also reflected in the difference spectrum, in which the curve has a maximum at $\delta \approx 178$ ppm. This frequency was thus used for imaging with optimized MT conditions, which gave a strong MT ratio (MTR) in the area not containing LDC and weak signal changes in the other compartment (Figure 3 b–d). The MTR image (Figure 3 d) clearly demonstrates that the enzyme-active compartment can be easily identified through a pronounced decrease in the MTR signal. The superior performance of the MT experiment without a conventional CEST pool resonance frequency becomes most apparent in direct comparison with MR imaging by conventional Hyper-CEST, which gave much less image contrast (Figure SI-10). Moreover, the typical effort to avoid spillover effects and preserve specificity for the selected frequency by avoiding strong saturation pulses for fast exchanging systems is not an issue for MT experiments.

These results show that the concept of a dynamic equilibrium, in which Xe is gradually displaced from its host as an enzymatic reaction progresses, can be used to map enzyme activity by Xe MRI. The decreased spectral resolution in a complex cellular lysate with CB6 inspired the application of MT in combination with the first-time use of CB7 as an even faster exchanging host for Xe NMR experiments. This led to improved signal contrast compared to that from conventional Hyper-CEST acquisition and shows that current challenges in molecular imaging as well as biological applications of supramolecular systems can be accounted for by a thoughtful experimental design. Future developments include the extension to other enzyme classes as previously demonstrated for optical sensing,[9] as well as improvements inspired by ratiometric probes in which a reference signal provides direct information about the local biosensor concentration,[15] and by developments in supramolecular chemistry, such as the first demonstration of fluorescent supramolecular displacement assays in living cells.[16]

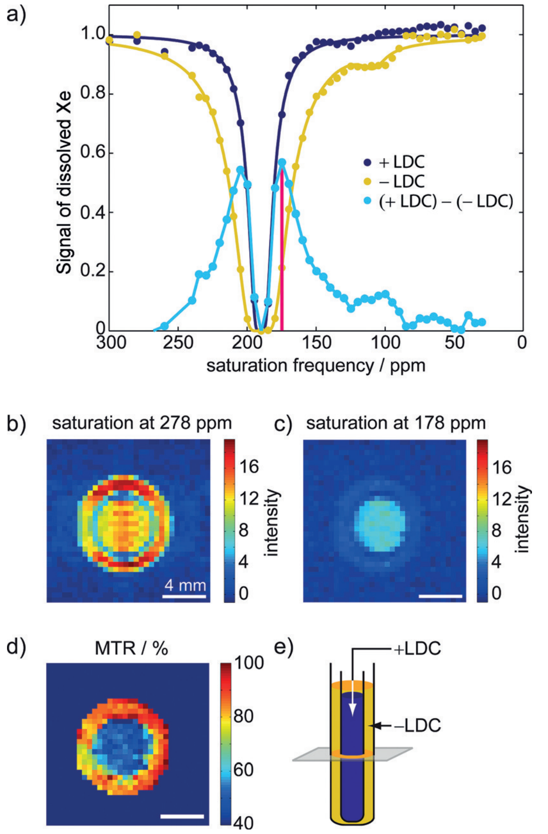

Figure 3. a) Hyper-CEST spectra of CB7 in the presence of lysed macrophage cells and 700 μM Lys before (yellow) and 45 min after adding 50 $\mu g\,mL^{-1}$ LDC (dark blue), and the difference spectrum (light blue). b)–e) Transversal Xe MT MRI of two nested NMR tubes with 50 $\mu g\,mL^{-1}$ LDC in the inner tube only (incubation time 45 min). Saturation at b) $\delta = 278$ ppm and c) $\delta = 178$ ppm, and d) resulting MTR image. e) Schematic representation of the setup. Hyper-CEST images were recorded using a RARE pulse sequence with an in-plane resolution of 0.14 mm^2 and 20 mm slice thickness. Saturation parameters: $B_1 = 20$ μT, $t_{sat} = 4$ s. Conditions: 500 μM CB7, 10 mM NH_4OAc (pH 6.0), 22 °C, 25×10^6 lysed macrophage cells/mL (ca. 9 $mg\,mL^{-1}$ protein), [Xe] ≈ 975 μM. No. of averages: 10. Total acquisition time: ca. 7.5 min.

Acknowledgements

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