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The Synthesis of Rotaxane Probes for Magnetic Resonance Imaging (MRI)

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THE SYNTHESIS OF ROTAXANE PROBES FOR MAGNETIC RESONANCE
IMAGING (MRI)

BY
SCOTT MACGILL KARAS

THESIS SUBMITTED IN PARTIAL FULLFILMENT OF REQUIREMNTS FOR
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MASTER OF SCIENCE THESIS

OF

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ABSTRACT

Rotaxanes are simple molecules made by an interaction between a host and a guest.¹ Research has shown that rotaxanes have the capability of exchanging a hyperpolarized inert gas atom for use in Magnetic Resonance Imaging (MRI).² Currently, MRI contrast is enhanced by the injecting a magnetic gadolinium (III) [Gd(III)] ions into the human body prior to the imaging experiment.³ Unfortunately, these contrast agents are expensive and toxic; as a result, there is need for a cheaper and less toxic imaging agent. Additionally, it would be beneficial to develop targeted contrast agents, i.e. macromolecules that bind specific analytes, proteins, or cellular receptors within the body. By combining rotaxanes with Hyperpolarized ^{129}Xe Chemical Exchange Saturation Transfer (HyperCEST), ^{129}Xe MRI technology should be capable of imaging specific areas in the human anatomy, thus facilitating the study and diagnosis of diseases or injuries. Currently ^{129}Xe MRI is being used to generate images of the lungs and the brain, but with a synthetic molecule we hope to broaden this capability to include high-resolution molecular imaging.

The manuscript, “Cyclodextrin-based Pseudo-rotaxanes: An Easily Conjugatable Scaffold for Hyperpolarized Xenon Magnetic Resonance Imaging Biosensors” is the result of our preliminary work to develop a viable molecular probe. The manuscript focuses on the development of a new class of xenon-129 MRI contrast agents based on rotaxanes of γ -cyclodextrin, and the application of this new technology to the synthesis of a potential biosensor for imaging the β -amyloid plaques that are associated with Alzheimer’s disease..

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I want to thank my girlfriend **Dana DiScenza** for always being there for me, always listening to my frustrations with research, and for being the best friend any one could ask for. You are so smart, tons of fun, and a real character to hang out with. There is never a dull moment with you.

A big thanks goes out to my parents, **Robert Karas** and **Dr. Debbie Karas**. They have been such huge role models and support throughout my entire life. I would not be where I am without you. You have set the bar high for myself and all of my brothers. Additionally, I need to thank my three older brothers: **Jason**, **Andrew**, and **Ben**. You three have been such great role models too. When I needed guidance you were always there to help me out. I wouldn't have taken the path I have in life if it wasn't for my parents and my brothers. Thank you all so much!

PREFACE

The following work is presented in manuscript format according to the guidelines presented by the University of Rhode Island Graduate School. The thesis will consist of one manuscript entitled, “Cyclodextrin-based Pseudo-rotaxanes: An Easily Conjugatable Scaffold for Hyperpolarized Xenon Magnetic Resonance Imaging Biosensors” that has recently been submitted to the *Journal of the American Chemical Society*.

Before joining this project, my researched focused was on aromatic C-H amination through palladium catalysis. A wide variety of research has been focused on the synthesis of aromatic C-N bonds because of their prevalence in pharmaceuticals, herbicides, and dyes.⁴ My work attempted to use oxime esters as electrophilic aminating agents to synthesize various aromatic C-N bonds (Scheme 1). After trying a wide variety of palladium catalysts and ligands, I was unsuccessful in producing any C-N bonds. From there, I moved on to the new project of synthesizing molecular probes to be used as potential biosensors.

Scheme 1: Attempted aromatic C-H amination

The first potential probe I tried to synthesize involved Folic Acid (FA) because of its high binding affinity to folate receptors found in tumors.⁵ The plan was to bind an alkyne to the primary amine of FA (Scheme 2A) to eventually do copper catalyzed azide-alkyne cycloaddition (CuAAC) onto a C₈ or C₁₀ diazide linker molecule.⁶ The synthesis of the functionalized FA was a tedious process that ended up not working. Next, I tried *N*-hydroxysuccinimide (NHS) to selectively bind to the γ -carbonyl group. NHS activates FA allowing for selective binding of primary amines.⁷ Like Scheme 2A, Scheme 2B was proved to be difficult, and the final product was unable to be observed through ¹H NMR.

Scheme 2: Folic acid probe. A: Functionalized folic acid. B: Activated folic acid.

Along with FA, Thioflavin-T (ThT) was another molecule I worked with to synthesize a potential probe for Alzheimer's disease. I chose one of several ThT derivatives to use as the target moiety. From there I developed three different methods to make the ThT probe (Scheme 3). First, I bound a propargyl group to the amine of ThT to perform CuAAC to either a C₈ or C₁₀ diazide linker. This method proved to be

ineffective due to low yields of the propargylated ThT. The second method I tried involved the NHS ester of sebacic acid; unfortunately, this reaction didn't work. The final method used sebacoyl chloride as the linker to successfully synthesize the ThT probe in high purity and yields.

Scheme 3: ThT probe methods

Subsequent work by me, Ashvin Fernando and others in the group focused on the synthesis and NMR analysis of the pseudo-rotaxanes that could be made from the compounds that I synthesized. All of the xenon-129 NMR that is discussed herein was performed by our collaborators at the Thunder Bay Regional Research Institute in Ontario, Canada.

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MANUSCRIPT

**Cyclodextrin-based Pseudo-rotaxanes: An Easily Conjugatable Scaffold for
Hyperpolarized Xenon Magnetic Resonance Imaging Biosensors**

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**Cyclodextrin-based Pseudo-rotaxanes: An Easily Conjugatable Scaffold for
Hyperpolarized Xenon Magnetic Resonance Imaging Biosensors**

Abstract

Hyperpolarized (HP) xenon-129 (Xe) magnetic resonance (MR) imaging has the potential to detect picomolar concentrations of molecular biosensors with sub-millimeter spatial resolution. This high sensitivity and high resolution gives Xe MR imaging biosensors the potential to act as a molecular medical imaging modality similar to positron emission tomography (PET) but without the use of ionizing radiation. A number of challenges have hampered the translation of Xe MR imaging biosensors from theoretical and *in vitro* demonstration to *in vivo* testing. One of these challenges is the difficulty in conjugating the MR active Xe encapsulating cage molecule with an affinity tag which binds to the molecule of interest within the body at sufficiently high yields to be feasible. In this study we demonstrate that a pseudo-rotaxane based on a γ -cyclodextrin macrocycle with a 10-carbon axle, is easily synthesized and is detectable using HP Xe MR spectroscopy. A pseudo-rotaxane containing Thioflavin T was designed and synthesized, as a potential biosensor capable of binding the amyloid fibrils that are associated with Alzheimer's disease (AD). We refer to this potential biosensor as Xenon Cage Amyloid Ligand-1 (XCAL-1).

Introduction

Hyperpolarized (HP) xenon-129 based magnetic resonance imaging (MRI) biosensors have the potential to become a molecular imaging modality with similar sensitivity to positron emission tomography (PET), but with theoretically better spatial resolution, no ionizing radiation and lower cost. HP gas MRI takes advantage of the signal enhancement provided by the hyperpolarization of gas, that is, the aligning of the spins of a majority of nuclei with an external magnetic field, providing a signal enhancement of up to 100,000 times above thermally polarized nuclei.⁸ HP gas MRI is an ideal imaging modality for imaging of the lungs.⁹ Xe diffuses throughout the whole body following inhalation, and because Xe is hydrophobic, it deposits particularly well in lipid-rich tissues. High-resolution, three-dimensional images are obtained by detecting the HP Xe that is deposited in various fatty tissues, such as the brain.^{10–12} HP Xe atoms cannot, by themselves, be tuned to target particular regions in the body; however, targeted HP Xe molecular probes that are capable of binding both biochemical receptors and xenon atoms *in vivo* have been postulated as a way to perform molecular imaging, and numerous prototypes have been developed, though targeted HP Xe biosensors have yet to be used for imaging in a living animal.

