

DESIGN AND SYNTHESIS OF TARGETED CRYPTOPHANE BIOSENSORS FOR
XENON-129 NMR/MRI

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NMR/MRI
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Out of sheer luck and many footsteps, I made it.

-Markus Zukus, The Book Thief

“Do you think things always have an explanation?”

"Yes. I believe that they do. But I think that with our human limitations we're not always able to understand the explanations. But you see, Meg, just because we don't understand doesn't mean that the explanation doesn't exist."

Madeleine L'Engle, A Wrinkle in Time

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ABSTRACT

DESIGN AND SYNTHESIS OF TARGETED CRYPTOPHANE BIOSENSORS FOR

XENON-129 NMR/MRI

Brittany A. Riggle

Ivan J. Dmochowski

To expand the utility of hyperpolarized (hp) ^{129}Xe NMR for sensitive biodetection, a cryptophane host molecule can be specifically targeted to analytes of interest. This system has the potential to be used in conjunction with proton MRI for the diagnosis and staging of disease. Xenon was found previously to have high affinity ($K_D = 20\text{-}30\ \mu\text{M}$ at room temperature) for trifunctionalized, water-soluble cryptophanes. Cryptophanes have been designed for increased water solubility and xenon affinity, and provide a broad ($\sim 300\ \text{ppm}$) chemical shift window. Importantly, new synthetic methods have enabled conjugation of a variety of targeting and solubilizing ligands, and increased access to enantiopure cryptophane. This dissertation reports my progress in three areas. The first involves studies with carbonic anhydrase, a useful model system for the design and characterization of xenon biosensors targeted to enzymes indicated in disease progression. Cryptophane functionalized with a benzenesulfonamide ligand and two water-solubilizing moieties bound to carbonic anhydrase II (CAII) with nanomolar affinity; Zn^{2+} coordination in the active-site channel was confirmed by X-ray crystallography. Using xenon biosensors tailored for CA, progress has been made in manipulating and better resolving the ^{129}Xe NMR chemical shift, as required for multiplexing studies. The second study details the

development of a biosensor that labels cells in an acidic microenvironment. Cryptophane was conjugated to a peptide that undergoes a conformational change from random coil to alpha-helical as the pH decreases from 7.5 to 5.5. This conformational change stimulates membrane insertion, as validated with HeLa cell studies. With this compound we demonstrated the engineering of the largest published chemical shift change for cryptophane biosensor. We also utilized hp ^{129}Xe chemical exchange saturation transfer (Hyper-CEST) NMR methods to achieve low-picomolar cryptophane detection, which creates new opportunities for bio-analysis and molecular imaging. Finally, a peptido-cryptophane was developed that binds to calmodulin (CaM) protein. With this work we hope to not only demonstrate the development of a biophysical probe sensitive to changes in protein conformation but also generate a Ca^{2+} sensor. These three studies highlight the broad utility of cryptophane- ^{129}Xe NMR biosensors, and synthetic methods described herein lay the groundwork for future *in vivo* studies.

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Chapter 1: Introduction

§ 1.1 Preamble

Medical imaging is widely employed for visualizing normal and abnormal anatomy and physiology. The importance of imaging in the diagnosis and subsequent treatment of diseases like cancer has resulted in the development of a wide variety of imaging techniques. At the nexus of medicine and the physical sciences, the imaging field includes x-ray, ultrasound, positron emission tomography (PET), computed tomography (CT), single photon emission computed tomography (SPECT) and magnetic resonance imaging (MRI) as well as optical imaging and many more recent imaging modalities. Each of these techniques offers specific attributes with regard to spatial and temporal resolution, sensitivity, contrast, and level of radiation exposure. One important area of medical imaging—*molecular imaging*—overlaps with drug discovery efforts and focuses on targeting biomarkers with specific roles in disease pathology. Thus, by monitoring changes in number and distribution of specific biomarkers of interest, clinicians can monitor disease progression and tailor treatment appropriately. This thesis describes my research into the design of xenon-binding cryptophane biosensors, and additional steps on the path to developing hyperpolarized ^{129}Xe MRI as a molecular imaging modality. This general introduction is meant to provide overall motivation for the development of targeted ^{129}Xe biosensors as well as describe in broad strokes the chemistry previously accomplished by our research group and others. These studies culminated in the work described in Chapters 2-4, and a consideration of future studies is provided in Chapter 5. Brief discussions of the techniques used to observe and characterize both Xe-cryptophane and biosensor-target interactions are first provided here in Chapter 1.

Authors Note: The sections of Chapter 1 denoted with an asterisk (*) were originally published in the chapter: *Cryptophane-Based ^{129}Xe NMR Biosensors* and have been adapted here with permission from the Royal Society of Chemistry:

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§ 1.2 Nuclear Magnetic Resonance Spectroscopy[†]

Nuclear magnetic resonance (NMR) spectroscopy and magnetic resonance imaging (MRI) are a form of absorption spectroscopy in which nuclei in a magnetic field absorb and reemit electromagnetic radiation at radiofrequencies specific to the characteristics of a sample. In more general terms, NMR spectroscopy is a technique used to characterize the chemical structure and composition, as well as biological properties, of a sample. NMR, as it is applied to medical imaging, typically employs concepts of proton NMR. The long term goal of this work is to simultaneously employ proton MRI with xenon MRI to achieve both high resolution spatial context and ultrasensitive disease identification. Thus, this section

[†] Material in this section follows references: ¹⁻³ Figures are adapted from: ^{1, 2}

of the introduction will introduce the concept of proton NMR as well as compare and contrast it with the use of exogenously supplied xenon-129 nuclei.

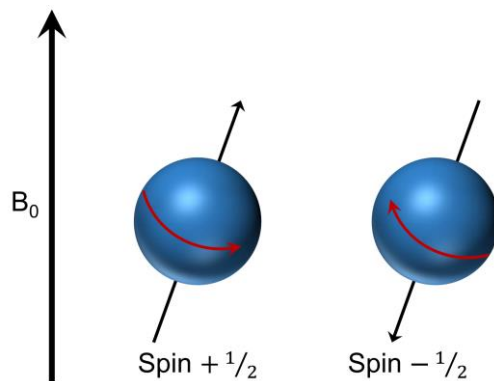


Figure 1.1. The two allowed spin states for a spin $\frac{1}{2}$ nucleus.

NMR exploits the magnetic properties of nuclei. Nuclei with either or both an odd atomic number or odd mass have a charge that spins on the nuclear axis thus generating a magnetic dipole along that axis. The quantum spin number I describes the angular momentum of this spinning charge where I is a physical constant corresponding to a given nucleus. There are $2I + 1$ allowed spin states from $-I$ to I which denote the number of orientations a nucleus may assume in an external and uniform magnetic field.² When the nucleus has a non-spherical charge distribution that results in a magnetic moment (μ). For protons $I = \frac{1}{2}$ and the allowed spin states are $+\frac{1}{2}$ and $-\frac{1}{2}$ which are clockwise and counterclockwise, respectively, as shown in Figure 1.1. For xenon-129 $I = \frac{1}{2}$, but this spin state arises from an unpaired neutron.⁴ Because each nucleus is a charged particle, in an applied magnetic field any moving charge generates its own magnetic field, thus these spin states are not equal in energy. For protons, the lower energy spin state is $+\frac{1}{2}$ because it is

aligned with the applied magnetic field (B_0) and $-1/2$ is higher in energy as it is counter aligned with the field, see Figure 1.2a.

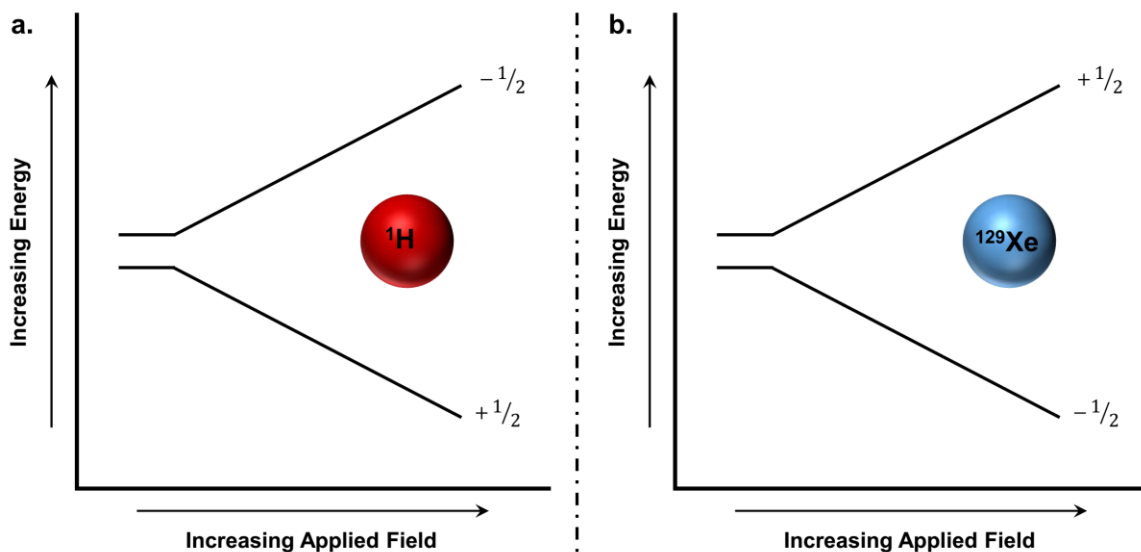


Figure 1. 2. Spin-state energy separation as a function of the applied magnetic field. For protons (a) and xenon-129 (b).

These two energy states are populated in accordance with the Boltzmann distribution where a slight excess of nuclear spins is found in the lower energy state. Nuclear magnetic resonance occurs when the nuclei aligned with an applied magnetic field absorb energy and change their spin orientation with respect to the applied field. The energy absorbed is equal to the energy difference as given by the equation:

$$E_{abs} = (E_{-1/2} - E_{+1/2}) = h\nu \quad (1.1)$$

The magnitude of the energy level separation is directly dependent on the nucleus in question and the strength of the magnetic field. As Figure 1.2 depicts, the energy difference between the two spin states increases as a function of the magnetic field strength as described by the equation:

$$\Delta E = \left(\frac{h\gamma}{2\pi} \right) B_0 \quad (1.2)$$

Where the change in energy is proportional to the magnetic field strength because h (Planck's constant), γ (the gyromagnetic ratio), and π are all constants. The gyromagnetic ratio is a constant for a given nucleus, in the case of protons $\gamma = 42.6 \text{ MHz/T}$.⁵ However, in xenon-129, $\gamma = -11.8 \text{ MHz/T}$.⁴ The negative value of the gyromagnetic ratio accounts for the different energy for the $+\frac{1}{2}$ and $-\frac{1}{2}$ spin states. As shown in Figure 1.2b, the $-\frac{1}{2}$ spin state is lower in energy than the $+\frac{1}{2}$ spin state for xenon-129. Thus, with protons the spin and magnetic moment are pointed in the same direction but with xenon the spin and magnetic moment point in opposite directions, but the understanding of how these nuclei function in an applied magnetic field is consistent. The term given in 1.2 can also be described with respect to frequency as:

$$\nu = \left(\gamma / 2\pi \right) B_0 \quad (1.3)$$

Since:

$$\Delta E = h\nu \quad (1.4)$$

The system is said to be in resonance when the frequency equals the applied B field and the energy is then absorbed by the proton raising it to the higher energy state. To describe it in another way, nuclei absorb energy because, as a result of the earth's gravitational field, they precess in the applied magnetic field of the NMR spectrometer. This precession is usually likened to a spinning gyroscope (as shown in Figure 1.3) as the nucleus 'wobbles' about its axis. The angular velocity ω by which the nuclei precess is termed its Larmor frequency and is directly proportional to the strength of the applied magnetic field. Only

when the applied radiofrequency waves (ν_1) are equal to the Larmor frequency (ν_L) can energy be absorbed and a spectrum be recorded. The radio frequency energy is introduced with a single burst covering the entire range of available frequencies for a given nuclei of interest thereby exciting the entire sample. As the excited nuclei return to the ground energy state, they radiate the previously absorbed energy which is recorded by a detector as a free induction decay (FID). The FID represents the entire energy radiated over time and can be converted to a spectrum as a function of frequency by a Fourier transform.

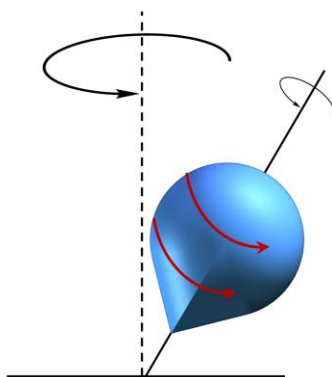


Figure 1. 3. The precession of a spinning nucleus can be equated to that of a spinning toy top or gyroscope.

It is helpful to consider the precessing nuclei in terms of Cartesian coordinates with a rotating reference frame. As the Boltzmann distribution of spins precesses about the z axis, aligned with the applied magnetic field, a net magnetization $\mathbf{M}_0$ is generated as shown in Figure 1.4. Again considering our Cartesian reference, the detection coil is in the xy plane thus $\mathbf{M}_0$ must be tipped into the xy plane for detection. To achieve this, ν_1 is applied in the xy plane such that the applied magnetic field $\mathbf{B}_1$ is orthogonal to the main magnetic field $\mathbf{B}_0$. The signal is recorded after the applied pulse. The caveat being that the signal can only be detected until the nuclei relax back to their ground state.

There are two main types of relaxation processes termed longitudinal or spin-lattice relaxation (T_1) and transverse or spin-spin relaxation (T_2), shown in Figure 1.5. As the name implies, T_1 relaxation results in the transfer of absorbed energy to the surrounding nuclei as thermal energy. This is indicated in the return of magnetization ($\mathbf{M}$) to $\mathbf{M}_0$ in a decreasing spiral as depicted in Figure 1.5a, T_1 relaxation results in a loss of signal intensity. The rate equation for the decay of magnetization ($\mathbf{M}$) with time (t) is given by:

$$\mathbf{M} = \mathbf{M}_0(1 - e^{-t/T_1}) \quad (1.5)$$

Thus the magnetization giving rise to the signal decays faster with decreasing T_1 . We can refer back to the rotating frame of reference where the $\mathbf{M}$ is represented by a single stationary arrow in the yz plane with θ representing the angle between the magnetization vector and the applied field, thus as θ decreases the signal intensity decreases until $\mathbf{M}$ has relaxed to alignment with the applied magnetic field, Figure 1.5a. Variation in T_1 is observed in different compounds and materials and is reduced by thermal motion increasing energy transfer in the sample, thus T_1 is inversely proportional to temperature.

Conversely, T_2 transverse relaxation is an entropic process that results from the dephasing of nuclear spins, see Figure 1.5b. The components of the precessing nuclear spins begin to spin at slightly different frequencies. This fanning out reduces the net $\mathbf{M}$ along the y axis resulting in signal decay. T_2 relaxation results in line-broadening, the rate of which is described by:

$$\mathbf{I} = \mathbf{I}_0(e^{-t/T_2}) \quad (1.6)$$

It is worth noting again that T_1 and T_2 are highly dependent on chemical composition and chemical structure and that T_2 can approach, but not exceed T_1 .

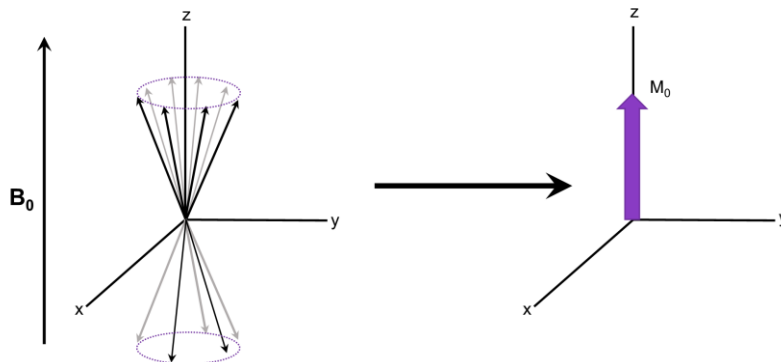


Figure 1. 4. The sum of the precessing nuclei in an applied magnetic field have a net magnetic moment, M_0 aligned with the stationary magnetic field, B_0 .

Proton NMR

For ^1H NMR sample acquisition, the sample is typically pure and well solubilized in a solvent containing no protons, is inert, has a low boiling point and is inexpensive. Deuterium labeled solvents are used to negate solvent proton signal. NMR spectrometers have a deuterium channel that can be locked on the B_0 field. The field inhomogeneities are minimized by either manually or automatically adjusting 20-30 small “shim” magnets to bend the main magnetic field thereby tuning the field.

The spectrum of a given compound is measured in frequency and is usually given either in hertz (Hz) or parts per million (ppm). The frequency of a nucleus is given by equation 1.3. While this represents the resonant frequency of a specific nuclei, the frequencies of various protons within a sample are complicated by shielding and deshielding effects. Thus the chemical shift of a specific nucleus in a sample is given by:

$$\nu_{eff} = (\gamma/2\pi)B_0(1 - \sigma) \quad (1.7)$$

Where σ is the shielding constant. This shielding constant arises from the fact that electrons within a magnetic field circulate and generate their own magnetic field. Thus, the degree of shielding is directly reflective of the density of circulating electrons along with the inductive effect of other groups attached to, in the case of proton NMR, the primary carbon. The difference in the frequency of a proton from the frequency of a reference proton is termed chemical shift.

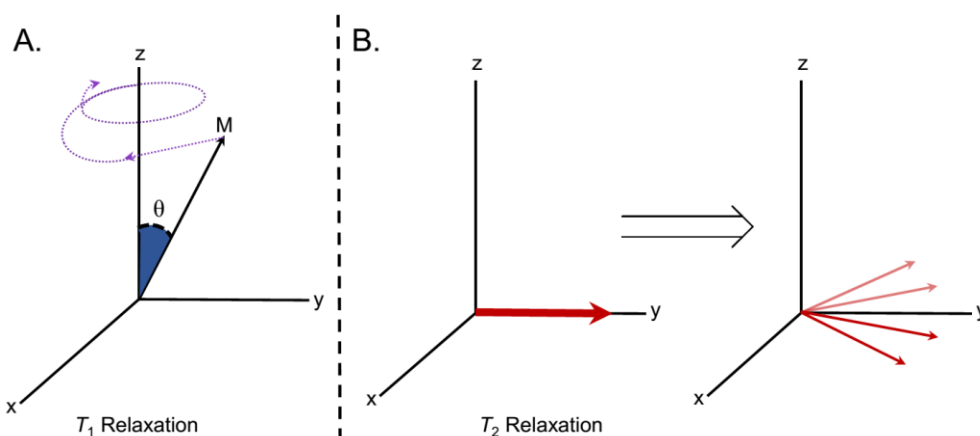


Figure 1. 5. Stationary Cartesian reference of A. spin-lattice and B. spin-spin relaxation.

