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After enzymatic reaction, $^{129}$Xe-MRI signal in CB6 was lighted.
An intracellular Diamine Oxidase Triggered Hyperpolarized $^{129}$Xe Magnetic Resonance Biosensor

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Here a novel method was developed for suppressing $^{129}$Xe signal in cucurbit[6]uril (CB6) until the trigger is activated by a specific enzyme. Since its noncovalent interactions of amino-group and CB6, putrescine dihydrochloride (Put) was chosen for blocking interactions between $^{129}$Xe and CB6. Upon adding diamine oxidase (DAO), Put was released from CB6 and $^{129}$Xe@CB6 Hyper-CEST signal emerged. This proposed $^{129}$Xe biosensor could be tested in small intestinal villus epithelial cells.

As an alternative to $^1$H MRI, hyperpolarized (HP) $^{129}$Xe nuclear magnetic resonance NMR and MRI are studied in an increasingly wide range of applications. There is no natural background competing against with exogenous HP signal of $^{129}$Xe. Using spin-exchange optical pumping (SEOP) for $^{129}$Xe, signals of $^{129}$Xe can be increased by several magnitude orders. Since the electron cloud of $^{129}$Xe atom is sensitive to the environment, $^{129}$Xe NMR can be used for liquid crystal ordering, $^3$P, $^4$H, and temperature changes detection. Detection sensitivity can be further improved by the Hyper chemical exchange saturation transfer (Hyper-CEST) technique. Furthermore, as chemical exchange occurs much quickly than $^1$T$_1$ relaxation in Hyper-CEST, depolarized $^{129}$Xe accumulates in the solvent, allowing for indirect but highly sensitive detection.

The hosts of $^{129}$Xe can be functionalized variously. Meanwhile, chemical shift becomes an indicator for diverse applications such as analyte detection and marker imaging. Typically, cryptophane-A (Cry-A) and its derivatives are the most studied $^{129}$Xe binding cages for these applications. However, the multistep synthesis and extremely low yields of Cry-A and its derivatives hamper their preparation. CB6 has also been reported as an excellent $^{129}$Xe host for Hyper-CEST NMR, which is characterized by higher solubility in water and better exchange parameters for Hyper-CEST in comparison with Cry-A. Various CB6 supramolecular host-guest interactions have been well studied and the participation of CB6 in protein binding or enzymatic reactions has been explored. Studies were based on competitive binding, and an unexpected inhibition of the $^{129}$Xe@CB6 interaction occurred in the presence of excess target. It would be beneficial to combine the well-known host-guest properties of CB6 with HP $^{129}$Xe and diseases related biochemical reactions. However, “OFF-ON” switch for $^{129}$Xe@CB6 biosensor for enzyme detection has not been developed.

Here a new CB6-based biosensor (Scheme I) that allows for selective and sensitive enzyme detection was developed using...
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129Xe Hyper-CEST NMR. In order to design 129Xe@CB6 "OFF-ON" NMR biosensor successfully, the affinity between a substrate and CB6 is required to significantly differ from that of the enzymatic reaction product and CB6. In other words, a strong CB6 binding substrate was converted into product that cannot bind to CB6 via an enzymatic reaction. The 129Xe@CB6 Hyper-CEST response could be suppressed by a strong CB6 binding substrate until its enzymatic oxidation to product that cannot bind to CB6. Since the binding constant of substrate@CB6 was much bigger than that of 129Xe@CB6, whereas the product of enzymatic reaction that cannot bind to CB6, the CEST response emerged after the enzymatic reaction.

As show in Figure S1, CB6 (a strong 129Xe binding host) was chosen as a cage of contrast agent. Putrescine dihydrochloride (Put) was chosen as a substrate owing to its strong noncovalent interactions of amino-group with CB6. For the enzymatic oxidation of Put to aminobutyraldehyde (Ami), diamine oxidase (DAO) was chosen. Then, Ami was converted into an end product (Δ1-pyrroline), which had no amino-group combining with CB6. After this process, the cavity of CB6 changed from occupied to empty, with the concomitant HP 129Xe exchange inside and outside the cavity. As a result, the NMR signal of the caged 129Xe turned from "OFF to ON".

DAO, playing an important role in polyamines metabolism, is over expressed in small intestinal villus epithelial cells of mammals. An increase in the activity of DAO in serum is closely related to many clinical diseases, including histamine intolerance (HIT), chronic idiopathic urticarial (CIU), small bowel Crohn disease (SBC), and ruptured fetal membranes. A widely known methods fluorescence spectrophotometry have been developed to DAO. Nevertheless, this method have poor tissue penetration and tissue fluorescence, thereby making it impossible to obtain DAO spatial distributions within a region of interest in vivo. The 129Xe@CB6 "OFF-ON" MRI biosensor offers the opportunity to map the DAO enzyme reaction and the changes in its distribution over time in vivo.