Like all molecular probes, HP Xe MRI biosensors consist of two functional parts, a binding component and a detection component that are joined by a covalent tether (Figure 1A). The binding component is an affinity tag or antibody that binds to a specific analyte or biochemical receptor, allowing for the detection and characterization of specific biochemical phenomena. The detection component for a HP Xe biosensor is usually a supramolecular cage-like structure that can encapsulate a

xenon atom. For *in vivo* imaging, the magnetically active HP Xe can be inhaled by the subject and the imaging biosensor can be administered via an injection. After the dissolved Xe gas has circulated through the subject's body and the targeted biosensor has attached to the desired molecular target, the Xe will be reversibly encapsulated by the biosensor in a traditional host-guest interaction. If the reversible encapsulation is slow on the NMR time scale, it will produce a unique chemical signal in the ^{129}Xe NMR spectrum which can be transformed into a three-dimensional image.

The Hyperpolarized gas Chemical Exchange Saturation Transfer (HyperCEST) pulse sequence takes advantage of the continual diffusion of Xe atoms in and out of a Xe-encapsulating cage molecule, and allows for the detection of picomolar concentrations of a HP Xe biosensor.^{13–15} Because the binding of the Xe is reversible, but slow on the NMR timescale, the ^{129}Xe spectrum of a biosensor contains two peaks, one for unbound Xe and one for the xenon that is encapsulated by the biosensor. By exciting the HP Xe atoms inside the supramolecular cage at their unique chemical shift offset frequencies, the Xe atoms inside the cage molecules become depolarized. When these depolarized Xe atoms exchange with the HP Xe atoms from the pool of dissolved Xe atoms, there is a reduction in signal from the pool of dissolved phase Xe atoms (Figure 1B). The HyperCEST technique, combined with hyperpolarization of the nuclei, provides a theoretical signal enhancement of up to a billion times above thermally polarized nuclei.¹⁶

Figure 1: **A/** Schematic of a targeted HP Xe MRI biosensor. **B/** Predicted ^{129}Xe spectrum of dissolved HP Xe (orange) in the presence of a Xe biosensor (blue) and its detection via HyperCEST.

Despite the seminal work of Pines and colleagues over two decades ago,¹⁴ HP-Xe MRI biosensor technology has yet to be translated into a clinical imaging technique. We recently disclosed the first *in vivo* images of the MR contrast portion of a xenon MR imaging biosensor in a live animal using the non-targeted Xe cage, CB6.¹⁷ In our opinion, the development of targeted HP Xe MRI biosensors has not been slowed by a lack of interest in the techniques or by barriers in MRI technology. The problem is more fundamental: the supramolecular hosts that encapsulate xenon are difficult to synthesize and derivatize, so sufficient quantities of biosensors for *in vivo* imaging are simply not available.

Herein, we describe the development on a new class of water-soluble HP Xe biosensors that use a pseudo-rotaxane to encapsulate HP Xe and can be imaged using a HyperCEST pulse sequence. We have developed a new biosensor based on this scaffold that has the potential to image the amyloid fibrils that are associated with Alzheimer's disease (AD), which we call Xenon Cage Amyloid Ligand-1 (XCAL-1). The modular

nature of the synthetic scheme should allow for the rapid synthesis of not only XCAL-1, but a wide variety of biosensors, thus paving the way for numerous clinical applications.

Design of pseudo-rotaxane HP Xe probe

Our group, as well as others, have tested a number of different xenon hosts including cryptophanes,^{18–21} cucurbiturils,^{17,22–24} liposomes,^{25,26} gas vesicles,²⁷ and bacterial spores.²⁸ Of these xenon-capturing scaffolds, cryptophane-A has been studied the most. For example, cryptophane-A derivatives have been conjugated to affinity tags to bind a wide variety of targets,^{29–31} such as CD14 cancer cells,³² the cancer-associated HER2 receptor,²⁷ zinc,³³ toxic metal ions,³⁴ and the inflammation-marking peripheral benzodiazapene receptor (PBR)³⁵. These reports have not come without a great deal of effort. In one recent report, the Dmochowski group conjugated a cryptophane-A to folic acid to yield a potential biosensor for cancer.³⁶ While notable not only for its scientific novelty, the tenacity of the research associate who conducted 20 non-linear steps to synthesize the final product is remarkable in and of itself. Surely there must be a simpler and higher yielding synthesis of a HP-Xe biosensor!

Until recently, the hollow, ball-shaped molecular cages known as cryptophanes appeared to be privileged structures in the field of HP-Xe imaging. Cryptophane-A contains a hydrophobic core with a volume of 95 Å³,³⁷ and is capable of reversibly binding xenon with a k_a of 3 kM⁻¹, with a residence time of 0.5–8 ms at room temperature³⁸. Despite its more tube-like structure, cucurbit[6]uril (CB[6]) is also capable of binding xenon with a comparable affinity ($k_a = 200 \text{ M}^{-1}$),³⁹ but its larger derivatives, cucurbit[7]uril (CB[7]) and cucurbit[8]uril (CB[8]), do not show any affinity for xenon,

as observed by ^{129}Xe NMR. Additionally, α , β and γ -cyclodextrins (CD), which are truncated cone-shaped macrocycles composed of six, seven or eight D-glycopyranoside units, have hydrophobic cavities with minimum diameters of 5.3 Å, 6.5 Å and 8.3 Å, respectively (Figure 2A). CDs are some of the most commonly used hosts in supramolecular chemistry,^{13a} and they would be ideal components of ^{129}Xe biosensors because they are non-toxic and water-soluble. Unfortunately, α , β and γ -CD either fail to bind xenon in aqueous media, or the reversible binding has too fast of an exchange rate to be observed by ^{129}Xe NMR at room temperature.^{40b}

We hypothesized that macrocycles that were too large to bind xenon on their own, such as the cyclodextrins, could be threaded with long alkyl chains to create rotaxane-type complexes that were capable of forming a ternary complex with xenon (Figure 2B).^{13c} Rotaxanes are well-known supramolecular species composed of a molecular axle that is threaded through a tube-shaped host, creating a non-covalently bound structure. In order to serve as a molecular probe, the inner diameter of the rotaxane's macrocycle must be large enough to fit both the molecular axle and a xenon atom in its hydrophobic core. However, a macrocycle that is too large would not be detectable using HyperCEST because the xenon would exchange in and out of the host at a rate that is too high to support HyperCEST detection. Prior to this work the only precedent for the formation of this kind of ternary complex with xenon was reported by Cohen, who showed that a CB6 derivative could simultaneously bind hexane and xenon.⁴⁰ Consequently, we designed three classes of pseudo-rotaxanes to determine their potential as the imaging component of HP-Xe biosensors: CB-based pseudo-rotaxanes, pillararene-based pseudo-rotaxanes, and CD-based pseudo-rotaxanes.

Figure 2: A/ Electrostatic potential maps and chemical structures of cucurbiturils and cyclodextrins. B/ Proposed binding of xenon via formation of a ternary complex.

In addition to the novelty of the ternary xenon complexes, the design shown in Figure 2B could also expedite the development of a wide variety of biosensors because they would not be synthesized by covalently tethering the affinity tag to the xenon host; rather, the affinity tag would be conjugated to the axle of the rotaxane, which is presumably a straightforward process, and the tethering of the affinity tag to the macrocycle would then be accomplished via classic supramolecular chemistry relying primarily on hydrophobic interactions, which, coincidentally, are the same forces that are required for efficient xenon binding. For these preliminary studies, we defined two criteria for success: firstly, the molecule had to be readily synthesized and conjugatable.

Secondly, the molecule had to be MR detectable by displaying a HyperCEST effect. Towards the first goal, we found that pillararenes were relatively easy to synthesize and CB and CD macrocycles are both commercially available. Each of the host molecules were threaded with five, eight and ten-carbon molecular threads that contained terminal ethylimidazolium groups, which served to enhance the water solubility of the greasy alkanes and enabled facile detection by mass spectrometry (Scheme 1). Furthermore, one can easily imagine methods to create similar moieties that were attached to affinity tags. In all cases studied, the rapid formation of threaded complexes was observed by NMR, though most cases showed rapid host-guest exchange on the NMR time scale. Subsequent analysis by ^{129}Xe NMR quickly identified the promising scaffolds that combined the desired attributes of facile synthesis with MR detectability via HyperCEST.