Xenon NMR

For ^{129}Xe NMR sample acquisition, background signal from the buffer/sample is not an issue since ^{129}Xe is not typically found in laboratory or medical settings. Similar to proton NMR, field inhomogeneities are minimized with the shim magnets, however, because of the lack of deuterium in buffered samples a standard is shimmed to generate a shim file that can then be applied to the xenon experiment.⁶ Unlike ^1H NMR, ^{129}Xe has a much larger frequency window approaching 300 ppm in water, which is highly dependent

on solvent and local environment (to be described in greater detail in Section 1.5 of this chapter). ^{129}Xe is also highly sensitive to pressure ($\Delta\delta = 0.9$ ppm/atm) and temperature ($\Delta\delta = 0.4$ ppm/K).⁷ These attributes make xenon an excellent probe of solution physical properties. Exploiting environmental sensitivity is a key feature of hyperpolarized ^{129}Xe as a contrast agent for MRI. Thus understanding shielding effects on xenon chemical shift is key for designing xenon based probes.

Relaxation mechanisms in ^{129}Xe NMR are somewhat different from the standard T_1 and T_2 of proton NMR. For the longitudinal relaxation of xenon there are 5 component factors in non-solid phases that add up to give the overall T_1 :

$$\frac{1}{T_1} = \left(\frac{1}{T_1}\right)_{It} + \left(\frac{1}{T_1}\right)_{Ip} + \left(\frac{1}{T_1}\right)_{Ew} + \left(\frac{1}{T_1}\right)_{Eg} + \left(\frac{1}{T_1}\right)_{Eo} \quad (1.8)$$

Where E and I refer to extrinsic and intrinsic mechanisms which are further categorized as transient Xe_2 dimers, persistent Xe_2 dimers, wall collisions, magnetic field gradients, and oxygen interactions. For solution phase ^{129}Xe NMR we can ignore the second term in the above equation and it is generally held that both intrinsic terms can be disregarded in spin-exchange optical pumping experiments (described later). The two main sources of T_1 relaxation are terms 3 and 5, wall and oxygen relaxation for gaseous xenon in a chamber. For biological NMR experiments, however, the most important factor is Xe-solvent interactions where by the presence of paramagnetic species, including O_2 attenuate xenon signal. Table 1.1 details variations in T_1 values in different solvents and biological fluids. Molecular oxygen serves to catalyze xenon relaxation via collisional dipolar coupling because of its permanent electronic magnetic dipole.⁸ Counterintuitively, Albert *et al.*

found the blood oxygenation *increased* T_1 values relative to deoxygenated blood whereas in plasma oxygenation *decreases* the T_1 value as a result of the paramagnetic nature of oxygen. This unexpected finding is a result of the strong paramagnetic properties of deoxyhaemoglobin which serve to induce attenuated relaxation times.⁹ The reverse findings in blood foam was attributed to hyperpolarization exchange dynamics.^{9, 10} Due to their long xenon relaxation times, the use of Intralipid solutions (20%), $T_1 = 40$ s,¹¹ or perfluorooctyl bromide (PFOB) emulsions, $T_1 = 110$ s, have been proposed for *in vivo* imaging.¹²⁻¹⁴ That being said, transport times from areas of initial xenon introduction, such as the lungs, to areas of interest, e.g. the brain, are in the range of 5-7 s, thus T_1 values in various tissue types and in both oxygenated and deoxygenated blood should be sufficiently long for imaging purposes.⁹

T_1 values are longer at higher fields which serve to better resolve different chemical shifts resulting from xenon exchanging between different environments (i.e. blood vs lipids).¹⁵ However, a strong field is not needed to detect a signal from hyperpolarized ^{129}Xe because the polarization level is independent of field strength.¹⁵ This allows for “one-shot” imaging experiments with large signal-to-noise ratios at field strengths on par with that of Earth’s.¹⁵ This attribute has cost advantage where inexpensive spectrometers can be used to detect xenon signal even though field strength requirements would preclude the detection of proton signal on reasonable timescales.¹⁵ Aside from cost effectiveness, ultra-low field MRI could expand the field for patients with pacemakers and other metal implants for whom this imaging technique is currently not available.¹⁵

Table 1.1. T_1 for ^{129}Xe in different solvents and biological fluids

Tissue/solution	$T_1^{\S}$	Field Strength
Water	26.3 s	2.35 T
Saline/deutrated saline	66 s/1000 s	9.4 T
Intralipid (20%)	40 s	3.4 T
Red Blood Cells	4.5 s	2.35 T
Plasma	9.6 s	2.35 T
Blood foam with O_2	21 s	4.7 T
Blood foam without O_2	>40 s	4.7 T
Oxygenated (arterial) blood	13 s	4.7 T
Deoxygenated (venous) blood	4 s	4.7 T
Brain tissue	6 s	--
Lungs tissue	12 s	--
Blood solution in open air	5 s	9.4 T
Atomospheric O_2	11.9 s	2.35 T
Perfluorooctyl bromide emulsions	110 s	4.7 T

$\S$ These values aren't directly comparable because T_1 is influenced by magnetic field strength

Transverse relaxation in xenon-129 NMR is typically described by T_2^* to account for all sources of dephasing.¹⁶ As with proton NMR there are static field effects from field inhomogeneities that result in the xenon dephasing with increased rates of dephasing being observed at higher field strengths.¹⁶ Additionally, the rapid exchange of xenon between different sites (such as a host molecule and the bulk) also effects transverse relaxation.¹⁶ A final point of note on different aspects affecting xenon relaxation is that if this system is used *in vivo* different considerations have to be made depending on what area of the body a clinician is imaging. Xenon can dissolve in tissue (solid-phase considerations), blood (solution-phase considerations), and air pockets, like in the lungs, (gas-phase

considerations). Thus a variety of pulse sequence programs will be needed to tune the resulting signal from different areas of interest.

§ 1.3 Magnetic Resonance Imaging[‡]

Magnetic resonance imaging (MRI) is a versatile and commonly employed technique for the diagnosis and staging of disease. More than 30 million scans are performed in the United States every year. Importantly, MRI utilizes non-ionizing radiation and offers sub-millimeter spatial resolution and excellent soft tissue contrast and depth penetration. In the late 1960's Raymond Damadian observed by NMR that malignant tissue had a different spectrum than normal tissue. Then, in 1973, Lauterbur proposed a method of converting NMR signal into an image. Magnetic resonance imaging is characterized by high spatial resolution, which is the ability to readily discern one object as separate from another object. There are three main factors that determine the chemical shift of a given proton in NMR: spin-density, longitudinal relaxation, and transverse relaxation. Images can be measured by changing pulse programs to weight these contributing factors differently. When considering tissues in general and humans (or animals) specifically, the proton concentration is directly affected by the water and lipid content of a given area. The relative differences are only on the order of 10-15% and thus these "spin-density" images suffer from poor contrast. It should be noted, however, that grey-white matter

[‡] This section follows discussions in references: ^{5, 17-19}

differentiation achieved in spin-density images is superior to that of computed tomography (CT) images. For T_1 weighted images, increasing rigidity of the tissue reduces atomic collisions and typically leads to longer spin-lattice relaxation times. Because most tissues have high water content, typical T_1 values are in the hundreds of milliseconds range. T_2 depends on local non-uniformities in the magnetic field. Molecular motion disturbs the distribution of non-uniformities thus increasing T_2 relative to that of bulkier macromolecules. It may be helpful to compare the relative effects of different disease processes on the three variables influencing proton signal as seen in Table 1.2.¹⁸

The signal generated in NMR spectroscopy can be converted into an image in a variety of ways. Initially, it is important to understand how the signal of one type of tissue in one part of the body can be differentiated from the signal from the same tissue in a different part of the body. The principle first proposed for spatial localization was described by Lauterbur. Simply put, he proposed making the frequency of the emitted radio waves reflect the position of the emitting protons. To do this, MR imaging instruments contain gradient coils which cause variations in the linear field. Using these gradient coils, spatial localization can be achieved in a variety of manners but the most straightforward is slice selection.

Slice selection is achieved by applying a gradient field across the body along a coronal plane (orange gradient, Figure 1. 6). This generates an inhomogeneous field resulting in resonance frequency variations in the direction of the field gradient. The result is that each spin will each turn on an angle that is linearly dependent on the nucleus' position, termed a phase roll. Thus, the frequency needed for the applied radiowave to be

in resonance changes resulting in selective excitation of the protons within the selected sagittal slice (blue bar, Figure 1. 6). Then, different signals are generated for different positions within the sagittal plane by employing gradients.

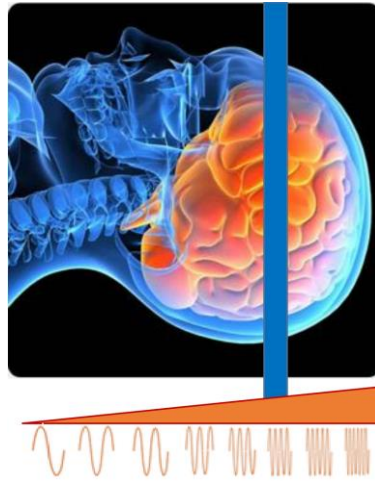


Figure 1. 6. Depiction of slice selection where a gradient (orange wedge) is applied along the plane orthogonal to the imaging slice. A rf pulse that matches the narrow range of frequencies (generated by the gradient) is simultaneously applied (blue rectangle) along the desired imaging slice.

The use of gradients to generate this phase roll results in a net decrease in signal. Because there are void areas from which there is no water signal (e.g., bone), the “wave length” of the phase roll directly corresponds to the distance between the areas generating water signal. Thus the magnitude of the signal for a particular phase roll pattern determines whether there is structural similarity between imaged areas. This description is simplified a bit to understand imaging in a one-dimensional plane but to extend the image to the 2-D images with which doctors are familiar, k-space must be considered. Spin patterns are generated from phase rolls and a vector $\mathbf{k}$ is assigned in the direction of change, the magnitude of which is indicative of spin density. Thus, the radio wave signal is presented

as a function of $\mathbf{k}$, a Fourier technique. In this manner images within a 2-D plane are generated but by applying gradients in different directions and strengths and then overlaying the slices it is possible to generate a meaningful image in a process termed signal reconstruction. For the generation of MR images then, the spatial limitation is determined by the movement of water molecules, gradient strengths, and relaxation times. To mitigate these limitations then a variety of gradients are used in addition to the introduction of compounds which function to perturb relaxation times, namely, contrast agents.

Table 1.2. Effects of Various Disease Processes on Proton Spin Density and T_1 and T_2

Disease Process	Spin Density	T_1	T_2
Cerebral infarction	Increase	Increase	Increase
Hemorrhage	Increase	Decrease	Increase
Edema	Increase	Increase	Increase
Multiple sclerosis	Decrease	--	Increase
Malignant tumor	Increase	--	Increase
Pituitary tumor	--	Decrease	--
Cyst	--	Strongly increase	Increase

Adapted from Magnetic Resonance Imaging Part I—Physical Principles

§ 1.4 Contrast Agents in Magnetic Resonance Imaging

Proton (^1H) magnetic resonance signals are abundant based on the high concentration of water, fat, and other biomolecules *in vivo*. Technicians can modulate the signal and contrast of the images with different pulse sequence programs in order to obtain drastically different images; highlighting differences in proton density, relaxation times, and chemical shift (lipids vs water).²⁰ However, high background, coupled with the very small magnetic resonance signals obtained from individual proton nuclei, generally limit

the sensitivity of this technique.²⁰ One consequence is that molecular signatures from protons in discrete environments (e.g., from specific biomolecules or acidic compartments in cells) are not readily detected. These challenges have motivated the development of MRI contrast agents to perturb the endogenous proton signals. All contrast agents decrease the longitudinal (T_1) relaxation time and the transverse (T_2) relaxation time, thereby increasing the relaxation rate. If an agent increases the longitudinal relaxation rate ($1/T_1$) more than the transverse relaxation rate ($1/T_2$) they are said to be T_1 weighted and are termed positive contrast agents because they give rise to increased signal intensity. Conversely, T_2 weighted images result from the negative contrast achieved by an increase in $1/T_2$, usually with ferromagnetic iron oxide particles. The majority of contrast agents, however, are chelated gadolinium compounds whose paramagnetic properties reduce T_1 . Indeed, four of the six clinically approved intravenous contrast agents are gadolinium chelates. A relaxation rate change of 0.5 s^{-1} can produce observable contrast; thus, gadolinium contrast agents with typical relaxivities ($r_1 = 3\text{-}10 \text{ mM}^{-1}\text{s}^{-1}$) require minimum concentrations $\sim 100 \text{ }\mu\text{M}$, shown in Figure 1. 7.²⁰ The design strategies employed for gadolinium-based agents must balance the need for strong organic ligand coordination (to mitigate toxicity) with the desire to markedly perturb relaxation times.²¹ Gadolinium serves to perturb water relaxivity by either directly (inner sphere) or indirectly (outer sphere) coordinating water molecule(s). Thus, an increase in the number of inner sphere waters coordinated (q) tends to increase relaxation rates. Again, because of the need to fully chelate the gadolinium ion, q is limited.²¹ One way to decrease longitudinal relaxation time is to slow the molecular rotational correlation time because slower tumbling leads to faster relaxation rates.^{20, 21}

Thus, to slow tumbling times a wide variety of bulky scaffolds have been appended to the chelation complexes.²² In order to allow the detection of much rarer analytes, “super-stoichiometric” strategies have been explored, which include incorporating numerous gadolinium agents within a single particle, as well as engineering enzyme reporters, where a single enzyme can change the coordination environment of many gadolinium agents.²⁰⁻²⁵ Targeted contrast agents are typically bifunctional ligands with the cyclen or diethylenetriamine Gd^{3+} chelating core with nitrogen or oxygen donor atoms that are then appended with a ligand specific to the protein or enzyme of interest.²⁵ Protein binding can serve to retard the tumbling time but can also affect the metal hydration by blocking the inner coordination sphere. The targeting must be achieved with high specificity while the complex must be inert and fully clear the body.²⁵ Thus, these smart agents have the potential to strongly improve contrast, but remain limited both because they modulate endogenous signal and because the use of a heavy metal necessitates strong chelation. A parallel and potentially complementary strategy involves the use of exogenously supplied “hyperpolarized” nuclei, which include ^{13}C , ^3He , and ^{129}Xe .

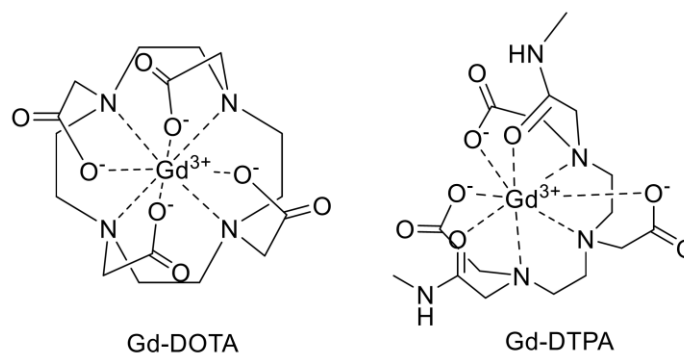


Figure 1. 7. DOTA and DTPA gadolinium chelates for magnetic resonance imaging.²¹

§ 1.5 Exogenous Nuclei in NMR/MRI: Advantages of ^{129}Xe *

The intrinsic NMR chemical shift sensitivity, exceptional signal-to-noise, and chemical inertness of hyperpolarized xenon-129 (hp ^{129}Xe) make it attractive for many magnetic resonance applications, and a natural complement to proton magnetic resonance imaging (MRI). For imaging studies, ^{129}Xe is a viable alternative to ^3He , which is rare and nonrenewable; moreover, among the noble gases, ^{129}Xe (with 54 electrons) possesses unique capabilities for host-guest chemistry based on its volume ($\sim 40 \text{ \AA}^3$), significant polarizability (which yields high affinity for void spaces and good water solubility, as well as local environment sensitivity),²⁶ and lack of radioactivity. These applications are enhanced by recent improvements in spin-exchange optical pumping methods that yield near unity ^{129}Xe hyperpolarization.²⁷ Pioneering biological imaging studies with hp ^{129}Xe targeted the pulmonary void spaces in rat lungs.²⁸ There have since been many additional xenon studies in the lungs, brain, and other tissues in both rat and humans, shown in Figure 1. 8.²⁹⁻³³

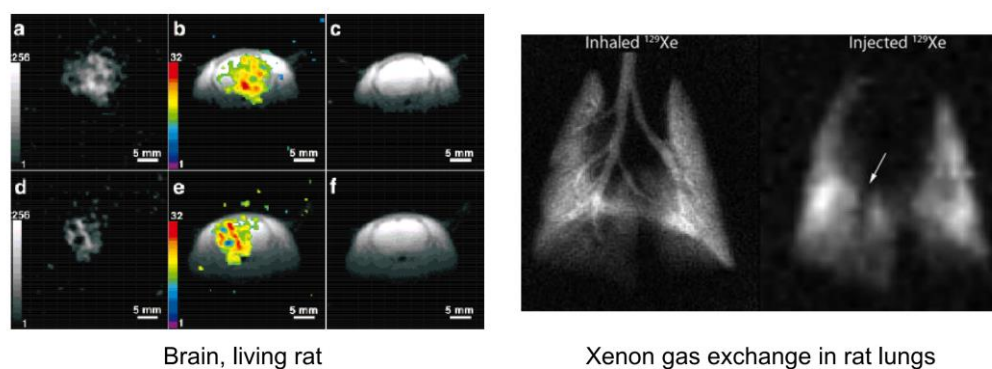


Figure 1. 8. Representative ^{129}Xe MR images from living rat brain and lungs. Reprinted with permission from references:^{34, 35}

These successes motivate greater use of hp ^{129}Xe in human imaging, with the caveat that xenon is limited to areas where the gas will readily diffuse, after inhalation or direct injection. Although very soluble in organic solvents and lipid environments, xenon exhibits low affinity for endogenous proteins and other biomolecules; attempts to engineer high-affinity xenon-binding sites into proteins have thus far been unsuccessful.³⁶ Therefore, xenon should not localize to biomolecular targets *in vitro* or *in vivo*, unless biosensors with high xenon affinity are employed. Here, we summarize lessons learned over the past decade as we have worked to develop cryptophane-based hp ^{129}Xe NMR biosensors for biological imaging applications.