To elucidate the mechanism of the enzymatic reaction between the CB6 cage and DAO, the products of this reaction were analyzed via mass spectrometry (MS; Figure S2). A peak at 70.0660 m/z corresponding to Δ1-pyrroline was found. Upon addition of DAO, 129Xe Hyper-CEST NMR spectra were obtained in the same sample at different times in Figure S4. The intensity of the 129Xe@CB6 Hyper-CEST signals at δ = 105 ppm was found increasing gradually over time, and recovered 8 days later compared with the CB6 alone. The above results demonstrated that the host-guest interactions between CB6 and Put did not prevent Put from being oxidized by DAO.

Previous research has confirmed that organic ammonium could enter the cavity of CB6 and form a 1:1 host-guest complex. Putrescine and cadaverine can inhibit the 129Xe@CB6 NMR signals in solution. To confirm if the Put can take effect as expected in the biosensor, its binding affinity with CB6 was measured by ITC at the following conditions: 298 K, pH 7.2, and 20 mM NH4OAc buffer (Figure S3). The result showed that the binding affinity between CB6 and Put was 2.62 × 106 (±3.66 × 105). This value is significantly greater than those reported was much higher value than those reported for the binding affinities between CB6 and 129Xe (Kd = 500–3000 M−1). So, this system can be an "OFF-ON" biosensor as we expected. Moreover, TAOBR (1951) et al. previous research has confirmed that the aldehyde afforded as a result of the oxidation of Put undergoes subsequent condensation to form Δ1-pyrroline. Δ1-pyrroline did not contain amino-group combining with CB6. Accordingly, the 129Xe@CB6 NMR signals can be lightened by DAO.

![Figure 1](image)

Figure 1. Details and overview of Hyper-CEST spectra and CEST effect in buffer and cell. 129Xe gas at δ = 0 ppm for reference. (a) Hyper-CEST spectra in buffer of only CB6, with Put, and DAO. Saturation parameters: B = 6.5 µT and t = 10 s. Conditions: 20 mM NH4OAc, 25 °C, pH 7.2. (b) Details of CEST effect @105 ppm in buffer. (c) Hyper-CEST spectra in cell of only CB6 co-incubation with IVEC, with Put co-incubation with IVEC, with Put, and Hyd. Saturation parameters: B = 20 µT and t = 15 s. (d) Details of CEST effect @122 ppm in cell.

Based on the result above, the reported system was explored with HP 129Xe NMR spectroscopy in buffer. First, 50 µM CB6 was dissolved in 20 mM NH4OAc buffer (pH 7.2). Continuous wave (CW) saturation pulses were scanned over the chemical shift range of δ = 60 – 220 ppm in 3 ppm steps. As shown in Figure 1(a), two saturation responses were displayed in the Hyper-CEST spectrum of CB6. One saturation response attributable to the direct saturation of free dissolved 129Xe was centered at δ = 193.5 ppm, whereas the other was centered at 105 ppm, stemming from the saturation transfer of 129Xe encapsulated in CB6. This result proved that the exchange kinetics of 129Xe was consistent with Hyper-CEST. Upon addition of 100 µM Put to the solution and incubation for 25 min, the 129Xe@CB6 Hyper-CEST signal at δ = 105 ppm disappeared completely. Due to the strong binding of CB6@Put, the exchange was completely blocked. As shown in Figure 1(a) and (b), when 2 mg/mL of DAO was added to the solution and co-incubated for 36 h, the 129Xe@CB6 Hyper-CEST signal "turned on" because of the CB6 cavity release by the oxidization of Put. Then, CB6 was available for the interaction with 129Xe. Taken together, these results indicate that DAO can be highly sensitive and specific detection due to the significant enhancement in the 129Xe@CB6 Hyper-CEST signal (30% CEST effect). Moreover, the intensity of the 129Xe@CB6 Hyper-CEST signals at δ = 105 ppm was found increasing gradually over time. The cavity of CB6, which was released by DAO progressively, was again occupied by 129Xe (refer to Figure S4). Simultaneously, the signal of the dissolved 129Xe Hyper-CEST at δ = 193.5 ppm widened progressively because of the off-resonance effect and release of CB6 cavity. To reduce foam.
formation when bubbling $^{129}$Xe in the gas mixture, 0.1% of Pluronic L-81 (by volume) was added right before the NMR and MRI measurements. To verify whether cellular DAO could be monitored using the proposed biosensor via $^{129}$Xe NMR, the Hyper-CEST method was applied in cell studies. In the first group, CB6 (2.5 mM) was dissolved in the DMEM medium (including 10% fetal bovine serum and 1% penicillin-streptomycin), followed by incubation with the intestinal villus and epithelial cells (IVEC) in a culture flask at 37 °C. After 20 h incubation, the cells were washed and resuspended in the culture medium and the cell concentration was maintained at $3 \times 10^6$ cells/mL. To detect the cellular $^{129}$Xe@CB6 NMR signal, a selective CW saturation pulse for 15 s with a 20 µT field was swept across the chemical shift range of 110 – 136 ppm in 2 ppm steps. As shown in Figure 1 (c) and (d), only one signal appeared around 122 ppm according to $^{129}$Xe@CB6 in cells. Then, 2.5 mM hydroxylamine hydrochloride (Hyd) was used to block cellular DAO in the control group to confirm whether the cellular $^{129}$Xe@CB6 signal could be suppressed by Put. CB6, Put, and Hyd were incubated for 20 h in the control group. The remaining procedures were identical to those of the first group (i.e., cell concentration held at $3 \times 10^6$ cells/mL). The cellular $^{129}$Xe@CB6 Hyper-CEST signal was “turned off” attributing to the close combination of CB6 and Put. For comparison with the control group, CB6 (2.5 mM) and Put (5 mM) were dissolved in the DMEM medium (including 10% fetal bovine serum and 1% penicillin, streptomycin) and the remaining procedures were identical to those of the first group. Interestingly, DAO lightened the cellular $^{129}$Xe@CB6 suppressed by Put, allowing for sensitive and specific detection of endogenous DAO by $^{129}$Xe Hyper-CEST NMR.