Scheme 4: Synthesis of various guests. A/ Synthesis of the C₁₀ diethylimidazolium bar (**1**) and its incorporation into γ -CD to form a pseudo-rotaxane. B/ Other bars tested in pseudo-rotaxanes: C₅ diethylimidazolium bar (**2**), C₈ diethylimidazolium bar (**3**), C₈ diazide bar (**4**) and C₈ diazide bar (**5**).

For the HP-Xe studies, we used a custom-built fritted phantom inside of a custom dual tuned $^1\text{H}/^{129}\text{Xe}$ radiofrequency (RF) coil to acquire all free induction decay (FID) spectra (Figure 3). Cage molecules, dissolved in water and/or DMSO, were placed inside the fritted phantom, and HP ^{129}Xe was introduced below the fritted phantom which created microbubbles that bubbled vertically through the sample. A series of saturation pre-pulses at a variety of chemical shift offsets were loaded into the user interface software of the GE Achieva 3T MR scanner. Spectra with different saturation pre-pulses were acquired approximately every six seconds.

Figure 3: Schematic of experimental setup. 10 mM of γ -cyclodextrin with a C_{10} diethylimidazolium bar ($1\subset\gamma$ -CD) dissolved in water was placed inside of a fritted phantom and placed inside an NMR RF coil. Hyperpolarized xenon was introduced into the phantom via a glass frit which produced microbubbles that dissolved in the solution. The HP-Xe atoms diffused in and out of the CD cage. An RF saturation pulse was applied at the Xe-CD chemical shift offset frequency depolarizing the ^{129}Xe within the CD. The depolarized ^{129}Xe diffused out of the CD and was replaced by a HP-Xe atom. This resulted in a reduction in the pool of HP Xe in solution thereby depleting the NMR signal which indicates the presence of the probe molecule. The absence of a Xe imaging probe in the solution would result in no depletion of the dissolved phase Xe signal.

Discovery of pseudo-rotaxane HP Xe probes

Our initial studies commenced with the analysis of cucurbiturils, as we have previously had success using the most common member of this family of macrocycles, CB6, in HyperCEST studies. As expected, irradiation at +128 ppm (relative to the peak corresponding to dissolved xenon) produced a 67% depletion, thus confirming that our experimental method was reliable. Various threaded complexes of CB6, CB7 and CB8 were then synthesized (see Supporting Information) and subjected to the same HyperCEST protocol. Unfortunately, none of the pseudo-rotaxanes could be detected by HyperCEST, indicating that there is not sufficient space in the cavity of the supramolecular complex (likely true for complexes like $1\subset\text{CB6}$) or that that xenon

exchanges too rapidly in and out of the complex to be detected (possibly true for larger complexes like **1**⊂**CB8**).

Two different pillararene structures were also tested (see Supporting Information for structures), but both suffered from poor water solubility. Consequently, organic co-solvents or non-ionic diazide bars (Scheme 4) had to be employed. All pillararene-derived threaded complexes failed to produce a HyperCEST signal. This was surprising because pillararene-based pseudo-rotaxane reported to be was capable of binding xenon.⁴¹ We attempted to further these studies by applying HyperCEST saturation pulses and acquiring a HyperCEST depletion spectrum, but we were unable to observe a HyperCEST effect in pseudo-rotaxanes based on the pillararene macrocycle. We were neither able to detect the presence of a peak corresponding to a xenon-pillararene complex, nor a HyperCEST effect.

Gratifyingly, we found that cyclodextrin-based pseudo-rotaxanes can reversibly encapsulate xenon and be detected by ¹²⁹Xe magnetic resonance spectroscopy. A HyperCEST depletion of 30% for the pseudo-rotaxane formed by mixing α-cyclodextrin with the C₅ diethylimidazolium bar (**2**⊂**α-CD**), and 52% for γ-cyclodextrin pseudo-rotaxanes with the C₁₀ diethylimidazolium bar threaded through the cavity (**1**⊂**γ-CD**). The maximum HyperCEST depletion for all three cyclodextrin-based rotaxanes occurred at approximately +128 ppm from the Xe gas phase signal. HyperCEST depletion spectra for **1**⊂**γ-CD** are shown in Figure 4. Importantly the HyperCEST depletion for (**1**⊂**γ-CD**) was comparable to that of CB6, a xenon cage that we have recently shown to be amenable to *in vivo* HP Xe MRI.

The data shown in Table 1 and Figure 4 were obtained using a Phillips Achieva 3T clinical whole-body MR scanner, which proves significant advantages over conventional NMR spectrometers, namely the ability to perform whole-body imaging experiments. Unfortunately, these advantages come with some trade-offs, specifically the ability to acquire HyperCEST depletion spectra with a Lorentzian fit line, such as in the data processed for cryptophane and cucurbituril agents as demonstrated by the Schroeder and Dmochowski groups using high-field, high-resolution NMR spectrometers.^{42–45} However, the use of a clinical scanner bodes well for eventual clinical translation of our techniques in *in vivo* experiments, which we recently demonstrated using a non-targeted ^{129}Xe contrast agent.¹⁷

Table 1: HyperCEST data for pseudo-rotaxanes

Pseudo-rotaxane (macrocycle + bar)	% Depletion ^a	δ (ppm) ^a
cucurbit[6]uril		
+ none	67%	+128
+ C ₈ diethylimidazolium bar (3)	0%	-
+ C ₁₀ diethylimidazolium bar (1)	0%	-
cucurbit[7]uril		
+ none	0%	-
+ C ₈ diethylimidazolium bar (3)	0%	-
cucurbit[8]uril		
+ none	0%	-
+ C ₈ diethylimidazolium bar (3)	0%	-
+ C ₁₀ diethylimidazolium bar (1)	0%	-
decamethyl-pillar[5]arene		
+ none ^a	0%	-
+ C ₈ diethylimidazolium bar (3) ^b	0%	-
decabromoethyl-pillar[5]arene		
+ none ^c	0%	-
+ C ₈ diazide bar (4) ^c	0%	-
+ C ₁₀ diazide bar (5) ^c	0%	-
α -cyclodextrin		
+ none	0%	-
+ C ₅ diethylimidazolium bar (2)	30%	+132
+ C ₈ diethylimidazolium bar (3)	0%	-
+ C ₁₀ diethylimidazolium bar (1)	0%	-
β -cyclodextrin		
+ none	0%	-
+ C ₅ diethylimidazolium bar (2)	0%	-
+ C ₈ diethylimidazolium bar (3)	0%	-
+ C ₁₀ diethylimidazolium bar (1)	0%	-
γ -cyclodextrin		
+ none	0%	-
+ C ₅ diethylimidazolium bar (2)	0%	-
+ C ₈ diethylimidazolium bar (3)	43%	+128
+ C ₁₀ diethylimidazolium bar (1)	50%	+128

^a Samples (2 mL, 10 mM) were dissolved in water, 1D ¹²⁹Xe spectrum were initially recorded and then a series of HyperCEST spectra were sequentially recorded using a series of off resonance pulses varying by 5 ppm. ^b Performed in H₂O/DMSO. ^c Performed in CHCl₃.

Figure 4: FID and HyperCEST depletion spectra of γ -cyclodextrin with the C_{10} diethylimidazolium bar (**1**- γ -CD). Free Induction Decay (FID) spectra of 10 mM γ -cyclodextrin with a 10-carbon ethyl imidazole bar following off-resonance (A) (+255 ppm) and on-resonance (+128 ppm) HyperCEST pulses (B). HyperCEST depletion z-spectrum (C) of 10 mM γ -cyclodextrin with the C_{10} diethylimidazolium bar (**1**- γ -CD). Each data point indicates the HyperCEST depletion when the molecule is irradiated with a HyperCEST pulse at a given chemical shift offset. A maximum HyperCEST depletion of 52% occurs.

^1H NMR also confirms the formation of the ternary pseudo-rotaxane-xenon complex [**(Xe·1)**- γ -CD Figure 5]. Formation of the pseudo-rotaxane with γ -cyclodextrin and **1** can be easily monitored by an upfield shift in the triplet corresponding to the proton attached to the C3 position in the in the cyclodextrin. This proton is positioned on the interior of the macrocycle, so it is shielded when the pseudo-rotaxane forms. Subsequent binding of xenon further shifts the C3-H peak upfield, indicating that the xenon also binds to the interior of the macrocyclic host.

