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Authors

Slack, Clancy C
Finbloom, Joel A
Jeong, Keunhong
et al.

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Rotaxane probes for protease detection by ^{129}Xe hyperCEST NMR†

Clancy C. Slack,‡[ab] Joel A. Finbloom,‡[a] Keunhong Jeong,[ab] Carson J. Bruns,[a] David E. Wemmer,[ac] Alexander Pines[ab] and Matthew B. Francis*[ab]

We report a CB6 rotaxane for the ^{129}Xe hyperCEST NMR detection of matrix metalloprotease 2 (MMP-2) activity. MMP-2 is overexpressed in cancer tissue, and hence is a cancer marker. A peptide containing an MMP-2 recognition sequence was incorporated into the rotaxane, synthesized *via* CB6-promoted click chemistry. Upon cleavage of the rotaxane by MMP-2, CB6 became accessible for ^{129}Xe@CB6 interactions, leading to protease-responsive hyperCEST activation.

Small molecule probes are promising tools for disease detection, and are used in a variety of platforms including fluorescence, ^{1}H nuclear magnetic resonance (^{1}H NMR) spectroscopy, and ultrasound imaging.[1–3] These methods rely on suppressing background signals until a chemical or biological event occurs that activates the probe, at which point a detectable signal is generated. This strategy has been used to detect enzymes,[2–4] signaling molecules,[3] and redox conditions,[5] among other targets. Proteases, in particular, are widely used markers for disease detection, as certain proteases are overexpressed in diseased tissues and cells.[3,6,7] Proteases have the added benefits of being catalytically active, such that only a small amount is required to activate the probe. They can also be highly specific in their substrate recognition motifs. While proteases have been used in several detection platforms, the reports of such probes for ^{129}Xe NMR have been limited.

^{129}Xe NMR is a versatile tool that can take advantage of host–guest interactions that can reflect the molecular environment.[8,9] By use of spin-exchange optical-pumping for hyperpolarizing xenon, signals can be increased by more than four orders of magnitude, allowing for extremely sensitive detection.[10,11] The polarizability of ^{129}Xe atoms makes their chemical shifts highly sensitive to their environment, and thus they can be used to detect changes in temperature, pH, and liquid crystal ordering.[12,13] Further developments using chemical exchange saturation transfer (CEST) with ^{129}Xe hyperpolarization (hyperCEST) created a means for ultrasensitive magnetic resonance molecular detection. HyperCEST exploits the facile exchange of the xenon in and out of a macromolecular host, exchange occurring more quickly than T_1 relaxation, to reduce the observed signal of the xenon dissolved in water. By saturating the signal from xenon that interacts with the molecular host (^{129}Xe@host) the overall dissolved signal is reduced, allowing for indirect but highly sensitive detection.[14]

Since the development of the ^{129}Xe hyperCEST approach, ^{129}Xe hosts have been conjugated to targeting groups and chemical shift agents for applications in cancer marker imaging and detection of other analytes.[15–17] Typically, cryptophane-A (CryA) has been the ^{129}Xe host of choice for these applications; however, the low solubility of CryA creates difficult procedures for conjugate synthesis, and has low synthetic yields. Cucurbit[6]uril (CB6) was also reported as a promising ^{129}Xe host for hyperCEST NMR.[18,19] Recent work has taken advantage of known CB6 supramolecular host–guest interactions[20] to alter the ability of CB6 to participate in CEST in response to enzymatic reactions or protein binding. This strategy enabled detection of avidin, carbonic anhydrase II, and lysine decarboxylase.[21,22] These studies relied on competitive binding such that the target needed to be in significant excess to inhibit xenon interactions, an undesirable requirement. For more complex biological systems, it is preferable for CB6 to be mechanically locked into a rotaxane such that CB6 can be effectively delivered to a region of interest without any background CB6 release. Our recent work described a model "turn-on" system wherein CB6 was released, and activated for CEST, upon hydrolysis of an ester bond.[23] By mechanically locking CB6 into a rotaxane, we were able to suppress the CB6 hyperCEST response until the chemical event cleaved the rotaxane, producing a CEST response *via* increased ^{129}Xe@CB6 interactions. In this work, we describe a new CB6 rotaxane system that allows sensitive and selective protease detection by ^{129}Xe hyperCEST NMR.

[a] *Department of Chemistry, University of California, Berkeley, Berkeley, CA, 94720, USA. E-mail: mbfrancis@berkeley.edu*
[b] *Materials Sciences Division, Lawrence Berkeley National Laboratories, Berkeley, CA, 94720, USA*
[c] *Physical Biosciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA, 94720, USA*

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‡ C. C. S and J. A. F. contributed equally to this work.