§ 1.6 Hyperpolarization of ^{129}Xe

Exogenously supplied nuclei that are not found *in vivo*, like xenon, already demonstrate an advantage over proton signal in that there is no “background”. Increasing the utility of this nucleus specifically is the ability to generate a pool of “hyperpolarized” nuclei. The hyperpolarized state is differentiated from Boltzmann spin distribution (see Figure 1. 9) and from a polarized state which refers to electron cloud perturbation as a result of local electric fields. While there are a variety of methods used to achieve a hyperpolarized state of various nuclei, spin exchange optical pumping (SEOP) is highly efficient for ^{129}Xe .

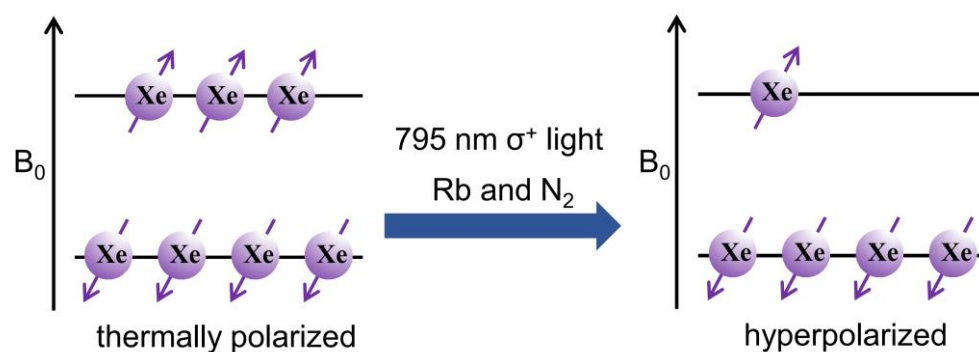


Figure 1. 9. Boltzmann spin distribution compared to hyperpolarized spin of ^{129}Xe .

SEOP was originally developed in the 1960s for hyperpolarization of noble gases.³⁷ This method embeds rubidium in a high pressure gas mixture, at 64 psi, containing 89% helium, 10% nitrogen, and 1% xenon-129 (natural abundance). The helium acts as a buffer and the nitrogen acts as a quencher. The percentage of xenon can be increased but this results in a significant cost increase. The elevated pressure of this gas mixture significantly broadens the optical transition of rubidium, which is compatible with the use of a broadband, high-powered, semiconducting laser (typically 50 W or greater) with line widths at 1-3 nm to excite the alkali metal. More recently, much narrower line width lasers centered on 795 nm have been developed that make it possible to work more safely with lower pressure (non-broadened) Xe gas mixtures. This process, termed optical polarization, relies on the absorption of circularly polarized light photons (σ^+) by Rb. The laser provided circularly polarized light is tuned to the D_1 transition of Rb at 794.7 nm. The quenching nitrogen gas prevents rubidium depolarization. The laser is directed through a beam expander and a $\lambda/4$ wave plate to generate circularly polarized light.³⁸ This process results in a ground state electron spin polarization of Rb. Next, as a result of the high pressure conditions, there are increased collisions between polarized Rb and Xe. These collisions

result in short-lived complexes generating isotropic hyperfine coupling or Fermi contact interactions which result in xenon spin polarization. This is depicted in Figure 1. 10, prepared by Yanfei Wang.

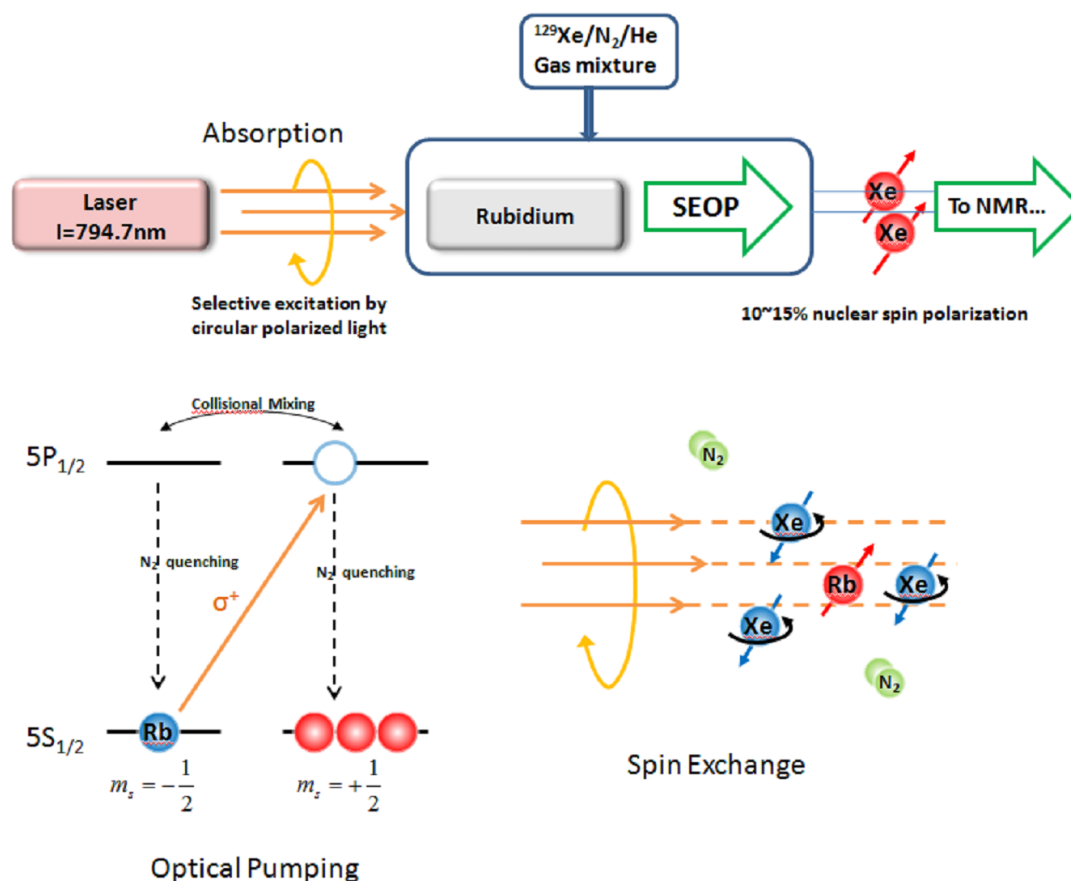


Figure 1. 10. Hyperpolarization of ^{129}Xe achieved via spin-exchange optical pumping.

§ 1.7 Host-Guest Chemistry and the Synthesis of Cryptophane-A Derivatives*

Most xenon biosensors developed to date employ an organic host molecule with a hydrophobic cavity that can reversibly bind xenon in aqueous solution. Host candidates with modest room-temp xenon association constants include: hemicarcerand ($K_A \approx 200\text{ M}^{-1}$), α -cyclodextrin ($K_A \approx 20\text{ M}^{-1}$), and calix[4]arene derivatives ($K_A \approx 14\text{ M}^{-1}$), shown in

Figure 1. 11.³⁹⁻⁴¹ These affinities are on par with xenon-protein association constants (the highest being myoglobin at 200 M^{-1}),⁴² which has limited their utility for most applications. Water-soluble cucurbit[6]uril has significantly higher xenon affinity ($K_A \approx 3000 \text{ M}^{-1}$) but presents synthetic challenges to achieve single-site functionalization for biomolecular targeting.⁴³ Cucubitr[6]uril itself has a xenon affinity of only 490 M^{-1} but is commercially available in gram quantities.⁴⁴ The most studied xenon-binding molecule to date is cryptophane-A (Figure 1. 12), in which two cyclotriguacylene caps are tethered by three ethylene linkers generating an approximately 1 nm diameter cage molecule with an internal volume of approximately $85\text{-}89 \text{ \AA}^3$.⁴⁵ Cryptophane-A was first synthesized in 1981 but it was not until 1998 that it was determined to have a room-temp xenon association constant of 3000 M^{-1} in a noncompeting organic solvent, $\text{C}_2\text{D}_2\text{Cl}_4$ (deuterated 1,1,2,2-tetrachloroethane).^{45, 46} Notably, cryptophane-A exhibits helical chirality (along the red axis depicted in Figure 1. 12) but is usually isolated and used as the racemic mixture of stereoisomers.

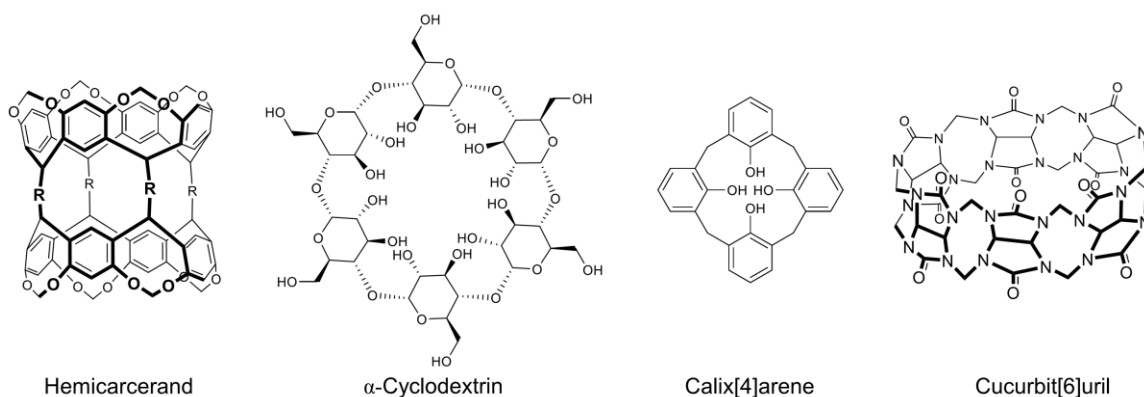


Figure 1. 11. ^{129}Xe host molecules with weak affinity. Hemicarcerand: $K_A \approx 200 \text{ M}^{-1}$; α -cyclodextrin: $K_A \approx 20 \text{ M}^{-1}$; Calix[4]arene: $K_A \approx 14 \text{ M}^{-1}$; and Cucubitr[6]uril $K_A = 490 \text{ M}^{-1}$.⁴⁴

The design of xenon-binding molecules is informed by the work of Mecozzi and Rebek, who tabulated the known host-guest interactions dominated by dispersion forces and determined that a guest-to-host volume ratio of 0.55 ± 0.09 optimally balances favorable van der Waals (enthalpic) contributions with the entropic cost of host crowding.⁴⁷ [Notably, this empirical formula was not derived for host-guest interactions in water, where the hydrophobic effect yields favorable entropic contributions.]

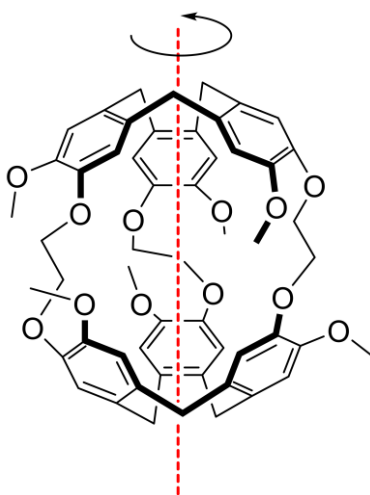


Figure 1. 12. Cryptophane-A. Macromolecular cage host molecule with axial chirality.

Indeed, in work using hexa-carboxylic acid cryptophane derivatives in D₂O, it was shown that increasing the size of the cryptophane cavity, going from two-carbon linkers (2,2,2) to three-carbon linkers (3,3,3), decreased the xenon association constant ($K_A \approx 6800 \text{ M}^{-1}$ for cryptophane-222 and $K_A \approx 1000 \text{ M}^{-1}$ for cryptophane-333, Figure 1. 13) but also shifted the Xe@cryptophane peak by 30 ppm upfield.^{48, 49} Conversely, decreasing the cage internal volume to $81 \text{ \AA}^3$ using one-carbon (i.e., methylene) linkers, increased the room-temp association constant to $10,000 \text{ M}^{-1}$.⁵⁰ These cryptophane derivatives have promising

features for use as xenon biosensors, including high xenon affinity and ease of manipulating ^{129}Xe NMR chemical shift through cage design.

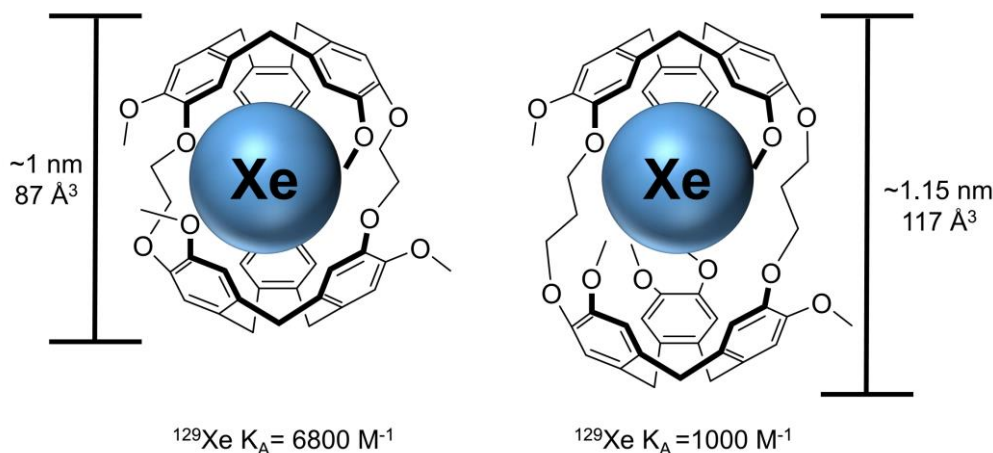


Figure 1. 13. Structures and relative sizes and xenon-129 binding affinity of cryptophane-222 (cryptophane-A) and cryptophane-333 (cryptophane-E).⁵¹

Synthetic Mechanism of Cryptophane Cyclization

The synthetic mechanisms for generating cryptophane cages have evolved steadily since their initial conception. In 1981, Gabard and Collet first published a “Synthesis of a D_3 -Bis(cyclotrimeratrylenyl) Macrocage by Stereospecific Replication of a C_3 -Subunit” where they determined that formation of cyclotriguiacylene (CTG) from vanillyl alcohol could only be achieved with a phenol protected derivative using 65% perchloric acid. Subsequent formation of a single product, the D_3 cage (in preference to the *meso* cage) was achieved at 90 °C in formic acid which they attributed to a “stereospecific replication” process. This illustrates a “template effect” where the new ring forms in the same chirality as the parent C_3 ring.⁴⁵ In 1985, Collet and coworkers coined the term “cryptophane” harkening to the [1.1.1]orthocyclophane structure of the top and bottom components of the

cage molecule. Cryptophane describes a spherical, hollow host molecule with a lipophilic cavity formed by two cyclotrimeratrylene (CTV) units joined by three alkoxy bridges, which results in three “windows” into the cage that allow for guest entry. In 1987, they strove to ascertain how different linkers and R groups affected the cage formation of which there are two options, *syn* or *anti* (Figure 1. 14a). The *anti*-conformation is chiral and has D_3 symmetry and, when viewed along the C_3 axis, the upper and lower CTV units are staggered by 50-60° whereas in the *syn* conformation, the CTV units are eclipsed and the cage has C_{3h} symmetry and is achiral.⁵² The *syn* cage is chiral, however, when $R \neq R'$. They found that $O(CH_2)_2O$ linkers and linker with *trans* alkenes preferentially led to *anti*-cryptophanes whereas $O(CH_2)_3O$ and *cis* alkenes yielded *syn* cages.⁵² This phenomenon is attributed to the position of the reactive veratryl ends with respect to the template ring.⁵² Subsequent studies determined that for many applications, including further derivatization, the template method is superior (better yielding and applicable to a wider variety of derivatives) than the direct, or two-step method.⁵³ The direct or two step method relies on the formation of a bis-vanillyl alcohol derivative followed by treatment with formic acid to form cryptophane. The template method, on the other hand, first promotes the intermolecular cyclization reaction between the vanillyl alcohol derivatives to form the corresponding CTV derivative, a comparison of these two methods is shown in Figure 1. 14, panel B. The direct method has the distinction of being simple and fast. It can be done on a large scale but is usually low yielding, resulting in large quantities of polymer. Additionally, only cryptophanes with hexa-symmetrical substituents can be generated. These cryptophanes have lower xenon affinities the reasons for which will be discussed in

detail later. The template method, on the other hand, is far more labor intensive with 5-6 step syntheses.⁵⁴ The final intramolecular cyclization is also low yielding and because it must be performed at ultra-low concentrations, is not readily scalable. Efficient intermolecular cyclization (for either method) requires electron-donating groups *meta* to the benzyl alcohol. In the template method the initial intermolecular reaction forms the CTV “cap” molecule that can then be coupled with a wide variety of benzyl alcohols (again conforming to the need for electron-donating groups at the *meta* position) before the final intramolecular, pre-organized cyclization via a Friedel-Crafts like mechanism, shown in Figure 1. 15.⁵² It should be noted that the steps depicting cation formation likely do not proceed in a concerted fashion but are depicted as such for the sake of clarity.