NMR titration studies were performed to assess the nature of the host:guest interaction for **1**- γ -CD.⁴⁶ An association constant of $8.8 \times 10^2 \text{ M}^{-1}$ for the 1:1 host:guest complex was measured. (see Supporting Information for details.) Despite this modest affinity, the formation of both the binary (**1**- γ -CD) and ternary [**(Xe·1)**- γ -CD] complexes is favorable and detectable by ^1H and ^{129}Xe NMR.¹⁴

Figure 5: ^1H NMR evidence for the formation of the ternary complex. **A/** ^1H NMR spectrum of γ -cyclodextrin. **B/** ^1H NMR spectrum after addition of 1 equiv of **1**. **C/** ^1H NMR spectrum after addition of 1.5 atm xenon.

Application to the synthesis of XCAL-1

To demonstrate the utility of this new class of xenon-binding agents for the synthesis of targeted biosensors, we synthesized a potential molecular probe using Thioflavin T (ThT). ThT is a fluorescent dye that binds to the β -amyloid plaques that are associated with Alzheimer's disease (AD).⁴⁷ Using HP Xe biosensors like XCAL-1, we envision potential clinical applications for studying the progression of AD or the efficacy of treatments for it (Scheme 5).

The synthesis of the biosensor involves a simple acylation, followed by the formation of the pseudo-rotaxane. Both steps are nearly quantitative. As before, formation of the 1:1 complex was confirmed by ^1H NMR studies, and the association constant for the pseudo-rotaxane was determined to be $2.0 \times 10^4 \text{ M}^{-1}$. Thus far, we have synthesized XCAL-1 on a scale of hundreds of milligrams, but the reactions are simple

and high-yielding. There is no reason to believe that biosensors like XCAL-1 could not be synthesized on a gram scale, or larger, thus paving the way for *in vivo* imaging studies.

Scheme 5: Facile synthesis of the potential biosensor, XCAL-1.

Summary

In conclusion, we have discovered a novel method for synthesizing potential HP Xe biosensors by using pseudo-rotaxane structures of γ -CD. These supramolecular complexes form a novel ternary structure in the presence of HP Xe which can be detected via ^{129}Xe MR spectroscopy and imaging experiments. Future work by our collaborative team will involve *ex vivo* and *in vivo* analysis of the XCAL-1 probe for imaging β -amyloid plaques. Furthermore, the ease of synthesis of these pseudo-

rotaxane probes should allow for the rapid development of numerous other HP Xe biosensors.

Experimental Methods

Nuclear Magnetic Resonance (NMR) HyperCEST Detection. Natural abundant ^{129}Xe gas was polarized to 26-30% using a Xemed polarizer (Xemed, Durham, NH, USA). 1.0 mL of sample was drawn into the glass frit cell using a syringe. The cell was then inserted into a custom RF coil tuned to the Larmor frequency of ^{129}Xe (35.33 MHz) at 3T, where HP ^{129}Xe gas was introduced to the vessel from the Tedlar bag in the pressure chamber which was pressurized at 35 kPa above atmosphere. The solution was mixed with HP ^{129}Xe gas as it passed through the fine fritted disc and produced several microbubbles, which continuously dissolved into solution and exited the vessel through the outflow tube. The concentration of ^{129}Xe at any point during the experiment was between 1-10 mM. A Philips Achieva 3T clinical scanner was used to collect all NMR spectra. The RF pulse length was determined with the use of the Ref B₁, a parameter of Philips MR scanners. The pulse length and flip angle were used to calculate the amplitude of the RF pulse and field strength. In this study, the B₁ field strength was determined by the scanner to be 15.9 μT . In the acquisition of NMR spectra, a pulsed saturation pre-pulse train consisting of 96- 20 ms 3-lobe sinc pulses with 0 ms pulse intervals was applied at various chemical shift offsets. Free induction decay (FID) spectra were acquired at various chemical shift frequency offsets, approximately 5 ppm apart. Each FID spectra was acquired approximately 6 seconds apart. Off-resonance FID spectra were obtained quarterly in this series and acquired with a saturation pre-pulse at +271 ppm off resonance from the gas phase peak. A HyperCEST depletion spectrum

was collected for each sample (See Supplemental Information) by measuring HyperCEST depletion at various frequency offsets from the Xe gas phase peak. A minimum of 3 spectra were obtained at each of the various chemical shifts and a plot of the mean signal depletion as a function of the frequency of the chemical shift offset (z-spectrum) was produced. The mean signal-to-noise ratio (SNR) obtained from all control spectra for individual samples were used in the measurement of signal depletion. The SNR for each spectrum was calculated using MATLAB (MathWorks, Natick, MA, USA). To measure signal depletion, the mean HyperCEST saturation spectrum SNR was subtracted from the mean control spectrum SNR. This difference was then divided by the mean control spectrum SNR to produce the signal depletion by the HyperCEST effect.

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APPENDIX

Background Information

Nuclear magnetic resonance imaging (MRI) is a common tool used for visualizing deep tissue in real time *in vivo*.^{2,48} MRI is the detection of protons found in the body through the alignment of their nuclei with the magnetic field to produce a signal.⁴⁹ This method has allowed medical professionals to study injuries in deep tissue such as torn ligaments or large tumors.⁴⁸ Although MRI is only capable of imaging molecules with a concentration of 10^{-3} to 10^{-5} moles per liter while the molecular receptors or genes that are often responsible for disease have a concentration of 10^{-6} to 10^{-12} moles per liter.² The use of contrasting agents has helped increase the spatial and temporal resolution of the MRI. A popular contrast agent currently being used is gadolinium (III) (Gd(III)). A drawback with Gd(III) is its very expensive, because it is a rare earth metal mined in only six countries, and if unligated, it is toxic to the patient being administered.

Figure 6: Example of a Gadolinium (III) Chelate $[Gd(DTPA)(H_2O)]^{2-}$

The development of cheaper and less toxic contrast agents have been researched by multiple groups.⁵⁰ One of the emerging methods in this field is the use of

hyperpolarized (HP) noble gases, Helium-3 (^3He) and Xenon-129 (^{129}Xe). Both of these atoms are viable alternatives to Gd(III) because they are non-toxic and easily removed from the body naturally. ^3He is only used for imaging the lungs but not for deep tissue because ^3He isn't lipophilic. ^{129}Xe , on the other hand, diffuses throughout all regions of the body, so it is capable of deep tissue imaging. It has a long resonance relaxation time that increases the sensitivity of ^{129}Xe NMR by five orders of magnitude.⁴¹ Unlike Gd(III), ^{129}Xe has a low binding affinity to proteins in the body; as a result, it is much less toxic.^{2,41,48,51}

The hyperpolarization (HP) of ^3He or ^{129}Xe aligns nearly all of the nuclei with the NMR or MRI magnet; as a result, increasing the sensitivity of the instrument.⁵² Without HP, the magnet can only align a small number of the nuclei.⁵¹ A noble gas becomes polarized through a process called spin-exchange optical pumping (SEOP).⁵³ SEOP is the transfer of electron spin through the collision of polarized alkali-metal atoms to noble gas atoms.⁵⁴ In small amounts, ^3He and ^{129}Xe can have a 95% and 65% degree of polarization, respectively.⁵⁵ Currently hyperpolarized ^{129}Xe MRI has been used *in vivo* to image the distribution of ^{129}Xe in lungs and brain.⁵³

Figure 7: Example of ^{129}Xe Biosensor

With HP ^{129}Xe and MRI capabilities, the option to detect specific tumors, molecules, and proteins are available with the help of a ^{129}Xe biosensor. The biosensor

consists of a cage molecule, linker, and targeting moiety (Figure 6).^{16,52} The ^{129}Xe will exchange quickly in and out of the cage to produce a defined NMR signal different to that of free HP ^{129}Xe .^{14,52,56,57} The caged molecules can be functionalized to bind to specific biological receptor.¹⁴ Previously, cryptophane-A has been functionalized and shown exchanging HP ^{129}Xe (Figure 8).^{16,52} A ^{129}Xe biosensor allows for the study and diagnosis of various biochemical phenomena.