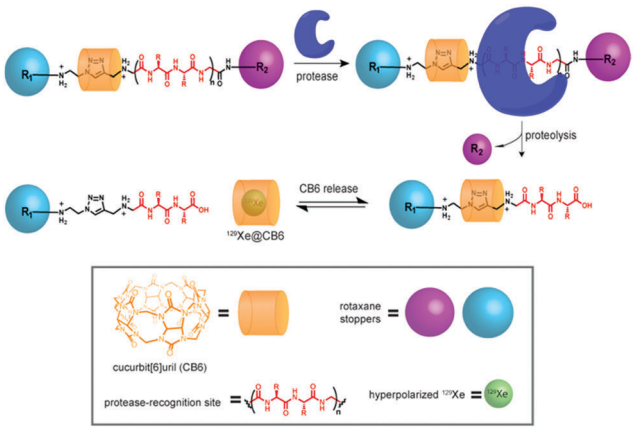

Fig. 1 CB6 rotaxanes probes are activated by proteases to produce a hyperCEST response. CB6 rotaxanes containing a protease cut sequence are cleaved specifically in the presence of an enzyme of interest. Upon cleavage of the rotaxane, CB6 becomes more accessible to host ^{129}Xe and produce a hyperCEST response.

To create CB6 rotaxane probes for protease detection, our design incorporates peptide sequences into the axle component of the rotaxane (Fig. 1). These peptides can be synthesized to contain any sequence, including protease recognition sequences of interest. After proteolysis events, a portion of the peptide remains on the axle component of the rotaxane, leaving a post-cleavage product mechanically unlocking CB6, which can be released from the rotaxane becoming CEST active. In theory, this design can be used to detect any of a wide variety of proteases using ^{129}Xe hyperCEST NMR if the CB6 becomes more accessible for ^{129}Xe interactions following proteolysis.

MMP-2 represents an interesting disease-related target for this rotaxane probe platform. Rotaxane **2** was synthesized with pyrene and (5,6)-carboxytetramethylrhodamine (TAMRA) stoppers, and an axle containing the PLG-LAG recognition sequence of matrix metalloprotease-2 (MMP-2). MMPs are overexpressed in a wide range of metastatic tumors,[4,24,25] and are present in heart tissue after myocardial infarction.[26] MMP-2 has been used successfully as a target for a variety of drug delivery and imaging applications.[4,7,24,25] Previously, a xenon sensor for MMP was developed with a peptide recognition sequence for MMP-7 covalently attached to CryA.[27] Upon cleavage by MMP-7, a change of approximately 0.5 ppm in the ^{129}Xe@CryA chemical shift was observed. In contrast, rotaxane **2** offers the opportunity to detect MMP-2 by measuring the increase in CEST effect increase upon cleavage. This on/off change could allow better detection in biologically complex environments than measuring a small shift in a broad line.

Rotaxane **2** was synthesized using CB6-promoted azide–alkyne cycloaddition (Fig. 2a). This synthetic strategy is widely used in rotaxane synthesis and provides a one step, straightforward, and modular route to a variety of CB6 complexes.[28–30] Furthermore, many types of bulky molecules can be used as stoppers on these rotaxanes, allowing diverse functionalities for localization or uptake to be coupled with hyperCEST NMR detection.

We previously observed that CB6 bound to triazole–diammonium guests without any bulky stoppers or additional axle components