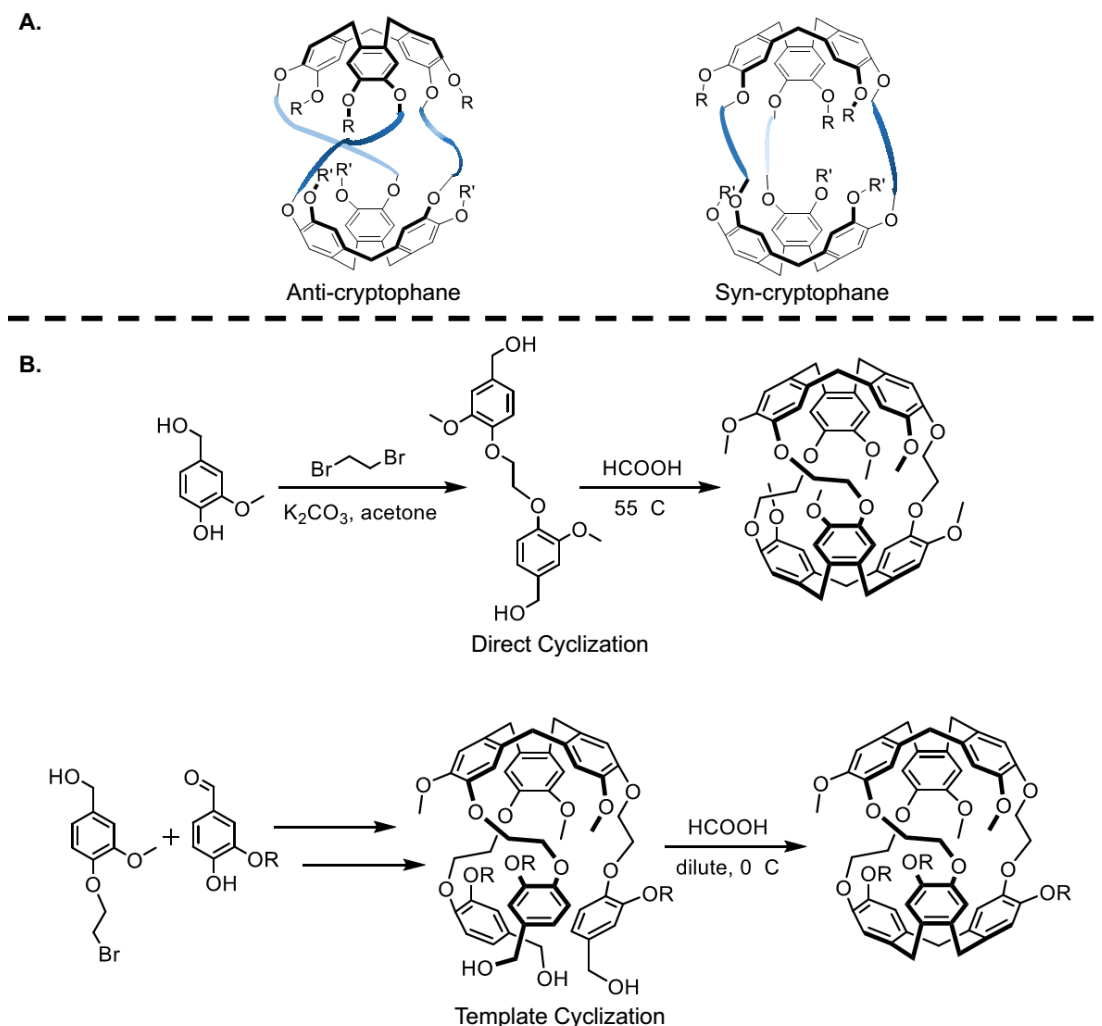


Figure 1. 14. Possible conformations of cryptophane host molecules either *anti* or *syn* shown in panel A. and the examples of Direct vs Templated cyclization for the synthesis of cryptophane-A and its derivatives in panel B.⁵²

A limitation of cryptophane host molecules is their poor water solubility. In 2007, our lab published a templated, 10-step synthesis for a water-soluble tri-functionalized cryptophane via tripropargyl cryptophane and a subsequent Cu(I)-catalyzed [3+2] azide-alkyne cycloaddition (CuAAC) with azido-propionic acid which was prepared in one step from β -propiolactone (Figure 1. 16e).⁵⁵ The resulting compound, termed tris-(triazole propionic acid)cryptophane (TTPC), had a room-temp xenon association constant of

17,300 M⁻¹ in water, significantly higher than previously published cryptophane derivatives (Table 1.3).^{48, 55}

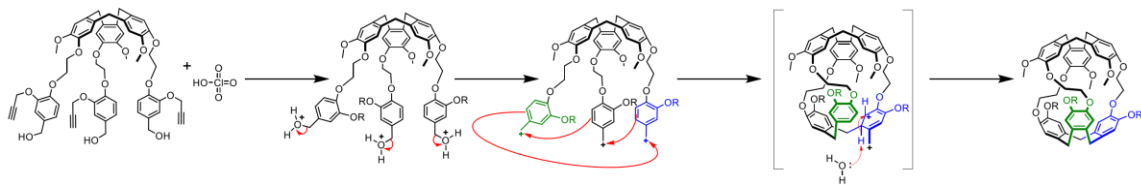


Figure 1. 15. Intramolecular cyclization mechanism to form cryptophane host molecules.

We postulated that desolvation of xenon in water as well as displacement of water from the cryptophane cavity entropically drives Xe-cryptophane association. In order to investigate how the solubilizing linkers affect xenon binding, we synthesized, in 13 steps, a triacetic acid cryptophane-A derivative or TAAC (Figure 1. 16d), with the carboxylates ~5 Å closer to the cavity.⁵⁶ Interestingly, TAAC exhibited a marked increase in xenon binding affinity (Table 1.3). To investigate how the charge on the solubilizing groups affects xenon binding, we synthesized a tris-(triazole ethylamine)cryptophane (TTEC), following the same protocols as TTPC, while conjugating 3-azidoethylamine in the last step.⁵⁷ As shown in Table 1.3, the amine groups afforded a cryptophane host with nearly 1.3-fold improved xenon affinity, producing the highest known xenon affinity for any host molecule. For all three water-soluble cryptophanes, the Gibbs free energy had very similar enthalpic and entropic components (Table 1.3).

Table 1.3. Thermodynamic parameters of water-soluble derivatives of cryptophane-A

Cryptophane	K_A ($M^{-1} * 10^4$)	ΔG (kcal mol ⁻¹)	ΔH (kcal mol ⁻¹)	$T\Delta S$ (kcal mol ⁻¹)
TAAC	3.33 ± 0.28	-6.06	-4.34 ± 0.66	1.72
TTPC	1.70 ± 0.17	-5.69	-3.14 ± 0.20	2.55
TTEC	4.20 ± 0.20	-6.20	-3.58 ± 0.93	2.62

Measure by ITC in buffer at pH 7.0 and at 293 K

The favorable entropy term likely results from the dissolution of the clathrate shell surrounding xenon in water along with the release of water molecules from the cryptophane cavity. A key feature of this system is the use of three solubilizing moieties, which helps to keep the cryptophane in an open, xenon-accessible conformation. Furthermore, the type of solubilizing moiety and their distance from the cryptophane interior are postulated to modulate the number of waters binding inside the cavity, which compete with xenon binding.⁵⁸

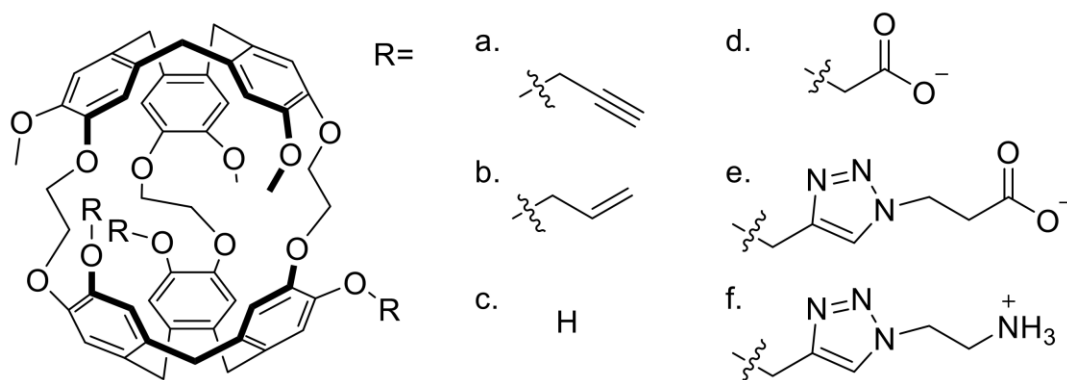


Figure 1. 16. Derivatives of cryptophane-A. a. tripropargyl cryptophane (TPC); b. triallyl cryptophane (TAC); c. trihydroxy cryptophane (THC); d. triacetic acid cryptophane (TAAC); e. tris(triazole propionic acid) cryptophane (TTPC); and f. tris(triazole ethylamine) cryptophane (TTEC).⁵⁴⁻⁵⁷

In 2011, we reported a shorter six-step synthesis of trifunctionalized cryptophanes shown in Figure 1. 16a and b with an improved yield of 6%.⁵⁴ This built on the work of

Brotin *et al.* who reported a milder $\text{Sc}(\text{OTf})_3$ cyclization for cyclotriguiacylene formation, removing the need for low concentrations and protection/deprotection steps.⁵⁹ The use of either tripropargyl cryptophane (Figure 1. 16a) with CuAAC or trihydroxy cryptophane (Figure 1. 16c) with amide coupling (following acetate addition) affords a facile means of functionalizing the cage with solubilizing or targeting moieties. It is notable that the tri-functionalized cryptophanes TTPC, TAAC, and TTEC show similar water solubility to the reported hexa-functionalized cryptophanes and exhibit significantly better xenon-binding affinities. From our work we have postulated this is a result of cation chelation by the six carboxylates, which may block xenon entry and also stabilize water inside the cavity. Very recent work from the Saven laboratory at the University of Pennsylvania has demonstrated through molecular simulations that a key component in binding equilibrium is the removal of water confined within the cryptophane cavity.⁵⁸ By comparing xenon-cryptophane binding in a variety of cages, they determined that while internal cage volume played a role, Xe binding was not perturbed by that alone.⁵⁸ Indeed four cages with identical central cavities have wide range of Xe affinity with K_a values from 34,000 – 6800 M^{-1} .⁵⁸ Desolvation of xenon is known to make binding of cryptophane entropically favorable.^{56,}⁵⁸ They found that hexa-acid cryptophanes have greater water occupancies in their core than trifunctionalized cryptophanes. Additionally, having charged groups on either side of the cryptophane pores seems to promote the formation of water clusters occluding the cavity.⁵⁸ The cryptophane work performed to date has yielded a ~300 ppm chemical shift window for encapsulated xenon in water (by modulating the cage volume or appending ruthenium, see Figure 1. 29) as well as tens of micromolar xenon dissociation constants.^{50,}

^{57, 60, 61} Thus, our research focus involves coupling the use of exogenously supplied xenon with targeted molecular imaging techniques.

§ 1.8 Binding Characterization*

Methods for measuring xenon binding parameters to host molecules have been refined in recent years. Early work used either changes in ¹H NMR chemical shifts upon ¹²⁹Xe binding or integration of bound and free ¹²⁹Xe resonances to calculate the binding affinity of cryptophane.⁵⁵ These methods, however, suffer from large errors.⁴⁸ To develop a more sensitive method for measuring xenon binding affinity for water-soluble cryptophane, we took advantage of xenon's ability to quench fluorescence of organic chromophores by the heavy atom effect.⁶² This was previously shown by very efficient Xe quenching of pyrene bound to apomyoglobin.⁶³ Using 15 μ M TTPC (Figure 1. 16e), experiments were conducted at 293 K in 1 mM phosphate buffer at pH 7.2 by titrating a solution of 1-atm-saturated (5.05 mM) aqueous xenon. Fluorescence maximum intensity vs. xenon concentration (0-5.05 mM) data were fit to a single-site binding model:

$$\frac{[Xe@1e]}{[Xe] + [1e]} = \frac{[Xe]}{[Xe] + K_D} \quad (1.9)$$

We also demonstrated the utility of isothermal titration calorimetry (ITC) for measuring xenon-cryptophane association constants, which gives data in excellent agreement with the fluorescence quenching method.^{55, 56} ITC has the advantage of providing both enthalpic and entropic xenon-binding parameters for a single cryptophane concentration, but requires significantly more cryptophane sample, particularly as the enthalpic contribution to xenon-cryptophane binding is only a few kcal/mole.

We further investigated the interactions between cryptophane and guest molecules by X-ray crystallography. Cryptophane is known to encapsulate a large size range of guests from methane (28 Å³) to chloroform (72 Å³), and these guests bind with similar affinity ($K_A \approx 130\text{-}230\text{ M}^{-1}$).^{47, 64} In an effort to explain why xenon binds with so much higher affinity, we co-crystallized cryptophane-A derivatives, Figure 1. 16a, and b with methanol, xenon, and chloroform and found that the cavity internal volume (80–102 Å³) varied with guest size.⁶⁵ Importantly, it was observed that in the xenon-bound structure, van der Waals interactions were nearly optimized, with an interior cavity volume of 85-89 Å³ and guest:host volume ratio of 0.47-0.49.

§ 1.9 Cryptophane Biosensors: Targeted Detection*

In developing the first xenon biosensor, Pines, Schultz, and coworkers elegantly demonstrated how to functionalize cryptophane to achieve the dual aims of targeting streptavidin and improving water solubility. In cryptophane-A, one methoxy group was replaced with a free hydroxyl group which was then converted to a carboxylic acid. Coupling of the carboxylic acid with the amino-terminus of a protected peptide on resin and subsequent coupling with a biotin linker afforded a water-soluble, targeted biosensor. Avidin binding generated a ~2.3 ppm upfield chemical shift change for hp ¹²⁹Xe encapsulated in the biotin-conjugated biosensor.⁶⁶ In a follow-up study, one resonance was observed for mono-allyl-substituted cryptophane-A. However, upon conjugating the cryptophane with a chiral CKR peptide two peaks appeared, 0.15 ppm apart. These peaks were attributed to the RL and LL diastereomers. After the cryptophane was further derivatized with the biotin linker through maleimide chemistry a new racemic chiral center

was formed, generating RLR, RLL, LLR, and LLL combinations for which four “bound” peaks were assigned.⁴⁹ The observed sensitivity of xenon to diastereomerism is problematic for many biosensing applications, as it “dilutes” the xenon-biomarker signal, and complicates peak assignments as well as efforts to selectively irradiate ^{129}Xe in a specific environment, as required for many NMR experiments.

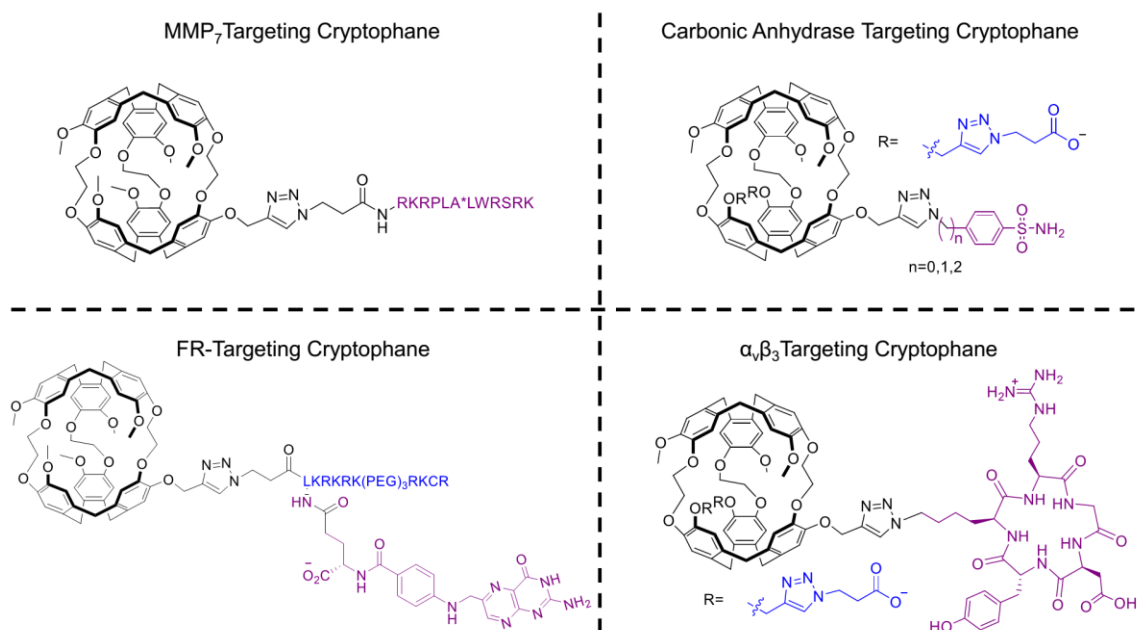


Figure 1. 17. Targeted cryptophane biosensors from the Dmochowski laboratory. Clockwise from top left: matrix metalloproteinase-7 (MMP-7) targeting, MMP-7 peptido-substrate cryptophane; carbonic anhydrase targeting, benzene sulfonamide cryptophane; $\alpha_v\beta_3$ integrin-receptor targeting, c[RGDyK] cryptophane; and folate receptor targeting, peptide-folic acid cryptophane.⁶⁷⁻⁷⁰

Following up on this research, our laboratory demonstrated the ability of ^{129}Xe to report on an enzyme cleavage event by appending racemic cryptophane with a peptide substrate for matrix metalloproteinase-7, a known cancer biomarker, shown in Figure 1. 17. The ^{129}Xe NMR spectra showed two peaks before cleavage and two new peaks after cleavage < 1 ppm upfield of the original peaks.⁶⁷ Because the xenon signal was effectively

split between the diastereomers, a subsequent work, by Dutasta, Berthault, and others, utilized enantiopure cryptophane-A grafted with a 20-mer oligonucleotide.⁷¹ ^{129}Xe NMR spectra were measured for the biosensor alone, the biosensor with a complementary DNA strand, and biosensor with non-complementary DNA strand. The biosensor plus complementary strand was shifted 1.5 ppm upfield of the other two samples, and all three samples exhibited one bound peak, as expected for single enantiomers.⁷¹ Surprisingly, however, when the concentration was increased, both the biosensor alone and the biosensor plus noncomplementary strand were seen to foam and exhibit multiple Xe@biosensor peaks. It was hypothesized that this was the result of increased microemulsions and micelle/vesicle formation.⁷¹ This observation highlights the importance of developing highly water-soluble xenon biosensors.

A subsequent work appended cryptophane-A with a peptide ligand to major histocompatibility complex (MHC). Specifically, Schlundt *et al.* used the hemagglutinin (HA) peptide which binds human leukocyte antigen (HLA) DR1. The design employed a solubilizing linker, which acted as a ~ 35 Å spacer between the cryptophane and the peptide binding moiety.⁷² ^{129}Xe NMR revealed one peak for the bound and unbound biosensor, with a $\Delta\delta$ of only 1 ppm.⁷² While the spacer improved solubility, it positioned the xenon too far from the peptide to generate a significant chemical shift change upon MHC binding. Heretofore, targeted xenon biosensors were mono-functionalized derivatives of cryptophane-A but our work with water-soluble TAAC, TTPC, and TTEC suggested that we could achieve both better xenon binding and simplified ^{129}Xe NMR spectra with a tri-functionalized cryptophane. Building on earlier work in our lab using mono-functionalized

cryptophane with a linear (RGD)₄ repeat,⁷³ we functionalized tripropargyl cryptophane (Figure 1. 16e) with a cyclic peptide RGDyK, known to have high affinity and specificity for $\alpha_v\beta_3$ integrin, and two 3-azidopropionic acids with CuAAC.⁷⁴ Interestingly, despite the cryptophane chirality and potential for diastereomerism, we observed only one ¹²⁹Xe@biosensor NMR peak when the biosensor bound to $\alpha_v\beta_3$ integrin and it was 4.1 ppm downfield from the free biosensor. This demonstrated that using a well-solubilized cryptophane, it is possible to engage protein targets using short tethers and still obtain well-resolved ¹²⁹Xe NMR spectra.