Figure 8: Cryptophane-A with a linker and target moiety having ^{129}Xe being encapsulated.

The synthesis of rotaxane probes can assist in broadening the capabilities of ^{129}Xe biosensors. Rotaxanes are in a category of mechanically interlocking molecules (MIM).⁵⁸ They consist of a cyclic molecule, host, and a linear molecule, guest, bound together by noncovalent forces forming a host-guest relationship.⁵⁸ Some common hosts are cucurbit[n]urils, pillar[n]enes, and cyclodextrins with each varying in size, shape, and capability. Biological applications of rotaxanes include optical bioimaging, drug delivery, and cell transport agents.⁵⁹ Rotaxanes can form a ternary complex with HP

^{129}Xe to create a molecular biosensor. We are capable to detect, in small amounts, the ^{129}Xe biosensors in the body using Hyperpolarized ^{129}Xe Chemical Exchange Saturation Transfer (HyperCEST).¹⁹ Right now we are trying to synthesize a Folic Acid probe to bind to cancer tumors and a Thioflavin-T probe to image cells found in Alzheimer's disease.

Thioflavin-T Molecular Probe

Thioflavin-T (ThT) is a fluorescent molecule with a high binding affinity to beta-amyloid ($\text{A}\beta$) plaques used to diagnose Alzheimer's Disease (AD), a type of dementia, in the brain.^{60–62} Amyloid plaques are developed from amyloid precursor proteins (APPs), also known as senile plaques, found exclusively in AD patients. $\text{A}\beta$ plaques are a type of amyloidosis, a disease, caused by the misfolding of peptide or protein that is unable to remain in its natural state.^{61,63} Along with AD, amyloidosis has been associated with Parkinson's Disease, type-II diabetes, and cataracts.^{61,63,64} As an individual ages, amyloid plaques build up inside neurons and blood vessels in the brain to reduce the functionality of the brain.⁶⁴ In order to diagnose AD, physicians need to detect the presence of $\text{A}\beta$ along with any cognitive deterioration.⁶⁰ Pharmaceutical companies have developed ThT derivatives to be used *in vitro* allowing for the identification and quantification of $\text{A}\beta$.⁶⁵ Once the ThT binds to $\text{A}\beta$, ThT fluoresces with an excitation at 440nm and with emission at 490nm. Unbound ThT has a very low fluorescence with a blue-shifted excitation at 350nm and emission at 440nm.⁶³

Using the capability to synthesize various ThT derivations, we wanted to synthesize a molecular probe, as stated earlier, to image $\text{A}\beta$ using MRI. We chose 4-aminobenzothiazole because it is the easiest derivative to synthesize (Scheme 1). Based

on the determined inclusion complexes, 8-carbon and 10-carbon alkyl guests were chosen to make up the core of our molecular probe.

The first approach was to bind ThT (compound **7**) to the alkyl chain by an azide-alkyne “click” reaction (Scheme 6)⁶⁶ or through a nucleophilic acyl substitution (Scheme 7).^{67, 68} The “click” reaction route required the synthesis of a propargylated ThT (**10**) and a diazido alkyl chain (**4** and **5**). Unfortunately the “click” reaction was unable to be purified or confirmed by NMR; as a result, the acyl substitution (Scheme 4) was attempted using ThT with sebacoyl chloride (route 1), and disuccinimidyl sebacate (**9**, route 2). Synthetic route 1 was able to produce compound **8** with a yield of 98%. Route 2 produced no product, only starting material. With the bar for the rotaxane (**8**) in hand, the next step was to perform binding studies with γ -cyclodextrin and ^{129}Xe to see if we have successfully created a new pseudo-rotaxane molecular probe.

Scheme 6: Synthetic route for propargylated thioflavin-T reaction and diazido alkyl chain.

Scheme 7: Synthetic routes for the thioflavin-T probe.

Folic Acid Molecular Probe

Folic acid (FA) was chosen to make a biosensor due to its high binding affinity to folate receptors (FR) which are upregulated in many tumors, particularly ovary, lung, breast, kidney, brain, endometrium, and colon cancers.⁵ Currently, FA conjugated drugs are being used as cancer treatments by binding to the FR located on cancer tumors. FA is water soluble and naturally forming, which makes it cheap, readily available, and applicable to a wide range of cancer tumors.⁵ Having a probe that specifically binds to one of these tumors would allow researchers and physicians to study and treat these diseases more efficiently.

Using the same approach as the ThT molecular probe, we wanted to bind FA to an alkyl chain by functionalizing FA. We have made several unsuccessful attempts to bind dibromopropionyl chloride (DBPC) to the primary amine in order to perform a “click” reaction to either compound 4 or 5 (Scheme 8).^{69,6} The next approach would be to perform a coupling reaction to activate γ -carboxylic acid of FA with *N*-

hydroxysuccinimide and to react this activated ester with 1,10-diaminodecane to form a molecular thread containing FA on both ends (Scheme 9).⁶⁷

The preliminary development of a ThT probe was successful. After performing the binding studies, we determined a pseudo-rotaxane, not a rotaxane, was formed. The pseudo-rotaxane allows for the guest molecule to slip in and out of the host molecule. Our probe can be altered to make a rotaxane by conjugating a large end cap to one side of the guest molecule. This should lock the ThT probe in place not allowing any exchange between the host and guest. When the FA probe is synthesized, there might be an issue of threading the probe into a host molecule due to the size of the FA. If there are complications threading the probe, the FAs will need to be bound *in situ* with the alkyl chain and host molecules.

Scheme 8: Synthetic route using functionalized folic acid.

Scheme 9: Synthetic route using activated folic acid.

Experimental

Known and novel compounds were synthesized according to the following procedures.

Reagents

Substrates, including methyl 4-amino-3-methoxybenzoate, 4-(hydroxymethyl)-2-methoxyphenol, dibromoethane, 4-hydroxy benzaldehyde, tris(2-aminoethyl)amine, iron(iii)perchlorate hydrate, phosphorous pentoxide, glyoxal, urea, p-tertbutyl phenol, 1,4-dimethoxybenzene, 1,4-bis(2-hydroxy)benzene, 1-ethylimidazole, 1,8-dibromooctane, 1,10-Dibromodecane, 1,5-dibromopentane, cucurbit[6]uril, cucurbit[7]uril, cucurbit[8]uril, sebacoyl chloride, polyphosphoric acid, 2-aminothiophenol, 4-aminobenzoic acid, sodium azide, alpha-cyclodextrin, beta-cyclodextrin and gamma-cyclodextrin were purchased from Sigma-Aldrich, Fisher Scientific, and TCI chemicals. Flash chromatography was performed using a Teledyne-Isco CombiFlash R_f with Redisep Gold silica cartridges. All reagents were stored under an inert atmosphere before use. All reaction was performed under N₂ atmosphere if not mentioned.

Instrumentation

NMR spectra were obtained using a Bruker Avance 300 MHz spectrometer and a Bruker Avance 400 MHz spectrometer. Low resolution mass spectrometry was performed using a Shimadzu LRMS-2020.

Synthesis of C₁₀ diethylimidazolium bar, 1

1-Ethylimidazole (1.94 mL, 20 mmol) and 1, 10-dibromodecane (1.79 mL, 8 mmol) were dissolved in toluene (15 mL) and refluxed for 12 hours. Once complete, the toluene was poured out while the crude product remained in the flask. The crude product was washed with diethyl ether (3 x 20mL) then dried under vacuum until a white solid product formed (3.78 g, 56% yield). Spectral data matched the reported values.⁷⁰

¹H NMR (400 MHz, D₂O) δ 8.80 (s, J = 1.7 Hz, 2H), 7.51 (d, J = 9.1, 1.9 Hz, 4H), 4.3 – 4.12 (m, 8H), 1.88 (m, J = 7.1 Hz, 4H), 1.52 (t, J = 7.4 Hz, 6H), 1.37 – 1.21 (m, 12H).

¹³C NMR (101 MHz, D₂O) δ 134.75, 122.26, 121.95, 49.57, 44.80, 29.14, 28.32, 27.96, 25.29, 14.50.