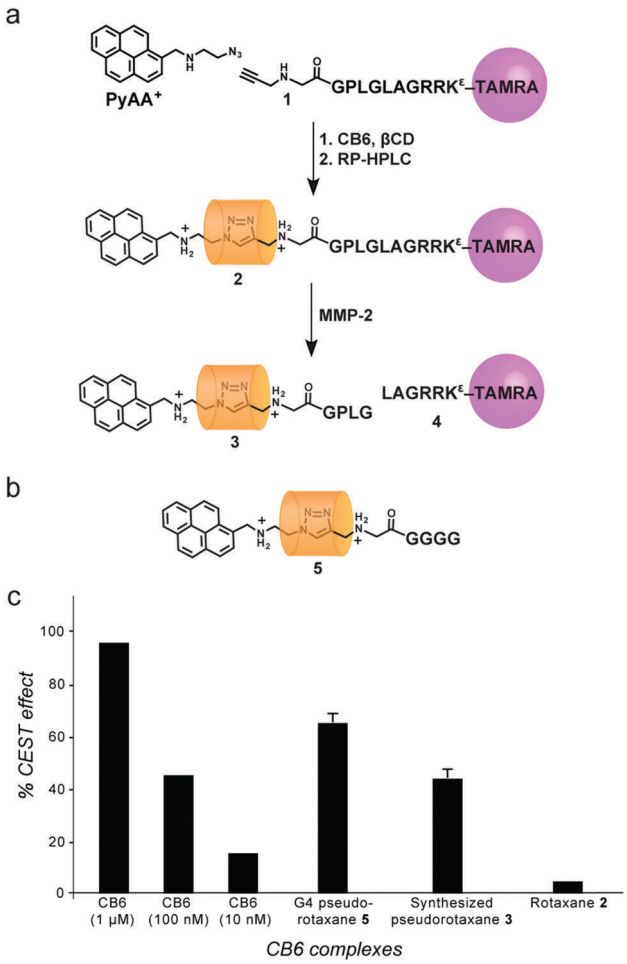

Fig. 2 Synthesis and hyperCEST response of CB6 complexes. (a) CB6 rotaxane **2** was synthesized using CB6-promoted azide–alkyne click chemistry in the presence of βCD to accelerate the reaction. Cyclodextrin is removed during purification by RP-HPLC. Rotaxane **2** contains the MMP-2 recognition sequence PLG-LAG. (b) Structure of G4 pseudorotaxane **5**. (c) The percent CEST effect was measured for various CB6 complexes. Oligoglycine pseudorotaxane **5** and pseudorotaxane **3** were synthesized to function as positive controls. Both pseudorotaxanes **5** and **3** demonstrated significant CEST effects, whereas capped rotaxane **2** showed minimal CEST effect. All measurements were performed at 10 μM rotaxane in pH 7 phosphate buffer. Error bars represent standard instrumental error for 4 repeats. The standard deviation of the baseline is 7% and thus the signal observed for **2** was not significant.

reduced the hyperCEST effect when compared to free CB6 in solution.[23] This is likely due to an equilibrium that exists between triazole-bound and unbound CB6 (Fig. 1). To confirm that the peptide component of the rotaxane would not prevent CB6 release and subsequent hyperCEST response of the CB6 rotaxane, we synthesized two different peptide pseudorotaxanes to compare to rotaxane **2**. Oligoglycine pseudorotaxane **5** was compared to the GPLG sequence remaining after cleavage by MMP-2 (pseudorotaxane **3**), as well as rotaxane **2** to determine the percent CEST effect of these CB6 complexes (Fig. 2c). The temperature was held constant at 25 °C, and 10 μM solutions of rotaxane or pseudorotaxane were used. A significant CEST effect was observed for pseudorotaxane **5** and a similar response was

observed for pseudorotaxane **3**. This indicates that for both these constructs, CB6 is able to leave the rotaxane axle and become available for ^{129}Xe@CB6 interactions and subsequent hyperCEST response.

A hyperCEST spectrum for intact rotaxane **2** was taken as a baseline to compare the varying peptide sequences, as there was no possibility for CB6 release from the rotaxane axle. No significant CEST effect was observed for the intact rotaxane **2**, which indicates that the CB6 is held in place by the bulky end groups (Fig. 2c). Comparative CEST responses for 1 μM, 100 nM, and 10 nM free CB6 suggest that on the order of 100 nM CB6 is being released from the oligoglycine (**5**) and GPLG (**3**) pseudorotaxanes.

To evaluate the construct for enzymatic cleavage, rotaxane **2** (100 μM) was incubated at 37 °C with MMP-2 (100 nM) and monitored by reverse phase high performance liquid chromatography (RP-HPLC) and matrix assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF MS).

Approximately 60% cleavage of **2** was observed after 24 h by RP-HPLC and post-cleavage products **3** and **4** were observed by MALDI-TOF MS (Fig. 3a). This confirmed the PLG-LAG cleavage by MMP-2. As a control, peptide **1** was subjected to identical MMP-2 cleavage conditions and was monitored by RP-HPLC and MALDI-TOF MS (Fig. 3b). Similar rates of proteolysis were observed for peptide **1** as were observed for rotaxane **2**. This indicates that the CB6 supramolecular component does not hinder binding to, or subsequent proteolysis of the rotaxane.