Targeting $\alpha_v\beta_3$ Integrin*

In order to investigate the cell compatibility of xenon biosensors we fluorescently labeled our c[RGDyK]-cryptophane (see Figure 1. 17) and performed cell uptake, viability, and specificity studies. The MTT assay demonstrated $\geq 60\%$ viability at 75 μM biosensor for three cell lines. Confocal microscopy studies with the cancer cell line, AsPC-1, and normal human fibroblasts, HFL-1, showed preferential biosensor uptake in AsPC-1 and further demonstrated that uptake could be blocked with antibody or excess c[RGDyK] peptide (Figure 1. 18). Flow cytometry confirmed 2-3-fold greater uptake over antibody and c[RGDyK] blocked cells and 4-fold greater uptake in AsPC-1 vs. HFL-1.⁷⁴ Our studies also demonstrated targeting of $\alpha_v\beta_3$ integrin and $\alpha_{IIb}\beta_3$ integrin with nanomolar affinity and specificity and low cytotoxicity at concentrations required for NMR experiments. This work paved the way for cellular NMR spectroscopy and imaging experiments, which are now underway in our laboratory and elsewhere.⁷⁵⁻⁷⁷

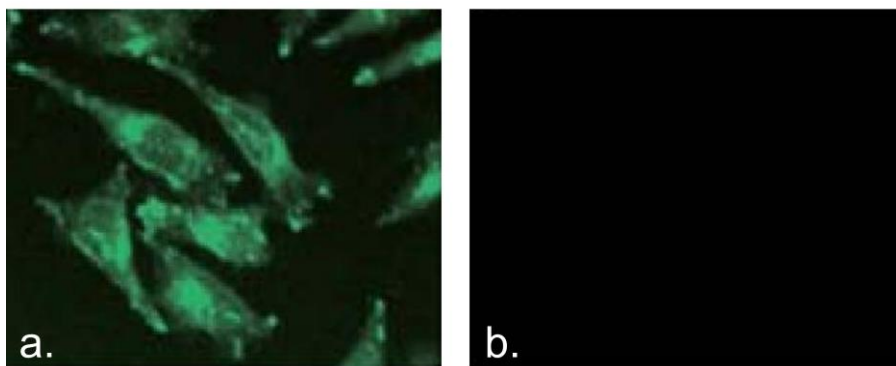


Figure 1. 18. Cellular uptake studies using confocal microscopy. Alexa-fluorophore 488-labeled c[RGDyK]-cryptophane (1 μ M) targeting ASPC-1 cells (a) after 1 h incubation and (b) with blocking co-treatment of 0.15 mg/mL anti- α_v antibody. Adapted with permission from Seward *et al.*⁶⁸

Targeting Carbonic Anhydrase*

A concurrent work sought to characterize how the proximity of cage binding modulated the xenon-129 chemical shift. For this study we synthesized a carbonic anhydrase-targeting cryptophane where a benzene sulfonamide ligand with three linker lengths (**C6B**, **C7B**, and **C8B**) was coupled to tripropargyl cryptophane (Figure 1. 17 and Figure 1. 20). Our laboratory has employed a carbonic anhydrase (CA) model system, because of its biomedical relevance for cancer detection.⁷⁸⁻⁸¹ CA is a ubiquitous zinc metalloenzyme that catalyzes a simple but physiologically important reaction: the reversible hydration of carbon dioxide to form bicarbonate and a proton.⁸²⁻⁸⁴ Five classes of evolutionarily unrelated CA exist (α -, β -, γ -, δ -, and ζ -CAs), with humans expressing fifteen isoforms of α -CA.⁸⁰ All fifteen isozymes share a conserved fold consisting of a central ten-stranded β -sheet surrounded by helical connections.⁸⁵ The catalytic active site of α -CA is positioned at the base of a conical cavity approximately 15 Å wide and 15 Å deep and is comprised of a catalytic Zn^{2+} ion exhibiting tetrahedral coordination with three

conserved histidine residues and a hydroxide ion, see Figure 1. 19. In this work we targeted CAI and CAII isozymes because unlike the isozymes CAIX and CAXII indicated in cancer, CA I and II are well characterized and express readily in bacteria, thus providing an ideal model system. Both are cytosolic isoforms of α -CA with high structural homology.

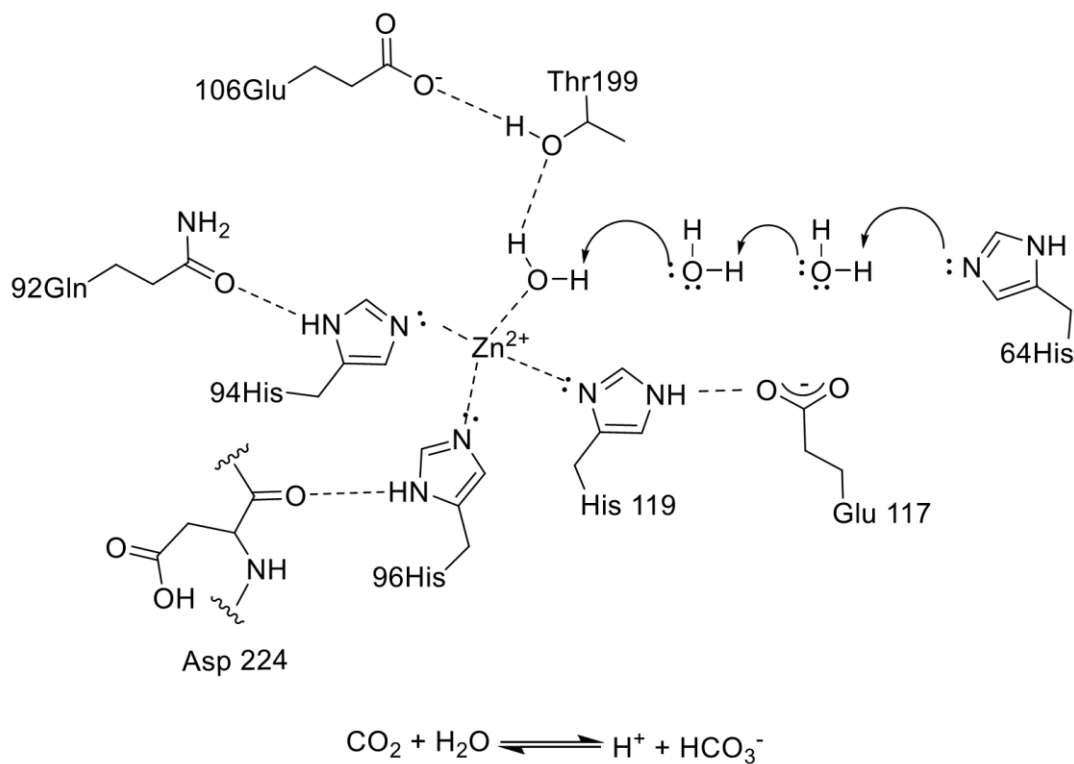


Figure 1. 19. Highly conserved active site residues of carbonic anhydrase. Shown is the His64 mediated proton shuttle mechanism that aids in the interconversion of carbon dioxide and bicarbonate plus proton.

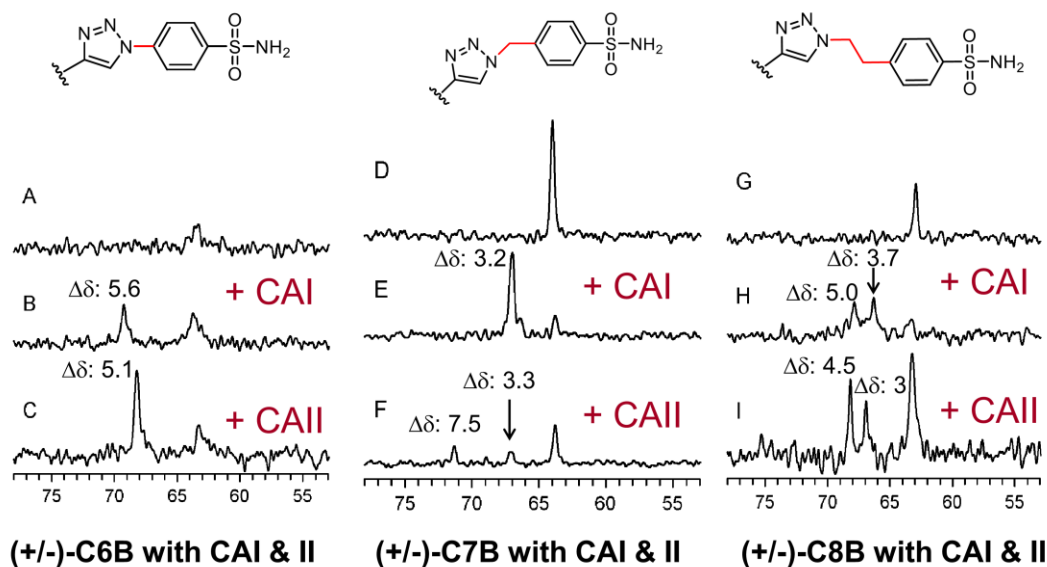


Figure 1. 20. ^{129}Xe NMR of CA targeting biosensors. ^{129}Xe NMR in 50 mM Tris, pH 8.0 of (a) C6B alone (96 μM); (b) C6B (188 μM) and CAI (141 μM); (c) C6B (148 μM) and CAII (123 μM); (d) C7B alone (186 μM); (e) C7B (136 μM) and CAI (100 μM); (f) C7B (132 μM) and CAII (105 μM); (g) C7B alone (121 μM); (h) C8B (189 μM) and CAI (141 μM); (i) C8B (189 μM) and CAII (153 μM). Data from Chambers *et al.*⁷⁰

Biosensor binding to CA was characterized by ^{129}Xe NMR spectroscopy and isothermal titration calorimetry (ITC).⁷⁰ ITC measurements of biosensor binding to CAI and CAII gave dissociation constants in the 10-100 nM range for all three biosensors. Interestingly, the dissociation constants for the full-length biosensors were comparable to those of the biosensors lacking the cryptophane. All three biosensors showed distinct and reproducible ^{129}Xe NMR chemical shifts upon binding CAI and CAII, respectively (Table 1.4). The hp ^{129}Xe NMR spectrum of racemic biosensors bound to CAI and CAII is shown as in Figure 1. 20. The unique ^{129}Xe NMR chemical shifts for biosensors bound to CAI or CAII demonstrate the potential of xenon biosensors to discriminate between isoforms of α -CA. CAI and CAII are structurally homologous, thus it is expected that xenon tethered in the active-site channel experiences similar steric environments. However, the

distribution of hydrophobic and hydrophilic residues lining the active-site channels varies between CAI and CAII; the large electron cloud surrounding the encapsulated xenon nucleus is sensitive to these differences, and thus the two isoforms are distinguishable by hp ^{129}Xe NMR spectroscopy. It is expected that CAIX and CAXII will also report characteristic ^{129}Xe chemical shifts upon binding xenon biosensor, thereby allowing for the selective detection of these cancer biomarkers by MRS/MRI. Interestingly, racemic **C7B** bound to wild-type CAII reports two ^{129}Xe NMR chemical shifts (Figure 1. 20). **C8B** also reports two chemical shifts when bound to CAI and CAII, respectively. It was initially hypothesized that the two observed ^{129}Xe chemical shifts were the result of single-site diastereomerism, with the (+) and (−) enantiomers of **C7B** binding to the same site but interacting slightly differently with residues in the chiral active-site channel of CAII.⁸⁶ However, the ^{129}Xe NMR spectra of enantiopure (+) and (−) **C7B** both report multiple “bound” chemical shifts for xenon.⁸⁶ This highlights the potential for multi-site binding, with the two enantiomers occupying distinct sites in the active site cleft. The cause of observed multiple “bound” cryptophane peaks is discussed further in Chapter 2.

Table 1.4. ^{129}Xe NMR chemical shifts of **C6B**, **C7B**, and **C8B** in solution, bound to CAI or CAII

Biosensor	CAI (ppm)		CAI Δ (ppm)	CAII (ppm)		CAII Δ (ppm)
	Unbound	Bound		Unbound	Bound	
C6B	63.7	69.2	5.6	63.1	68.2	5.1
C7B	63.7	66.9	3.2	63.7	71.2, 67.0	7.5, 3.3
C8B	63.3	67.9, 66.3	4.5, 3.0	63.2	68.2, 66.9	5.0, 3.7

The crystal structure of **C8B** bound to wild-type CAII was determined at 1.70 Å resolution by Aaron *et al.* in 2008 and is shown in Figure 1. 21.⁸⁷ The occupancy of **C8B**

was refined to 0.5 (with the M₀M₀ and P₀P₀ enantiomers having occupancies of 0.25, respectively). An encapsulated xenon atom was confirmed by a Bijvoet difference Fourier map calculated from anomalous scattering data and refined with occupancy of 0.5. The overall fold of CAII is largely unperturbed by binding of **C8B**, and only a few conformational changes are observed along the active-site rim. Close examination of the interface between the biosensor and protein revealed only a few cryptophane-protein interactions, suggesting that the cryptophane experiences a high degree of translational and rotational freedom. Such biosensor mobility is advantageous for ¹²⁹Xe NMR spectroscopy as it yields narrower line widths, thereby increasing the sensitivity of ¹²⁹Xe NMR measurements in solution.^{87, 88}

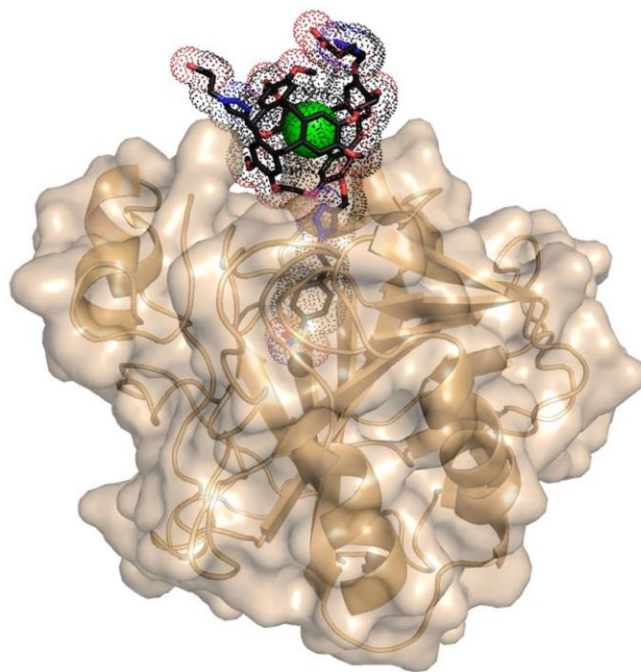


Figure 1. 21. X-ray crystal structure of xenon encapsulated in C8B (only M₀M₀ enantiomer is shown for clarity) bound to CAII (PDB: 3CYU). The Xe atom is shown in green; the active-site Zn²⁺ is grey; C8B is shown with carbon (black), oxygen (red), nitrogen (blue), and sulfur (green). Reproduced from Aaron *et al.*⁸⁹

Targeting Folate Receptor

A subsequent work sought to further characterize cell targeting and employed a different method of solubilization to ascertain the significance of types of solubilizing methods. In this study we used a mono-functionalized, PEGylated cryptophane with a leucine tethered folate recognition moiety (Figure 1. 17) synthesized in 20 nonlinear steps from four commercially available starting materials, depicted in Figure 1. 22a and b. The folate recognition moiety was synthesized in 5-steps as shown in Figure 1. 22a and then coupled to a PEGylated, azido peptide on solid support which was then “clicked” to mono-propargyl cryptophane, Figure 1.20b to yield the folate receptor-targeting biosensor. Here we again use a long chain to solubilize the compound but have the targeting portion in close proximity to the binding cage. In this work, two versions of the final compound were generated, the first, labeled “Folate receptor targeting cryptophane” in Figure 1. 22 is the compound proposed for use in NMR/MRI studies. A derivative compound for use in localization imaging studies was generated by TCEP deprotection of cysteine and conjugation with a maleimide Cy3 dye. Confocal microscopy studies demonstrated specific targeting of cells over-expressing folate receptor with the biosensor targeting the receptor and subsequently being taken up by an endocytotic mechanism, Figure 1. 23. Flow cytometry studies demonstrated a 10-fold specificity for FR overexpressing KB cells over HT-1080 cells, as shown in Figure 1. 24a while demonstrating low toxicity at concentrations relevant for ^{129}Xe NMR, Figure 1. 24b. The NMR again demonstrated two bound peaks, which exhibited unequal intensity. We attribute this to one of the

diastereomers of the poorly solvated cage maintaining a more collapsed conformation, thus reducing xenon affinity. The NMR of the folate-biosensor is shown in Figure 1. 25.