Spectrum 1 – ^1H NMR of Compound 1

Spectrum 2 – ^{13}C NMR of Compound 1

Synthesis of C₅ diethylimidazolium bar, 2

1-ethylimidazole (1.9 mL, 20 mmol) and 1,5-dibromooctane (1.1 mL, 8 mmol) were dissolved in toluene (50 mL) and refluxed overnight. The toluene was poured out while the crude product was left in the flask. The crude product was washed with diethyl ether (3 x 50mL) then dried by vacuum to form white solid product (2.5 g, 48% yield).

Spectral data matched the reported values.⁷¹

¹H NMR (300 MHz, D₂O) δ 8.74 (s, 2H), 7.45 (d, J = 9.8, 1.9 Hz, 4H), 4.22 – 4.11 (m, 8H), 1.92 – 1.83 (m, 4H), 1.44 (t, J = 7.4 Hz, 6H), 1.30 – 1.24 (m, 2H).

¹³C NMR (101 MHz, D₂O) δ 134.86, 122.21, 122.03, 49.19, 44.86, 28.71, 22.24, 14.51.

LRMS ESI (m/z): [M⁺] calculated for C₁₅H₂₆N₄Br₂ 131.11, observed 131.11.

Spectrum 3 – ^1H NMR of Compound 2

Spectrum 4 – ¹³C NMR of Compound 2

Synthesis of C₈ diethylimidazolium bar, 3

1-Ethylimidazole (1.06 mL, 11 mmol) and 1, 8-dibromooctane (808.7 μ L, 4.4 mmol) were dissolved in toluene (15 mL) and refluxed for 12 hrs. Once complete, the toluene was poured out while the crude product remained in the flask. The crude product was washed with diethyl ether (3 x 20 mL) then dried under vacuum until a white solid product formed (1.266 g, 38% yield). Spectral data matched the reported values⁷⁰.

¹H NMR (400 MHz, D₂O) δ 8.74 (s, 2H), 7.45 (d, J = 8.1, 1.9 Hz, 4H), 4.25 – 4.07 (m, 8H), 1.88 – 1.75 (m, 4H), 1.45 (t, J = 7.3 Hz, 6H), 1.29 – 1.20 (m, 8H).

¹³C NMR (101 MHz, D₂O) δ 134.79, 122.29, 121.99, 49.58, 44.86, 29.17, 27.85, 25.26, 14.57.

Spectrum 5 – ^1H NMR of Compound 3

Spectrum 6 – ¹³C NMR of Compound 3

Synthesis of C₈ diazide bar, 4

1,8-dibromooctane (1.84 g, 10 mmol) and sodium azide (1.95 g, 30 mmol) were dissolved in DMF (15 mL). The reaction was stirred at 60 °C for 24 hrs. Once complete, water (300 mL) was added then extracted with methylene chloride (3 x 100 mL). The organic layers were combined and dried with sodium sulfate. The sodium sulfate was filtered out and the solvent was removed under vacuum. The crude product was purified using column chromatography (hexane:ethyl acetate; 10:1) to produce a clear oil (1.3 g, 66% yield) Spectral data matches the reported values.⁶⁹

¹H NMR (400 MHz, CDCl₃) δ 3.26 (t, J = 6.9 Hz, 4H), 1.59 (p, J = 7.0 Hz, 4H), 1.45 – 1.27 (m, 8H).

¹³C NMR (101 MHz, CDCl₃) δ 51.44, 29.00, 28.80, 26.61.

Spectrum 7 – ¹H NMR of Compound **4**

Spectrum 8 – ^{13}C NMR of Compound **4**

Synthesis of C₁₀ diazide bar, 5

1,10-dibromodecane (2.24 mL, 10 mmol) and sodium azide (1.95 g, 30 mmol) were dissolved in DMF (15 mL). The reaction was stirred at 60 °C for 24 hrs. Once complete, water (300 mL) was added then extracted with methylene chloride (3 x 100 mL). The organic layers were combined and dried with sodium sulfate. The sodium sulfate was filtered out and the solvent was removed under vacuum. The crude product was purified using column chromatography (hexane:ethyl acetate; 10:1) to produce a clear oil (1.38 g, 62% yield). Spectral data matched reported values.⁷²

¹H NMR (400 MHz, CDCl₃) δ 3.25 (t, *J* = 6.9 Hz, 4H), 1.65 – 1.53 (m, 4H), 1.43 – 1.22 (m, 12H).

¹³C NMR (101 MHz, CDCl₃) δ 51.47, 29.35, 29.10, 28.83, 26.69.

Spectrum 9 – ^1H NMR of Compound 5

Spectrum 10 – ^{13}C NMR of Compound **5**

Synthesis of decadimethoxy-pillar[5]arene

To a solution of 1,4-dimethoxybenzene (1.38 g, 10mmol) in 1,2-dichloroethane (20 mL) was added paraformaldehyde (0.93 g, 30 mmol). Then, boron trifluoride diethyl etherate ($[\text{BF}_3\text{O}(\text{C}_2\text{H}_5)_2]$, 1.25 mL, 10 mmol) was added to the solution, and the mixture was stirred at 30 °C for 30 min. The solution was poured into ethanol (50 mL), and the resulting precipitate was collected by filtration. The obtained solid was recrystallized from acetone to give decamethyl pillar[5]arene as an off white solid (0.83 g, 71% yield).

Spectral data matched reported values.⁷³

^1H NMR (400 MHz, CDCl_3) δ 6.91 (s, J = 14.1 Hz, 10H), 3.79 (s, J = 12.6 Hz, 10H), 3.76 (s, J = 13.8 Hz, 30H).

^{13}C NMR (75 MHz, CDCl_3) δ 150.27, 128.18, 113.17, 55.32, 40.65, 29.18.

Spectrum 11 – ^1H NMR of decmethyl-pillar[5]arene

Spectrum 12 – ^{13}C NMR of decamethyl-pill[5]arene

Synthesis of decadibromoethoxy-pillar[5]arene

A solution of 1,4-bis(2-hydroxyethoxy)benzene (2.5 g, 12.6 mmol) and triphenylphosphine (7.8 g, 30 mmol) in dry acetonitrile (62.5 mL) was cooled with an ice bath. Under vigorous stirring, carbon tetrabromide (9.9 g, 30 mmol) was slowly added. The mixture was stirred at room temperature for 4 hrs. Cold water (50 mL) was added to the reaction mixture, producing a white precipitate. The precipitate was collected, washed with methanol/water (3:2, 3×25 mL), recrystallized from methanol, and dried under vacuum to afford the product as white crystals (1.99 g, 49% yield).

Spectral data matched the reported values.⁷⁴

^1H NMR (300 MHz, CDCl_3) δ 6.88 (s, 4H), 4.27 (t, $J = 6.3$ Hz, 4H), 3.64 (t, $J = 6.3$ Hz, 4H).

^{13}C NMR (101 MHz, CDCl_3) δ 152.82, 116.09, 68.71, 29.28.

A solution of 1,4-bis(2-bromoethoxy)benzene (3.37 g, 11.5 mmol) and paraformaldehyde (0.349 g, 11.5 mmol) in 1,2-dichloroethane (30 mL) was cooled with ice bath. Boron trifluoride etherate (3.26 g, 23.0 mmol) was added to the solution and the mixture was stirred at room temperature for 1 hour. The reaction mixture was then washed with water (2×30 mL) and dried with Na_2SO_4 . The solvent was evaporated to provide a crude product, which was purified by Combi-flash chromatography (eluent: petroleum ether/ethyl acetate, 100:1) to afford a white solid (4.83 g, 25% yield). Spectral data matched reported values.⁷⁴

^1H NMR (300 MHz, CDCl_3) δ 6.92 (s, 10H), 4.24 (t, $J = 5.7$ Hz, 20H), 3.85 (s, 10H), 3.64 (t, $J = 5.7$ Hz, 20H).

^{13}C NMR (101 MHz, CDCl_3) δ 149.67, 129.03, 116.10, 68.99, 30.71, 29.40.