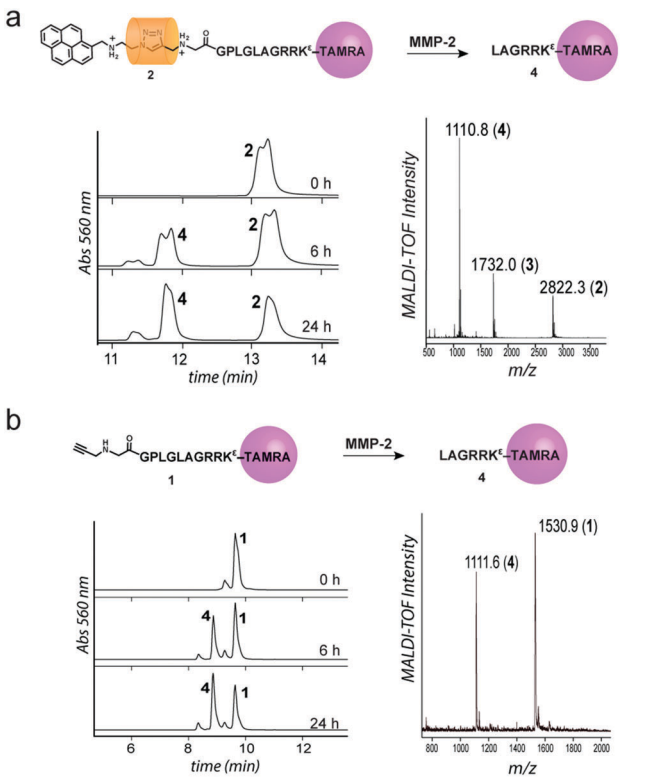

Fig. 3 MMP-2 recognizes and cleaves CB6 rotaxane **2** at similar rate as peptide **1**. (a) Rotaxane **2** contains a PLG-LAG MMP-2 recognition site. Upon cleavage by MMP-2, CB6 is available for ^{129}Xe host–guest interaction. RP-HPLC traces of 100 μM **2** (13 min) when exposed to 100 nM MMP-2 at 37 °C revealed approximately 60% cleavage to **4** (11.7 min) by 24 h. Further degradation (11.3 min) was also observed. MALDI-TOF MS confirmed both cleavage products **3** and **4**, as well as remaining rotaxane **2**. (b) HPLC traces of 100 μM **1** (9.5 min) when exposed to 100 nM MMP-2 at 37 °C. Peptide **1** contains the same MMP-2 recognition sequence as rotaxane **2**. Similar to rotaxane **2**, approximately 60% cleavage to product **4** was observed by 24 h. Double peaks are likely due to the 5,6 isomers of the TAMRA. MALDI-TOF MS confirmed the identity of the cleavage products.

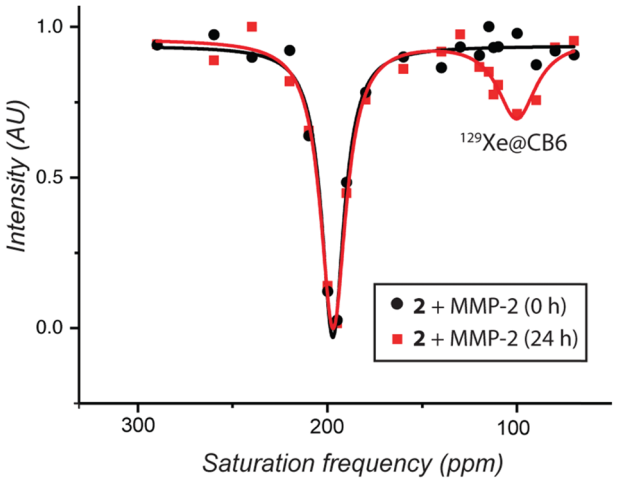

Fig. 4 CB6 rotaxane **2** functions as a responsive probe for MMP-2 *via* ^{129}Xe hyperCEST activation. Rotaxane **2** was incubated with MMP-2 for 24 h at 37 °C, and was diluted to 5 μM in ddH_2O before acquiring a hyperCEST spectrum. No detectable hyperCEST response was observed for **2** with MMP-2 immediately after incubation. After 24 h, a significant ^{129}Xe hyperCEST response was observed.

Rotaxane **2** cleavage by MMP-2 corresponded with an increase in hyperCEST response at 24 h (Fig. 4). Before hyperCEST spectra were obtained, the rotaxane-enzyme solution was diluted to 5 μM rotaxane and 5 nM enzyme. After cleavage by MMP-2, xenon was able to interact strongly with CB6, producing a significant increase in hyperCEST response (15% CEST effect) and allowing sensitive detection of MMP-2 *via* ^{129}Xe hyperCEST.

These results demonstrate that CB6 rotaxanes can be used as modular probes for protease detection. By tailoring the peptide axle to a protease of interest, these rotaxanes can be tuned for a wide range of disease detection applications. By cleaving the bulky end group, the CB6 can be released from the rotaxane axle and become available for hyperCEST detection. Future work to optimize the rotaxane guest for improved CB6 release upon cleavage could increase the observed CEST effect of CB6 rotaxanes, allowing the nanomolar concentration range required for enzymatic detection in biologically complex environments to be reached, and will help expand CB6 rotaxane probes for a wide range of disease detection applications.

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