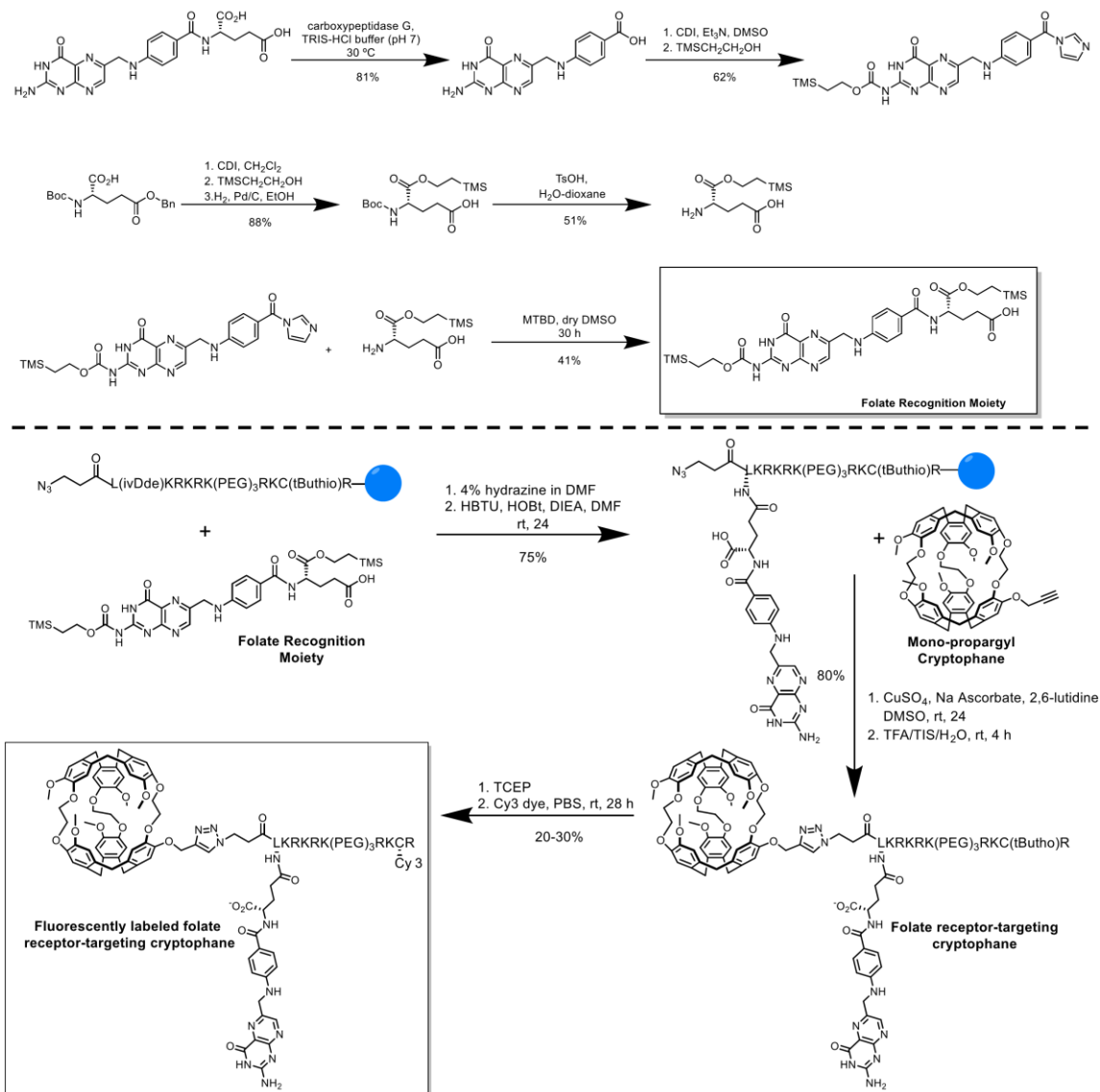


Figure 1. 22. Synthetic scheme showing the formation of the folate recognition moiety (top) and the fluorescently labeled folate receptor targeting cryptophane (bottom). The top scheme depicts the 5-step synthesis of [2-(trimethylsilyl)ethoxy]-2-*N*-[2-(trimethylsilyl)-ethoxycarbonyl]folic acid. The bottom scheme shows the synthesis of folate-targeting cryptophane and fluorescently labeled folate-targeting cryptophane, respectively. Monopropargyl cryptophane (synthesized in 12 steps with a 3% overall yield) was coupled to the folate conjugated azido-peptide on solid support via Cu(I) mediated [3+2] azide-alkyne cycloaddition and cleaved from solid support in 80% purified yield. Subsequent conjugation of the folate targeting cryptophane yielded the fluorescent derivative in 20-30% purified yield. Adapted with permission from Khan, Riggle *et al.*⁶⁹

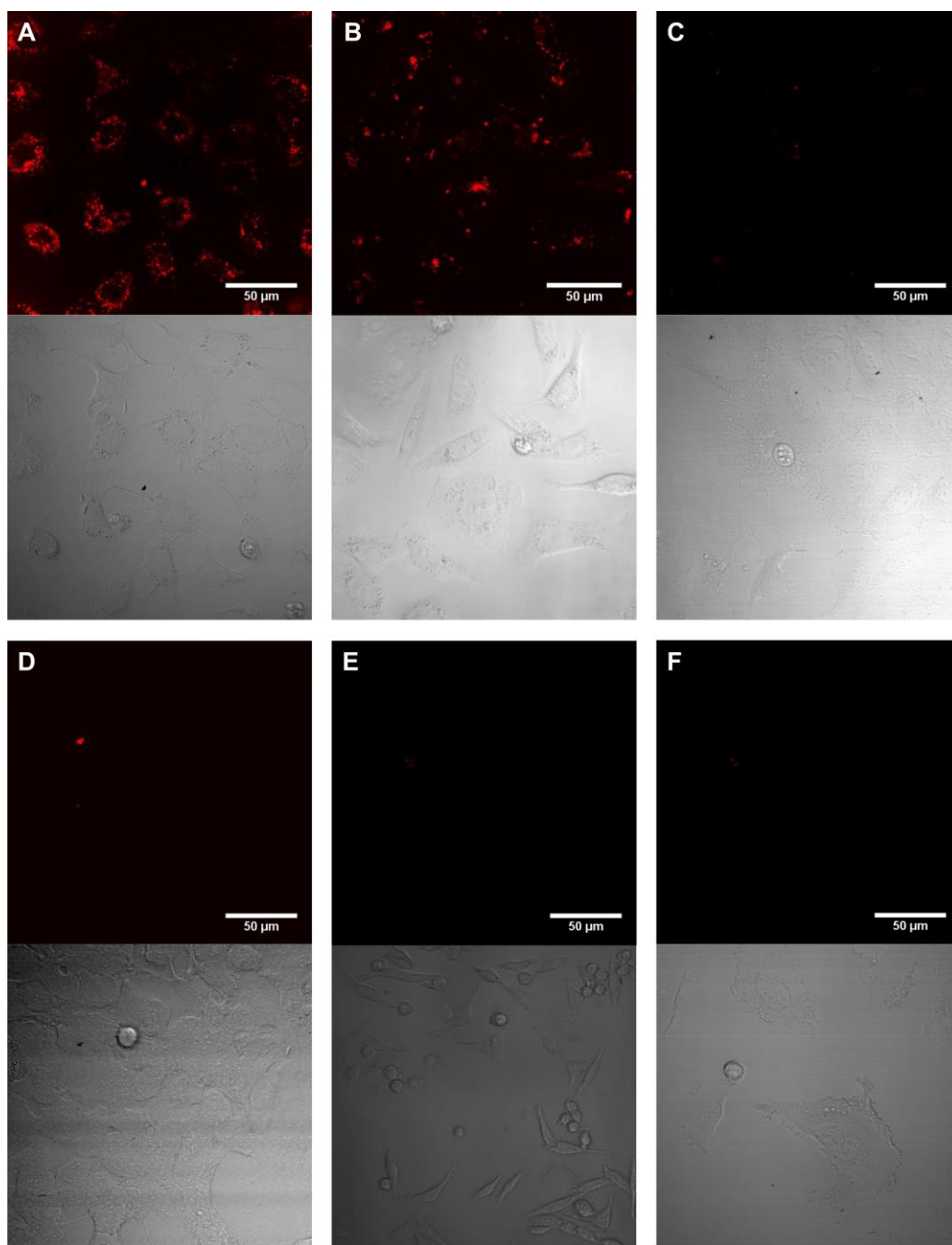


Figure 1. 23. Confocal micrographs and corresponding brightfield images of 4 μ M Cy3-labeled biosensor targeting FR α . Uptake was monitored in (a) KB; (b) HeLa; and (c) HT-1080 cells after 4 h incubation at 37 $^{\circ}$ C in folic acid depleted media. Uptake was blocked in (d) KB; (e) HeLa, and (f) HT-1080 cells pre-incubated in folic acid containing media. Adapted with permission from Khan, Riggle *et al.*⁶⁹

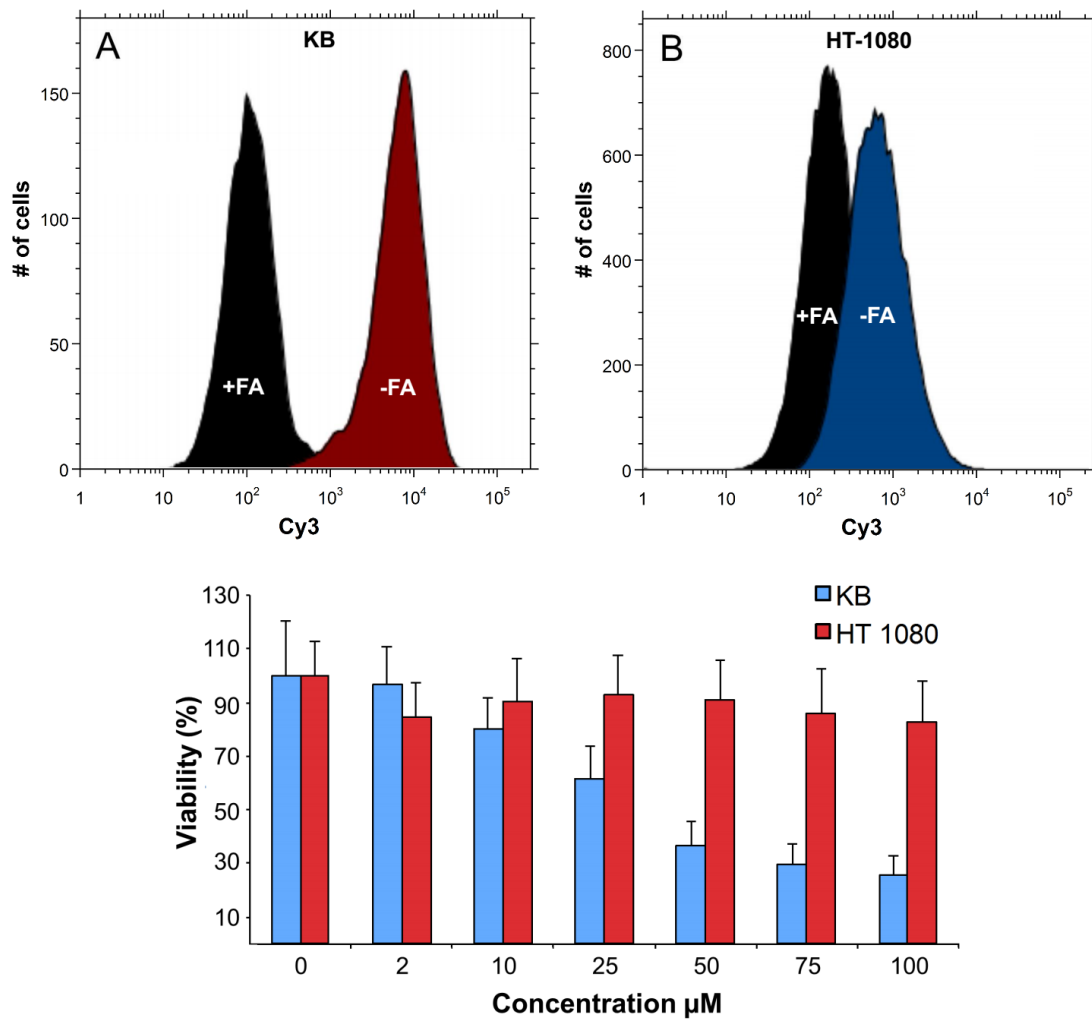


Figure 1. 24. Flow cytometry (top) and MTT viability assay (bottom). Flow cytometry quantified cell uptake of 4 μ M Cy3-labeled folate targeting cryptophane. Uptake in (a) KB (FR+, red) and (b) HT-1080 (FR-, blue) cells was compared to cells pre-incubated with folic acid (black). Cytotoxicity assays for folate-conjugated cryptophane (without dye) in KB (blue) and HT-1080 (red). Percent viability was determined via MTT assay after 24 h incubation with increasing concentrations of the biosensor. Adapted with permission from Khan, Riggle *et al.*⁶⁹

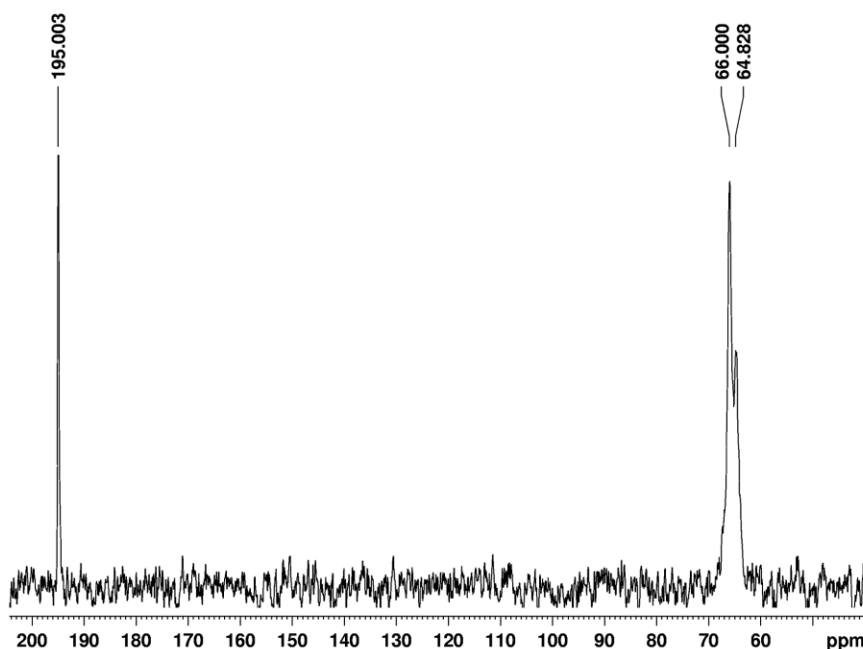


Figure 1. 25. Hyperpolarized ^{129}Xe NMR spectrum of folate receptor targeting cryptophane. Biosensor (60 μM) in acetate buffer at pH 5.0 (40 scans; S/N = 30:1 with 50 Hz line broadening). Adapted with permission from Khan, Riggle *et al.*⁶⁹

Paradigm Shift in Biosensor Design

In 2010, the Pines laboratory demonstrated that if they mixed diacid cryptophane-A with varying concentrations of Intralipid® they were able to achieve chemical shift changes (from that of the diacid cryptophane-A in buffer alone) approaching 10 ppm.⁹⁰ This work suggested that substantially larger chemical shifts could be obtained when the targeting biosensor was bound in close proximity to the cellular membrane. Indeed, in recent work the Schröder lab metabolically labeled sialic acid with a bioorthogonal azide.⁹¹ Employing a cryptophane scaffold containing a strained alkyne they labeled cell membranes with cryptophane host molecules. The cages tethered to cells exhibited a strong downfield chemical shift of 10 ppm relative to the free biosensor. Thus moving forward

our lab has endeavored to decorate cell membranes with our targeting biosensors, as demonstrated in Chapter 3.

Advantages of Targeted Xenon Biosensors

As these examples of targeted biosensors demonstrate, this new version of targeted or “smart” contrast agents can be made specific for bioreceptors or biomarkers of interest. This is an important consideration in the accurate diagnosis of malignant tissue. For example, mammography identifies masses that necessitate biopsy to determine whether they are malignant or benign. With targeted molecular imaging receptors strongly indicative of an aggressive cancer, like HER-2, could be targeted and invasive procedures only performed after positive identification. Mammography and other non-specific techniques also result in over-diagnosis, or the identification of small cancers that would have otherwise been asymptomatic and non-life threatening over the patient’s natural life span.⁹² Identification of these cancers may prompt chemotherapeutic or radiation treatments which carry their own adverse effects.⁹² Following this example, in cases where early positive identification of breast cancer is made, early treatment strongly improves survival.⁹³ Thus there is a strong need for *reliable* early diagnosis of cancer such that treatment efficacy is high but unnecessary treatment exposure is mitigated.

§ 1.10 Ultrasensitive Detection: HP Chemical Exchange Saturation Transfer

The use of exogenously supplied hyperpolarized ^{129}Xe improves detection limits of cryptophane-MRI contrast agents to the low micromolar range. However, the requirements of molecular imaging have made our lab and others endeavor to increase detection

sensitivity even further, approaching picomolar or even femtomolar detection regimes. One such paradigm, first described by Ward *et al.*, exploited the exchanging populations for selective saturation and was so named chemical exchange saturation transfer (CEST).⁹⁴ These experiments employed barbituric acid and 5,6-dihydrouracil, both of which contain exchangeable protons. A tuned radio frequency (rf) pulse was applied selectively to saturate the signal from the barbituric acid. As a result of the amide protons exchanging more rapidly than the time scale of spin relaxation with the bulk solvent (H_2O), the water signal was also depleted to a degree directly proportional to the concentration of the barbituric acid this is shown in **Error! Reference source not found.** Thus, these results demonstrated an indirect detection method for achieving increased detection sensitivity.

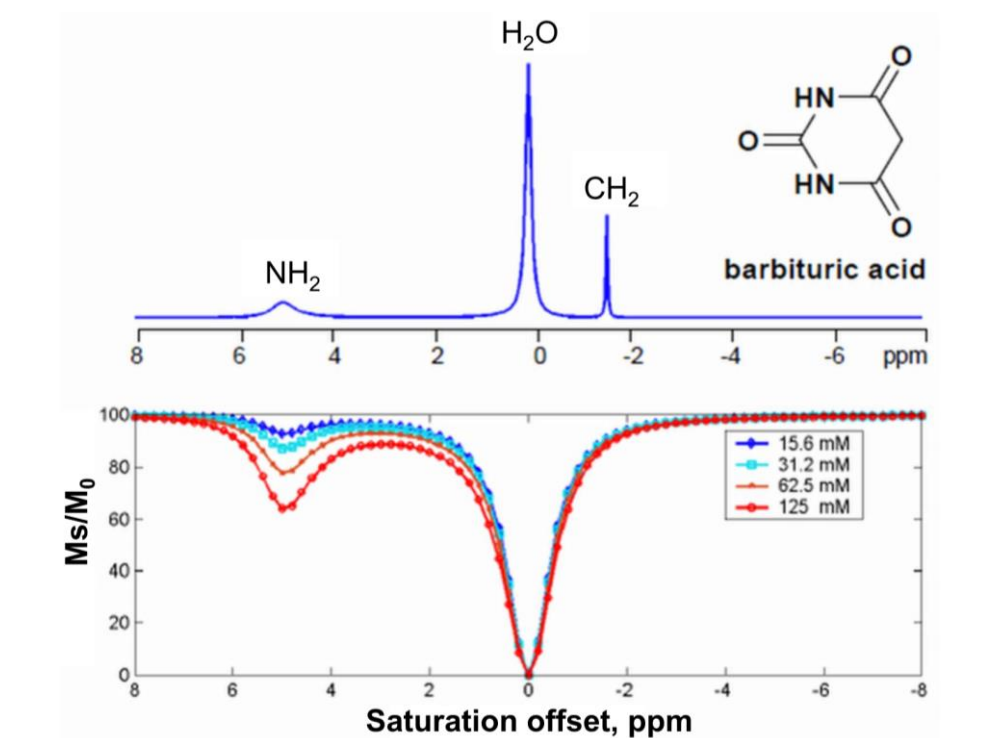


Figure 1. 26. Preliminary chemical exchange saturation transfer (CEST) experiment with barbituric acid. Direct detection ^1H NMR shown (top) and CEST spectrum (bottom) demonstrating concentration-dependent quenching. Adapted with permission from Bai *et al.*³⁸

The utility of this technique for hyperpolarized xenon NMR was first demonstrated by the Pines laboratory in 2006.⁹⁵ In this application the detection technique is termed hyper-CEST to account for the presence of hyperpolarized nuclei. In these experiments, the Pines group tethered biotinylated cryptophane to agarose beads and collected ^{129}Xe NMR spectra (**Error! Reference source not found.b**) and transverse ^1H NMR images (**Error! Reference source not found.a**) overlaid with transverse ^{129}Xe images (Figure 1. 27c). They demonstrated > 3300-fold reduction in image acquisition time as well as sensitivity $\sim 10,000$ times better than previous CEST methods. While these hyper-CEST experiments were performed at $\sim 5\ \mu\text{M}$, they postulate that several straight-forward optimizations could decrease the detection limit by at least 60-fold; namely, the use of isotopically enriched xenon and optimized polarization procedures. Thus hyper-CEST is a promising technique for drastically increasing sensitivity of ^{129}Xe NMR.