In addition to the Hyper-CEST $^{129}$Xe NMR analysis, the biosensor was studied by Hyper-CEST $^{129}$Xe MRI. The experiments for the $^{129}$Xe Hyper-CEST MRI study in 20 mM NH$_2$OAc buffer (pH 7.2) were shown in Figure 2 a, b, c, a FLASH sequence with a $10 \text{s}$, 6.5 µT CW saturation pulse was applied. The resonance frequency of the pulse was set at $-83.5$ ppm relative to free $^{129}$Xe in the solution, and the off resonance frequency was set at 83.5 ppm. The Hyper-CEST $^{129}$Xe MRI images (a) CB6, (b) CB6 + Put, and (c) CB6 + Put + DAO were obtained using a FLASH sequence. The difference between images (a) and (b) in Figure 2 shows that the $^{129}$Xe@CB6 MRI signals could be completely suppressed by Put. Moreover, the difference between images (b) and (c) illustrates that DAO could be specifically detected and localized by Hyper-CEST $^{129}$Xe MRI. This result demonstrated that high sensitive and selective DAO detection could be achieved by this “OFF-ON” biosensor through Hyper-CEST $^{129}$Xe MRI method. The experiments for the $^{129}$Xe Hyper-CEST MRI cell study were as follows: as shown in Figure 2 d, e, f, a FLASH sequence with a $10 \text{s}$, 13 µT continuous wave saturation pulse, whose resonance and off resonance frequencies were set at $-71.5$ and 71.5 ppm, respectively, relative to free $^{129}$Xe in solution at 0 ppm, was used to obtain the $^{129}$Xe Hyper-CEST MRI images of our biosensor: (d) CB6 in cell, (e) CB6 + Put + Hyd in cell, and (f) CB6 + Put in cell. Moreover, the difference between images (e) and (f) revealed that DAO in cell could be specifically detected and localized by $^{129}$Xe Hyper-CEST MRI. We set 0.2×maximum value as threshold to segment the image and interpolated it into 64×64 matrix in the post processing. These results clearly demonstrate that highly sensitive and selective detection of DAO in cell can be achieved with a HP $^{129}$Xe “OFF-ON” MRI biosensor. Thus, the proposed biosensor was found to extend the utility of CB6 for an ultrasensitive $^{129}$Xe NMR biosensor.

The Hyper-CEST response of CB6 was better compared to that of Cry-A. This response rendered CB6 more attractive and important for a variety of applications, including MRI and NMR detection. The results represented here prove that the $^{129}$Xe@CB6 NMR signals can be entirely inhibited by a substrate until a specific enzymatic reaction occurs. These signals appear again when the CB6 cavity is emptied. The proposed biosensor can be used to identify endogenous biomarkers in IVEC, enabling its easy handling in laboratories that circumvent tedious synthetic procedures. Consequently, this project represents the prospects for CB6 that are to be increasingly applied in $^{129}$Xe NMR detection together with multi-modality imaging, drug delivery, and ratio-meter probes. Our future research will be focused on the search of other CB6 guests that could be used with our model for its application in the detection of a wide range of diseases and in vivo detection.

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