Spectrum 13 – ^1H NMR of 1,4-bis(2-bromoethoxy)benzene

Spectrum 14 – ^{13}C NMR of 1,4-bis(2-bromoethoxy)benzene

Spectrum 15 – ¹H NMR of decadibromoethoxy-pillar[5]arene

Spectrum 16 – ^{13}C NMR of NMR of decadibromoethoxy-pillar[5]arene

Synthesis of Thioflavin-T (4-aminobenzothiazole, 7)

4-Aminobenzoic acid (0.960 g, 7 mmol), 2-aminothiophenol (0.790 mL, 7 mmol), and polyphosphoric acid (7.45 g, 87.5 mmol) were placed in a flask. The mixture was heated to 200°C for 4 hours. Once complete, the mixture was allowed to cool, and then poured into 200 mL of a 10% Na₂CO₃ solution. The solution was stirred until gas evolution was complete. The solid was isolated by filtration and washed with water (3 x 50 mL). The crude product was recrystallized with methanol and water to produce the final product (1.345 g, 85% yield). Spectral data matched reported values.⁶⁵

¹H NMR (400 MHz, CDCl₃) δ 8.01 (d, *J* = 8.2 Hz, 1H), 7.92 (d, *J* = 8.2 Hz, 2H), 7.85 (d, *J* = 7.9 Hz, 1H), 7.45 (t, *J* = 7.7 Hz, 1H), 7.33 (t, *J* = 7.6 Hz, 1H), 6.74 (d, *J* = 8.2 Hz, 2H).

¹³C NMR (101 MHz, CDCl₃) δ 149.36, 129.26, 126.16, 124.55, 122.41, 121.43, 114.79.

Spectrum 17 – ^1H NMR of Compound 7

Spectrum 18 – ^{13}C NMR of Compound 7

Synthesis of Thioflavin Probe (8)

4-Aminobenzothiazole (0.226 g, 1.0 mmol) was dissolved in anhydrous methylene chloride (10 mL) containing diisopropylethylamine (166 μ L, 0.95 mmol) and sebacoyl chloride (102 μ L, 0.47 mmol). The reaction stirred at 35°C overnight and monitored by TLC. Once complete, the suspension was centrifuged and the supernatant was discarded. The pellet was re-dissolved with methylene chloride and centrifuged again, twice, to produce a green solid (287.8 mg, 98% yield). Spectral data matched reported values.⁶⁸

¹H NMR (400 MHz, DMSO-*d*₆) δ 10.20 (s, 2H), 8.11 (m, *J* = 8.0 Hz, 2H), 8.02 (m, *J* = 7.3 Hz, 6H), 7.80 (d, *J* = 8.4 Hz, 4H), 7.52 (t, *J* = 7.7 Hz, 2H), 7.43 (t, *J* = 7.6 Hz, 2H), 2.35 (t, *J* = 7.5 Hz, 4H), 1.67 – 1.55 (m, 4H), 1.38 – 1.28 (m, 8H).

¹³C NMR (101 MHz, DMSO-*d*₆) δ 172.23, 167.48, 154.11, 142.65, 134.72, 128.46, 127.77, 127.02, 125.68, 123.01, 122.71, 119.65, 36.97, 29.15, 29.09, 25.45.

LRMS ESI (m/z): [*M*⁺] calculated for C₃₆H₃₄N₄O₂S₂ 619.0, observed 619.0.

Spectrum 19 – ^1H NMR of Compound 8

Spectrum 20 – ^{13}C NMR of Compound 8

Synthesis of pseudo-rotaxanes using cucurbit[n]uril and bars

Cucurbit[n]uril (n=6, 7, 8) was dissolved in deuterium oxide at 85°C with the following equivalents of different bars. ¹H-NMR was performed to see the peak movement of the bar when the inclusion complex was formed. Stacked plots are given below. CB [6], CB [7] and CB [8] are partially water soluble, once the guest is added, the complex becomes clear and homogeneous.

20 mg (0.020 mmol) of CB [6] + 30 mg (0.114 mmol) of C₅ diethylimidazolium bar **(2)**
+ 30 ml of DI water (Pseudo-rotaxane 1)

20 mg (0.020 mmol) of CB [6] + 40 mg (0.131 mmol) of C₈ diethylimidazolium bar **(3)**
+ 20 ml of DI water (Pseudo-rotaxane 2)

50 mg (0.050 mmol) of CB [6] + 160 mg (0.481 mmol) of C₁₀ diethylimidazolium bar **(1)**
+ 80 ml of DI water (Pseudo-rotaxane 3)

15 mg (0.013 mmol) of CB [7] + 35 mg (0.115 mmol) of C₈ diethylimidazolium bar **(3)**
+ 12 ml of DI water (Pseudo-rotaxane 4)

15 mg (0.013 mmol) of CB [7] + 30 mg (0.090 mmol) of C₁₀ diethylimidazolium bar **(1)**
+ 12 ml of DI water (Pseudo-rotaxane 5)

8.1 mg (0.006 mmol) of CB [8] + 17.6 mg (0.058 mmol) of C₈ diethylimidazolium bar **(3)**
+ 12 ml of DI water (Pseudo-rotaxane 6)

8.1 mg (0.006 mmol) of CB [8] + 17.6 mg (0.053 mmol) of C₁₀ diethylimidazolium bar **(1)**
+ 12 ml of DI water (Pseudo-rotaxane 7)

Spectrum 21 – ^1H NMR of Pseudo-rotaxane 1

Spectrum 22 – ^1H NMR (Magnified) of Pseudo-rotaxane 1 in the region of guest proton resonances

Spectrum 23 – ^1H NMR of Pseudo-rotaxane 2

Spectrum 24 – ^1H NMR (magnified) of Pseudo-rotaxane 2 in the region of the guest proton resonances

Spectrum 25 – ^1H NMR of Pseudo-rotaxane 3

Spectrum 26 – ^1H NMR (magnified) of Pseudo-rotaxane 3 in the region of guest proton resonances

Spectrum 27 – ^1H NMR of Pseudo-rotaxane 4

Spectrum 28 – ^1H NMR (magnified) of Pseudo-rotaxane 4 in the region of guest proton resonances

Spectrum 29 – ^1H NMR of Pseudo-rotaxane 5

Spectrum 30 – ^1H NMR (magnified) of Pseudo-rotaxane 5 in the region of guest proton resonances

Spectrum 31 – ^1H NMR of Pseudo-rotaxane 6

Representative synthesis of pseudo-rotaxanes using decadimethoxy-pillar[5]arene, decadibromoethoxy-pillar[5]arene and bars

Decadibromomethoxy-pillar-5-arene (Br-P-5-A) (25 mg, 0.015 mmol) and the 1,10-diazidodecane bar (**5**) (3 μ L, 0.015 mmol) were dissolved in CDCl_3 . The sample was mixed at room temp for 2hr, 60 $^\circ\text{C}$ for 2hr, then an additional 2 hr at 60 $^\circ\text{C}$ (total of 4hr at 60 $^\circ\text{C}$) to thread the C_{10} diazide bar (**5**) into decadibromoethoxy-pillar[5]arene. At each time interval, a ^1H -NMR was taken to observe any chemical shifts. ^1H -NMR stacked spectrum is shown below.

Spectrum 32 – ^1H NMR of Br-P-5-A and 5

Synthesis of pseudo-rotaxanes using alpha, beta, gamma cyclodextrin and bars

Cyclodextrin (alpha, beta and gamma) was dissolved in deuterium oxide at 40°C with following equivalents with different bars. ¹H-NMR was performed to see the peak movement of the bar and the peak movement of cyclodextrin and bars (specifically H-1 proton, H-3 proton of CD and bar protons) when the inclusion complex was formed. Stacked plots are given below.