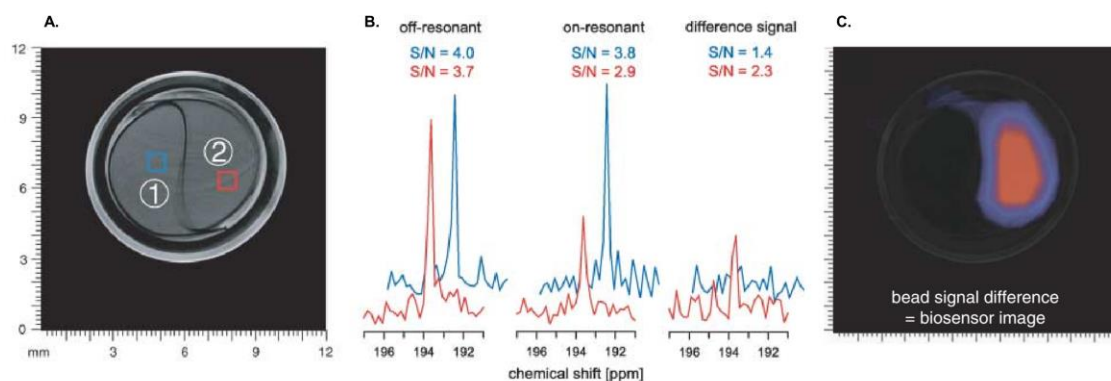


Figure 1. 27. Initial hyper-CEST experiment using agarose beads labeled with biotinylated cryptophane. Adapted with permission from Klippel *et al.*⁹⁵

Moving forward, advances in our lab and elsewhere produced significant improvements in detection sensitivity. Using TAAC (Figure 1. 16d), we demonstrated 1.4 pM detection limits in buffered solutions, shown in Figure 1. 28.⁹⁶

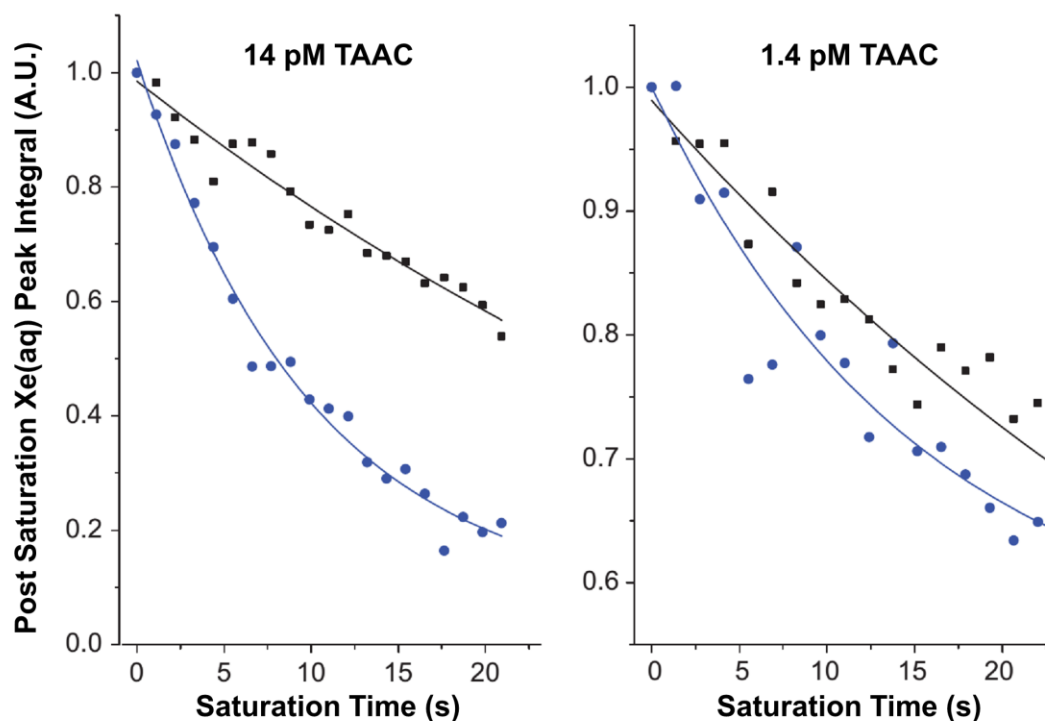


Figure 1. 28. Hyper-CEST experiments demonstrating 1.4 pM detection sensitivity of TAAC. Adapted from Bai *et al.*³⁸

§ 1.11 Multiplexing Applications*

Because of the large (~ 300 ppm) ^{129}Xe NMR chemical shift window for ^{129}Xe bound to cryptophanes in water, Pines *et al.* postulated in 2001 that hp ^{129}Xe NMR should be amenable to multiplexed detection using a cocktail of cryptophane biosensors.⁶⁶ One should ideally obtain only one “bound” peak for each biosensor, and these should be well resolved from each other (ideally > 5 ppm away) as well as from the “free” biosensor peaks.

This general idea is described in Figure 1. 29 where, in an MRI, signal from multiple targeted cryptophanes would be acquired simultaneously with proton anatomical reference images. The signal from multiple targets will help to negate false positives in tumor tissue identification. Already, large chemical shift changes ($\Delta\delta = 7.5$ ppm) upon target complexation have been achieved in our laboratory with carbonic anhydrase targeting cryptophanes as well as the 10 ppm chemical shift obtained from the metabolically incorporated cryptophane discussed in the previous section.^{70, 91} However, additional work is needed to probe how the nature of the xenon biosensor-protein interaction, diastereomerism, and cryptophane solubility affect the ^{129}Xe NMR spectrum. Recently, work in the Schröder laboratory demonstrated the first practical example of multiplexing with xenon host molecules where they mixed cryptophane and perfluorooctyl bromide (PFOB) nanodroplets and were able to label mammalian cells and demonstrate “two-color” contrast MRI shown in Figure 1. 30.⁹⁷

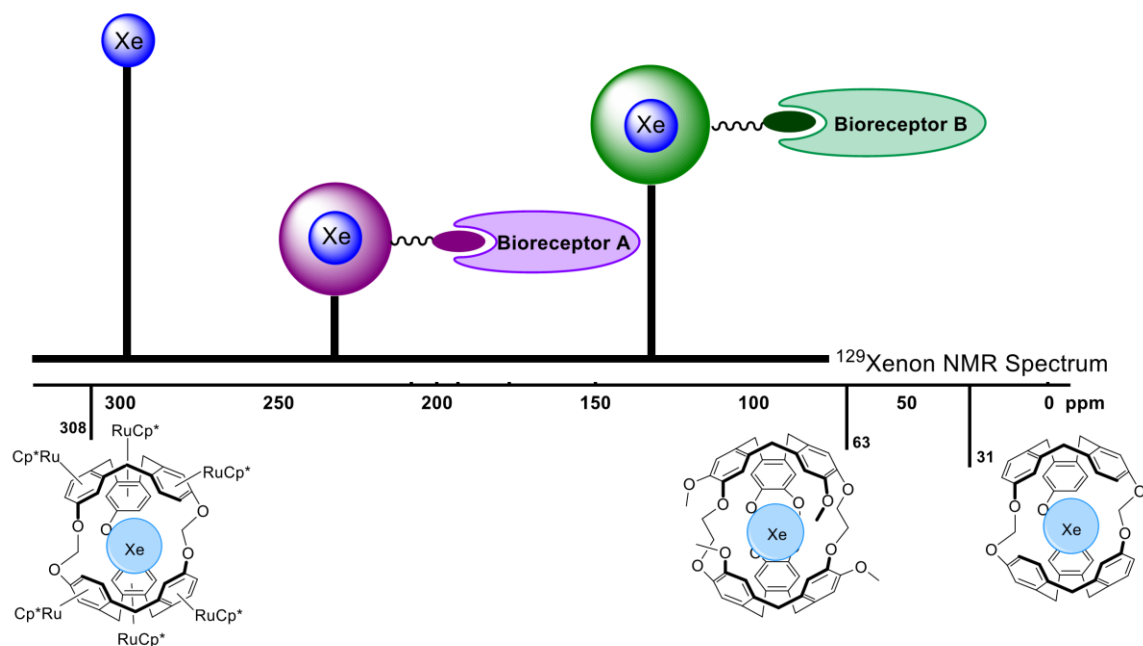


Figure 1. 29. Exploiting ^{129}Xe chemical environment sensitivity for multiplexing applications with cryptophane host molecules.

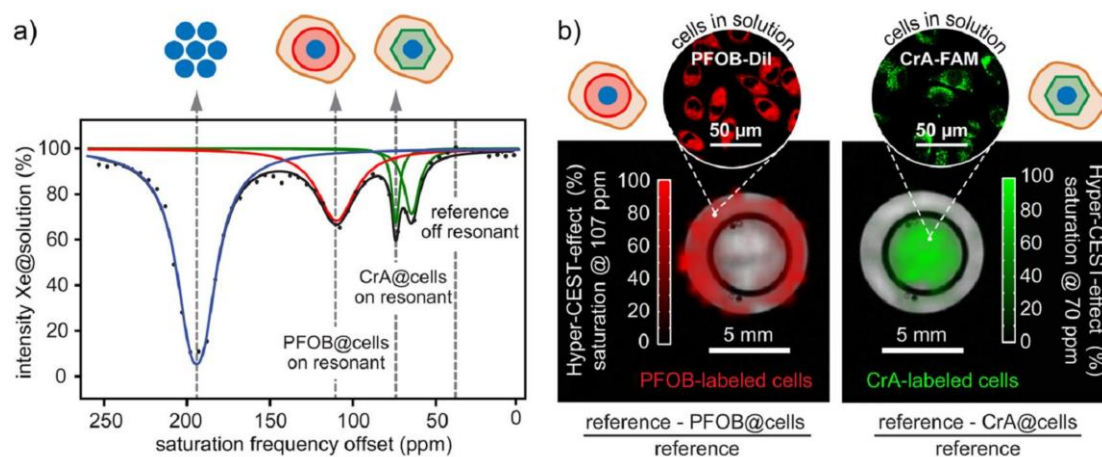


Figure 1. 30. First multiplexed detection experiment with cryptophane. Demonstrated the ability to readily discern between PFOB- and cryptophane-labeled cells. Adapted with permission from Klippel *et al.*⁹⁷

§ 1.12 Conspectus

This dissertation describes the advances in the development of disease-targeted cryptophane biosensors for the use in ^{129}Xe MRI/MRS. These biosensors couple targeted molecular imaging with the use of an exogenously supplied nucleus, ^{129}Xe . Cryptophane cages can be readily functionalized via “click chemistry” to target a wide variety of overexpressed biomarkers indicated in cancer progression. Our work has endeavored to develop synthetic strategies to generate biosensors with the highest degree of specificity and largest xenon association constants possible. We have used the biosensors we have generated to investigate the biological applicability and utility of targeted xenon biosensors for use as contrast agents in NMR. The results of some of these studies are discussed in the following chapters.

Chapter 2: Benzenesulfonamide Biosensor Binding Mechanisms in Carbonic Anhydrase

Section 3 of this chapter was originally published in *Organic Letters*. It has been adapted here with permission from the publisher:

Reprinted with permission from O. Taratula; M.P. Kim; Y. Bai; J.P. Philbin; B.A. Riggle; D.N. Haase; and I.J. Dmochowski. Synthesis of Enantiopure, Trisubstituted Cryptophane-A Derivatives. *Org Lett.* **2012**, *14*, (14) 3580-3583. Copyright 2012 American Chemical Society.

§ 2.1 Introduction

Ubiquitous zinc metalloenzymes, carbonic anhydrase isozymes are found in a majority of living organisms because they serve to catalyze the reversible hydration of carbon dioxide to bicarbonate and proton. The medical community has a special interest in carbonic anhydrase, however, because some of the 15 human isozymes are upregulated in specific disease states and are believed to aid tumor metastasis in certain forms of cancer, see Table 2.1.⁸⁰ Because of the high degree of homology in active-site conformation between the different isozymes of interest, the development of inhibitors and targeting ligands with isozyme specificity has been an area of considerable research. Our initial results with a carbonic anhydrase-targeting cryptophane demonstrated promising “bound” upfield chemical shifts of up to 7.2 ppm (see Chapter 1, Section 9) and a degree of isozyme discrimination between CAI and CAII. These data were convoluted, however, by the presence of multiple bound peaks. Thus a portion of my thesis work and ongoing studies in the laboratory have been devoted to elucidating the precise cause of the chemical shift variation in order to inform a variety of rationally designed biosensors specific to different isozymes of interest in the targeted molecular imaging of carbonic anhydrase. Additionally, this work may prove to further refine design specifications for a large variety of Xe-cryptophane protein sensors.^{49, 67}

Initial hypotheses for the cause of multiple bound peaks centered on the chiral nature of the cryptophane host molecule as shown in Figure 1. 12. Due to the chiral nature of cryptophane-2,2,2 once the biosensor binds to the protein active site, diastereomers are formed. Xenon, highly sensitive to its local environment, has been shown to be sensitive

to a diastereomeric host molecule.^{49, 67} Thus we first endeavored to develop an enantiopure synthesis of trifunctionalized cryptophane-A derivatives because although other enantiopure syntheses have been published these routes were not to the trifunctionalized cryptophanes, which exhibit superior xenon binding as a result of improved cage solubility and decreased water encapsulation.⁵⁶⁻⁵⁸ Thus Section 2.2 describes several routes we have developed to trihydroxy cryptophane which can then be, as described in Section 2.3, isolated into the corresponding (+) and (–) enantiomers and propargylated for further conjugation to form the desired carbonic anhydrase biosensor. [It should be noted that this route is not limited to producing CA biosensors, rather, it results in enantiopure tripropargyl cryptophane which is the starting material for all of our currently used cryptophane biosensors.]

Table 2.1. Human carbonic anhydrase isoforms as disease biomarkers

Isozyme	Organ/Tissue Distribution	Disease	Sulfonamide affinity
CA I	erhthrocytes, gastrointestinal tract, eye	retinal/cerebral edema	medium
CA II	erhthrocytes, gastrointestinal tract, kidney eye, bone osteoclasts, lung, testies, brain	glacoma, epilepsy edema, altitude sickness	very high
CA III	skeletal muscle, adipocytes	oxidative stress	very low
CA IV	kidney, lung pancreas, brain capillaries, colon, heart muscle, eye	glacoma, stroke, retintis pigmentosa	high
CA VA	liver	obesity	high
CA VB	heart and skeletal muscle, pancrease, kidney, spinal coard, gastrointestinal tract	obesity	high
CA VI	salivary and mam	cariogenesis	very high
CA VII	central nervous system	epilepsy	very high
CA VIII	central nervous system	neurodegeneration, cancer	--
CA IX	tumors, gastrointestinal mucosa	cancer	high
CA XII	kidney, intestine, reproductive epithelia, eye, tumors	cancer, glacoma	very high
CA XIII	kidney, brain, lung, gut, reproductive tract	sterility	high
CA XIV	kidney, brain, liver, eye	epilepsy, retinopathy	high

Data from Alterio *et al.*

§ 2.2 Synthesis of Trihydroxy Cryptophane[§]

In order to achieve trifunctionalized, enantiopure cryptophane we use trihydroxy cryptophane which can be achieved via three more efficient routes than from tripropargyl cryptophane.

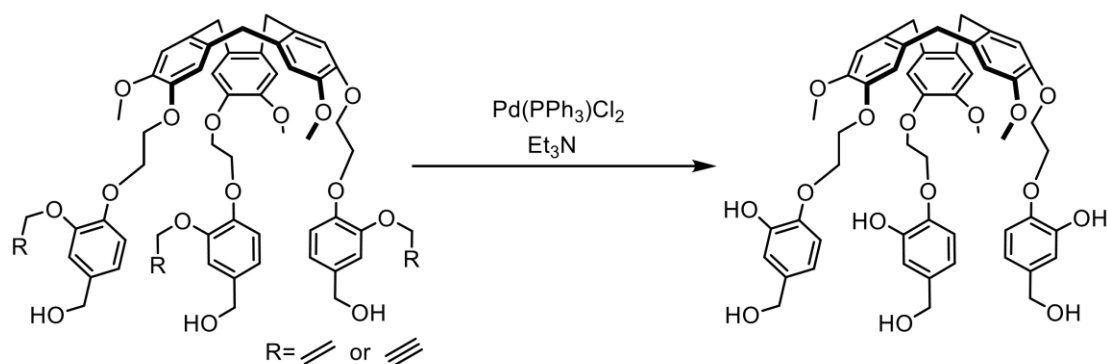


Figure 2. 1. Depropargylation of TPC or TAC precursor to form THC precursor.