Alpha CD (31.10 mg, 0.032 mmol) + **(2)** C₅ diethylimidazolium bar (40.50 mg, 0.154 mmol) in 3 ml of D₂O (Pseudo-rotaxane 10)

Alpha CD (31.10 mg, 0.032 mmol) + **(3)** C₈ diethylimidazolium bar (44.60 mg, 0.146 mmol) in 3 ml of D₂O (Pseudo-rotaxane 11)

Alpha CD (31.10 mg, 0.032 mmol) + **(1)** C₁₀ diethylimidazolium bar (47.30 mg, 0.142 mmol) in 3 ml of D₂O (Pseudo-rotaxane 12)

Beta CD (45.40 mg, 0.040 mmol) + **(2)** C₅ diethylimidazolium bar (50.70 mg, 0.193 mmol) in 6 ml of D₂O (Pseudo-rotaxane 13)

Beta CD (45.40 mg, 0.040 mmol) + **(3)** C₈ diethylimidazolium bar (55.70 mg, 0.183 mmol) in 6 ml of D₂O (Pseudo-rotaxane 14)

Beta CD (45.40 mg, 0.040 mmol) + **(1)** C₁₀ diethylimidazolium bar (59.10 mg, 0.178 mmol) in 6 ml of D₂O (Pseudo-rotaxane 15)

Gamma CD (51.90 mg, 0.040 mmol) + **(2)** C₅ diethylimidazolium bar (50.70 mg, 0.193 mmol) in 3 ml of D₂O (Pseudo-rotaxane 16)

Gamma CD (51.90 mg, 0.040 mmol) + **(3)** C₈ diethylimidazolium bar (55.70 mg, 0.183 mmol) in 3 ml of D₂O (Pseudo-rotaxane 17)

Gamma CD (51.90 mg, 0.040 mmol) + **(1)** C₁₀ diethylimidazolium bar (59.10 mg, 0.178 mmol) in 3 ml of D₂O (Pseudo-rotaxane 18)

Spectrum 33 – ^1H NMR of Pseudo-rotaxane 10

Spectrum 34 – ^1H NMR (magnified) of Pseudo-rotaxane 10 in the region of H-3

Spectrum 35 – ^1H NMR of Pseudo-rotaxane 11

Spectrum 36 – ^1H NMR (magnified) of Pseudo-rotaxane 11 in the region of guest proton resonances

Spectrum 37 – ^1H NMR of Pseudo-rotaxane 12

Spectrum 38 – ^1H NMR of Pseudo-rotaxane 13

Spectrum 39 – ^1H NMR (magnified) of Pseudo-rotaxane 13 in the region of guest proton resonances

Spectrum 40 – ^1H NMR of Pseudo-rotaxane 14

Spectrum 41 – ^1H NMR of Pseudo-rotaxane 15

Spectrum 42 – ^1H NMR (magnified) of Pseudo-rotaxane 15 in the region of guest proton resonances

Spectrum 43 – ^1H NMR of Pseudo-rotaxane 16

Spectrum 44 – ^1H NMR (magnified) of Pseudo-rotaxane 16 in the region of guest proton resonances

Spectrum 45 – ^1H NMR of Pseudo-rotaxane 17

Spectrum 46 – ^1H NMR (magnified) of Pseudo-rotaxane 17 in the region of guest proton resonances

Spectrum 47 – ^1H NMR (magnified) of Pseudo-rotaxane 17 in the region of H-3 proton of γ -CD

Spectrum 48 – ^1H NMR of Pseudo-rotaxane 18

Spectrum 49 – ^1H NMR (magnified) of Pseudo-rotaxane 18 in the region of guest proton resonances

Spectrum 50 – ^1H NMR (magnified) of Pseudo-rotaxane 18 in the region of H-3 proton of γ -CD

Association studies for Thioflavin-T probe

The NMR titration experiments were conducted per the following procedures. The host concentrations were kept constant while the guest concentration was increased periodically. Stock solutions of 10 mM host (gamma CD) and 100 mM guest (ThT-probe) were prepared. A series of NMR samples were prepared ranging from 1:1 (Host: guest ratios are mM concentrations) to 1:5, increasing the guest ratio by 1 mM for each sample. The aromatic proton of the ThT (circled in red) was monitored. Data was processed using Wolfram Mathematica software. The chemical shift values were used to determine the correct fit from the equations shown below (Table 2).⁷⁵ The plots are shown below. All samples were prepared at 45 °C.

Table 2: Correct fit equations

Stoichiometry	Equations
1:1 (equation-1)	$P_{obs} = \frac{P_{G_0} + P_{GH}K_{11}[H]}{1 + K_{11}[H]}$
1:2 (equation-2)	$P_{obs} = \frac{P_{G_0} + P_{GH_2}K_{12}[H]^2}{1 + K_{12}[H]^2}$
1:1+1:2 (equation-3)	$P_{obs} = \frac{P_{G_0} + P_{GH}K_{11}[H] + P_{GH_2}K_{11}K_{12}[H]^2}{1 + K_{11}[H] + K_{11}K_{12}[H]^2}$

In these equations G and H represent host and guest. P_{Go} and P_{GH} are constants. P_{obs} (NMR shift value) is the y-axis, and the guest concentration is used as the x-axis to plot the data. Curve fitting Equation 3 produced a negative value indicating the data set does not fit the given equation; hence, the combination of 1:1 and 1:2 stoichiometry won't be present in that system. Using the calculated values, the association constants of the complexes were obtained.

Spectrum 51 – ^1H NMR of ThT Probe with Gamma CD in the region of the aromatic protons of ThT

Spectrum 52 – Date fit for Equation-1; γ -CD and ThT-Probe
 K_{11} (association constant for 1:1 inclusion complex) = $2 \times 10^4 \text{ M}^{-1}$

Spectrum 53 – Data fit for Equation-2; γ -CD and ThT-Probe
 K_{12} (association constant for 1:2 inclusion complex) = 25.6 M^{-1}

Synthesis of Disuccinimidyl Sebacate (9)

Sebacic acid (1.01 g, 5 mmol) was dissolved in dry THF (15 mL). DCC (2.48 g, 12 mmol) and *N*-hydroxysuccinimide (1.38 g, 12 mmol) were dissolved in THF (5 mL) then added to the reaction at 0 °C. The mixture was stirred for 15 minutes then DMAP (73.3 mg, 0.6 mmol) was added. The solution warmed to room temperature and was stirred overnight. The white precipitate was filtered, and the solvent was removed from the filtrate using vacuum. The crude mixture was dissolved in methylene chloride (25mL) and washed with water (3 x 50 mL). The organic layer was dried with Mg₂SO₄, filtered and removed solvent. The crude product was purified using column chromatography (hexane:ethyl acetate) to produce a white solid (940 mg, 47% yield). Spectral data matched the reported values.⁶⁷

¹H NMR (400 MHz, DMSO-*d*₆) δ 2.81 (s, 8H), 2.65 (t, *J* = 7.2 Hz, 4H), 1.62 (p, *J* = 7.2 Hz, 4H), 1.40 – 1.25 (m, 8H).

¹³C NMR (101 MHz, DMSO-*d*₆) δ 170.72, 169.44, 30.64, 28.71, 28.35, 25.91, 24.69.

Spectrum 54 – ^1H NMR of Compound 9

Spectrum 55 – ¹³C NMR of Compound 9

Synthesis of Propargylated ThT (10)

4-aminobenzothiazole (142 mg, 0.626 mmol) was dissolved in dry acetone (10 mL) along with K₂CO₃ (461 mg, 3.337 mmol). The mixture was allowed to reflux for 30 minutes. Next, KI (104 mg, 0.626 mmol) and propargyl bromide (67 μ L, 0.751 mmol) was added. The reaction continued to reflux for 24 hrs. Once complete, the mixture was allowed to cool then filtered. The solvent was removed under vacuum. The product was purified using column chromatography (70:1, methylene chloride:methanol) to produce a yellow powder (23 mg, 14% yield). Spectral data matched reported values.⁶⁶

¹H NMR (400 MHz, DMSO-*d*₆) δ 8.05 (d, *J* = 7.9 Hz, 1H), 7.93 (d, *J* = 8.1 Hz, 1H), 7.87 (d, *J* = 8.4 Hz, 2H), 7.48 (t, *J* = 7.6 Hz, 1H), 7.37 (t, *J* = 7.6 Hz, 1H), 6.79 (m, *J* = 12.2, 7.2 Hz, 3H), 3.98 (dd, *J* = 6.3, 2.3 Hz, 2H), 3.15 (t, *J* = 2.6 Hz, 1H).

¹³C NMR (101 MHz, DMSO-*d*₆) δ 168.38, 154.33, 151.07, 134.28, 129.01, 126.75, 124.95, 122.42, 122.36, 121.58, 113.10, 81.96, 73.86, 32.19.

Spectrum 56 – ^1H NMR of Compound **10**

Spectrum 57 – ^{13}C NMR of Compound 10

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