Route 1:

Tripropargyl cryptophane precursor, achieved in 5 steps, (2,7,12-Tris-[2-[4-(hydroxymethyl)-2-propargyloxy-phenoxy]ethoxy]-3,8,13-trimethoxy-10,15-dihydro-2H-tribenzo[a,d,g]cyclononene) was depropargylated to form trihydroxy precursor (6,6',6''-(((3,8,13-Trimethoxy-10,15-dihydro-5H-tribenzo[a,d,g][9]annulene-2,7,12-triyl)tris-(oxy))tris(ethane-2,1-diyl))-tris(oxy))tris(3-(hydroxymethyl)phenol)), Figure 2. 1. An oven dried round bottom flask was changed with TPC Precursor (1.0 eq.), and Pd(PPh₃)₂Cl (0.10 eq.) and was dissolved in triethylamine (25 mL), tetrahydrofuran (25 mL), and water

[§] This section highlights the work of Taratula *et al.*⁵⁴

(12 mL) and stirred at 80 °C under inert atmosphere overnight. The work up entailed ethyl acetate extraction (3x) and the compiled organics were then washed with brine and dried over sodium sulfate and concentrated to a white powder under reduced pressure. The crude material was then purified by column chromatography ($\text{CH}_2\text{Cl}_2 \rightarrow \text{MeOH}:\text{CH}_2\text{Cl}_2$ 6:94, v/v) to yield 0.495 g (0.546 mmol, yield: 70% as a white solid). TLC (silica gel, 5% MeOH/ CH_2Cl_2): $R_f(7) = 0.16$. mp: 89-91 °C. ^1H NMR ($\text{DMSO}-d_6$) $\delta(\text{ppm})$: 8.88 (s, 3H), 7.16 (s, 3H), 7.09 (s, 3H), 6.87 (d, $J = 8.2$ Hz, 3H), 6.78 (s, 3H), 6.65 (m, 3H), 4.95 (t, $J = 5.8$ Hz, 3H), 4.70 (d, $J = 13.3$ Hz, 3H), 4.33 (m, 6H), 4.18-4.29 (m, 12H), 3.68 (s, 9H), 3.52 (d, $J = 13.3$ Hz, 3H). ^{13}C NMR ($\text{DMSO}-d_6$) $\delta(\text{ppm})$: 147.6, 146.8, 146.3, 145.3, 135.9, 132.6, 132.0, 117.1, 115.3, 114.4, 114.3, 114.0, 67.7, 67.5, 62.6, 55.9, 35.0. HRMS (m/z): $[\text{M}+\text{Na}]^+$ calculated for $\text{C}_{51}\text{H}_{54}\text{O}_{15}$, 929.3360; found, 929.3405. This method was very low yielding in my hands and difficult to purify as a result of the dark brown precipitate formed (from the catalyst) during the reaction. With the help of a talented undergrad, Mara Greenburg, it was determined that the reaction worked in an ~50% yield on an 80 mg scale, see Appendix B for ^1H NMR.

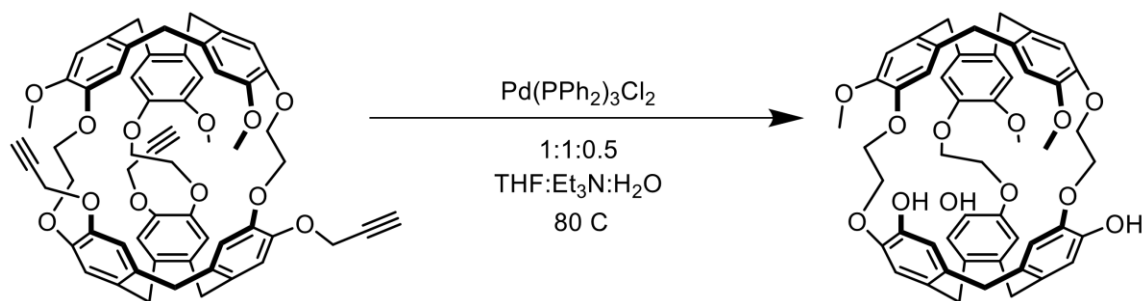


Figure 2. 2. Reduction of TPC to form THC precursor.

Route 2:

From tripropargyl cryptophane, achieved in 6 steps, trihydroxy precursor was synthesized. For 0.050 g TPC (0.05 mmol, 1.0 eq.) Pd(PPh₃)Cl₂ (0.1 eq), THF (5 mL), Et₃N (5 mL) and H₂O (2.5 mL) was used. The solution was heated to 80 °C and put to stirring under inert atmosphere overnight. The reaction was worked up by extracting 3x with ethyl acetate. The combined organics were washed with water and brine and dried over sodium sulfate. The resulting solvent mixture was concentrated under reduced pressure. Repeated trials with this method and then increasing the amount of catalyst up to 1 eq and/or substituting solvents for DMF/H₂O as well as allowing the reaction to run longer universally yielded only small amounts of product by TLC.

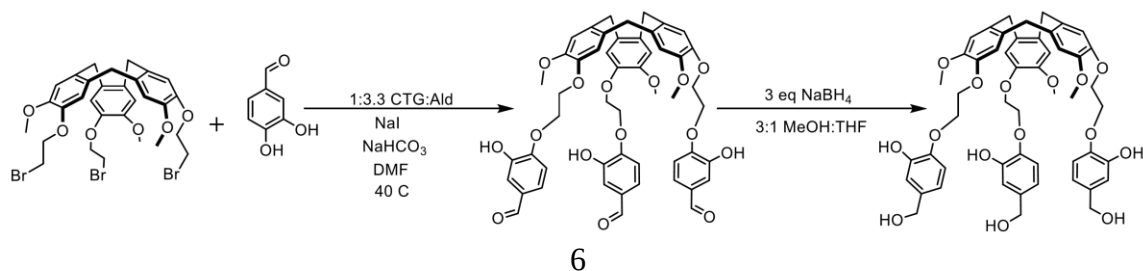


Figure 2. 3. Synthesis of THC aldehyde precursor and subsequent reduction to form THC precursor.

Route 3:

Trihydroxy aldehyde precursor is first achieved in 3 steps, as shown in Figure 2. 3. This route utilized regioselective protection of the 4-hydroxyl group in the 3,4-dihydroxybenzaldehyde as detailed by Plourde *et al* to couple the 3,4-dihydroxybenzaldehyde “hydroxyl linker” to the cap.⁹⁸ This method negates the difficult *meta* linker synthesis and purification and the redundancy of first protecting and then deprotecting the

meta alcohol. Relying on the increased acidity of the *para* proton for preferential reaction at this position. The reaction proceeded rapidly in respectable yield (crude, by TLC) purification and characterization is ongoing. Crude NMR shown in Appendix B. [This route not previously published]

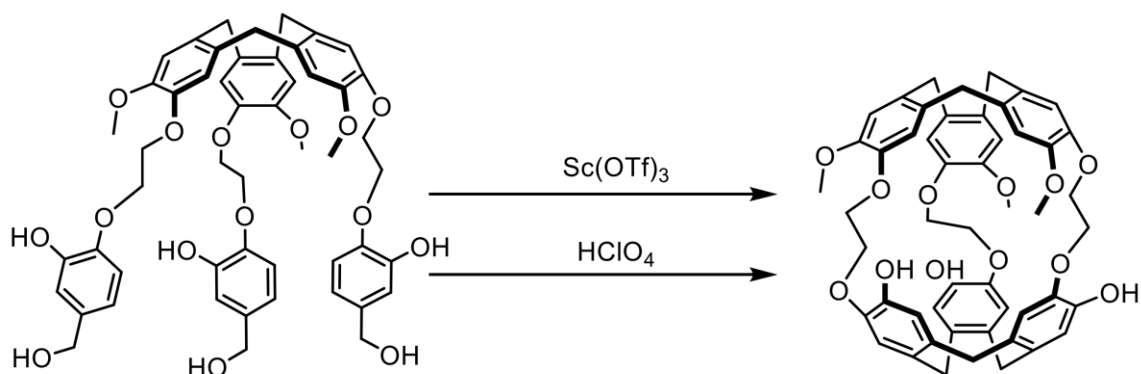


Figure 2. 4. Cyclization of THC precursor by two routes to form THC.

Cyclization to form trihydroxy cryptophane:

Trihydroxy aldehyde precursor (0.110 g, 0.121 mmol, 1.0 eq.) was dissolved in THF (10 mL) and methanol (250 mL) was added. Perchloric acid (60%, 250 mL) was then added dropwise into the cloudy solution at 0 °C. Scheme shown in Figure 2. 4. The reaction was allowed to warm to rt and stirred slowly for 24 h under N₂. The reaction mixture was diluted by CH₂Cl₂ and neutralized by 1 M NaOH solution at 0 °C. The reaction mixture was extracted with CH₂Cl₂. The combined organic extracts were concentrated and washed with NaHCO₃ solution and brine several times. The solution was filtered and dried over MgSO₄. After removal of the solvent under vacuum, the residue was chromatographed on a silica gel column (CH₂Cl₂:MeOH:CH₂Cl₂ 2:98, v/v) to yield 0.067 g (0.079 mmol, 65% yield) as a white powder. TLC (silica gel, 5% MeOH/CH₂Cl₂): R_{f(8)} = 0.33 mp >200 °C (decomp.).

^1H NMR (CDCl_3) $\delta(\text{ppm})$: 6.83 (s, 3H), 6.71 (s, 3H), 6.70 (s, 3H), 6.62 (s, 3H), 4.58 (dd, $J = 13.7$ Hz, 6H), 4.27-4.24 (m, 12H), 3.79 (s, 9H), 3.42 (d, $J = 13.8$ Hz, 3H), 3.36 (d, $J = 13.8$ Hz, 3H). ^{13}C NMR (CDCl_3) $\delta(\text{ppm})$: 149.9, 148.1, 145.7, 144.8, 134.3, 133.6, 131.7, 130.7, 122.1, 120.9, 115.7, 114.2, 70.1, 69.0, 53.5, 36.3, 36.2. HRMS (m/z): $[\text{M}+\text{Na}]^+$ calculated for $\text{C}_{51}\text{H}_{48}\text{O}_{12}$, 875.3043; found, 875.3062. The spectroscopic data matched those reported in the literature.⁵⁶ Although Dr. Taratula published two routes to trihydroxy cryptophane, in my hands only the perchloric acid route has been accessible.

§ 2.3 Synthesis of Enantiopure, Trisubstituted Cryptophane-A Derivatives

The need for imaging agents and analytical tools that can report on the concentration and activity of various biomolecules in complex media has motivated the development of ^{129}Xe NMR biosensors.^{53, 99} These agents have the potential to detect cancer and other diseases by localizing hyperpolarized (hp) ^{129}Xe to a diseased tissue and/or by multiplexed detection of different protein biomarkers.^{53, 99} To date, cryptophane-A organic cages, in which two cyclotriguaiacylene (CTG) units are connected by three ethylene oxide linkers, show the highest xenon binding affinity with dissociation constants of ~ 25 μM at physiological temperature in aqueous solution.^{53, 55, 99} Functionalized ^{129}Xe cryptophane biosensors can be targeted to different protein receptors and identified by changes to the frequency of the bound ^{129}Xe nucleus.^{66, 71, 72}

The use of enantiopure cryptophanes is preferred over racemic mixtures, which have been shown to produce multiple, diastereomeric peaks upon binding to chiral protein surfaces.^{49, 67, 70} Similarly, complex hp ^{129}Xe NMR spectra are observed when racemic cryptophanes are modified with chiral small molecules or peptides, based on

diastereomeric splitting.¹⁰⁰⁻¹⁰² For the sensitive detection of chiral biological analytes, enantiopure cryptophanes that offer well resolved “bound” and “free” ¹²⁹Xe NMR peaks should offer substantial advantages. Enantiopure cryptophanes have also been employed for chiral recognition of small guests.¹⁰³ Here, we report a new method for producing enantiopure cryptophanes for many different applications.

Results and Discussion

Until now, the resolution of chiral cryptophanes and hemicryptophanes has typically required expensive HPLC methods and yielded only small quantities of optically pure material.^{104, 105} Another approach has been the synthesis of enantiopure cryptophanes from the optically pure CTG units, but one limitation is possible racemization of CTG during the subsequent synthetic steps.¹⁰³ Recently, Dutasta and co-workers employed (–)-camphanic chloride as a chiral resolving agent to resolve monocryptophanol through separation of the resulting diastereomers.¹⁰⁶ The diastereomers were not separable by chromatography on silica gel or reversed-phase HPLC, but crystallographic resolution has recently been improved to give both enantiomers in 25% yield.¹⁰⁷ However, this crystallographic method is time-consuming. The low yield of pure cryptophane diastereomers limits the production of enantiomerically pure cages for uses in xenon biosensors and host guest chemistry, broadly defined.

Dutasta *et al.* previously demonstrated the chromatographic separation of trifunctionalized hemicryptophanes.¹⁰⁸ We hypothesized that a pair of cryptophane-A diastereomers substituted with three chiral auxiliaries would also result in a significant difference in polarity. Indeed, substitution with three chiral resolving groups allowed

efficient separation and isolation of cryptophane diastereomers using silica gel column chromatography. Deprotection of the isolated diastereomers yielded the enantiopure trisubstituted cryptophanes, whose chemical and physical properties can be tuned at the three positions.

According to Figure 2. 5a, diastereomers **2a** and **2b** were synthesized from trihydroxy cryptophane **1**, (as described in Section 2.2) which was obtained by a previously published six-step route.⁵⁴ Trihydroxy cryptophane **1** was reacted with 3.3 equivalents of (S)-Mosher's acid in the presence of DMAP/Et₃N. The Mosher's acid moiety was chosen as a readily available and sterically bulky chiral resolving agent. The reaction proceeded relatively slowly and went to ~70% completion after stirring for two days at 70 °C in DMF. The resulting cryptophane-A diastereomers **2a** and **2b** were successfully separated by column chromatography (silica gel, Et₂O/ CH₂Cl₂, 0.5:99.5, v/v) to give each enantiomer in 35% yield. Resolved diastereomers **2a**-(S)-(-) and **2b**-(S)-(+) were easily distinguished by ¹H NMR spectroscopy (Figure 2. 5b), each showing four singlets with different chemical shift values for aromatic protons. In contrast, the aromatic region of the diastereomeric mixture exhibited eight singlets in the same region (Figure 2. 5b). The enantiopurity of the isolated diastereomers was confirmed by electronic circular dichroism (ECD) spectroscopy showing the same peaks with opposite signs (Figure 2. 6a).

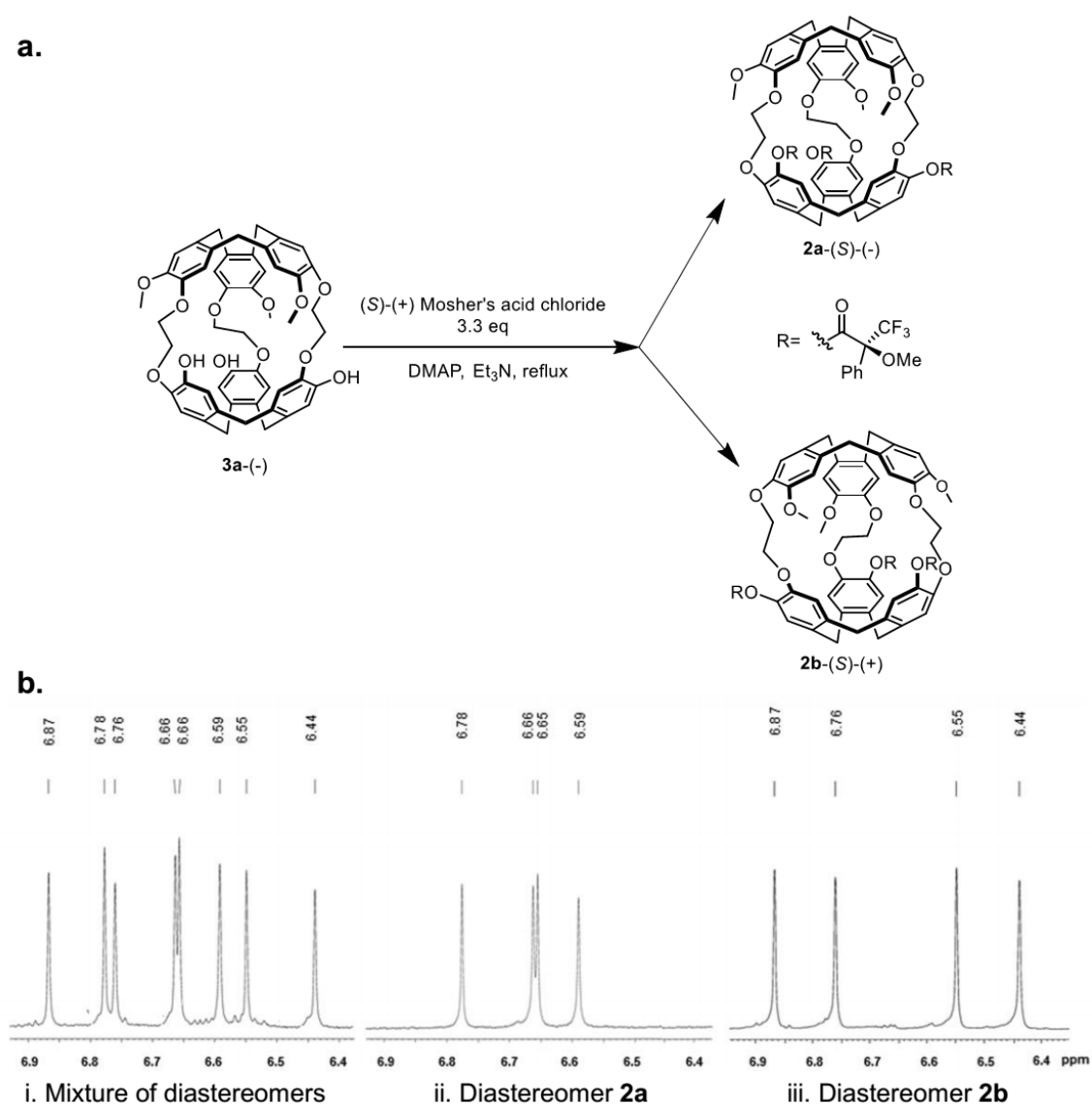


Figure 2. 5. Synthesis of trisubstituted cryptophane diastereomers of cryptophane from trihydroxy cryptophane (a) and aromatic region of ¹H NMR spectra for i. mixture of diastereomers; ii. **2a**; and iii. **2b**. Reprinted with permission from O. Taratula; M.P. Kim; Y. Bai; J.P. Philbin; B.A. Riggle; D.N. Haase; and I.J. Dmochowski. Synthesis of Enantiopure, Trisubstituted Cryptophane-A Derivatives. *Org Lett.* **2012**, *14*, (14) 3580-3583. Copyright 2012 American Chemical Society.

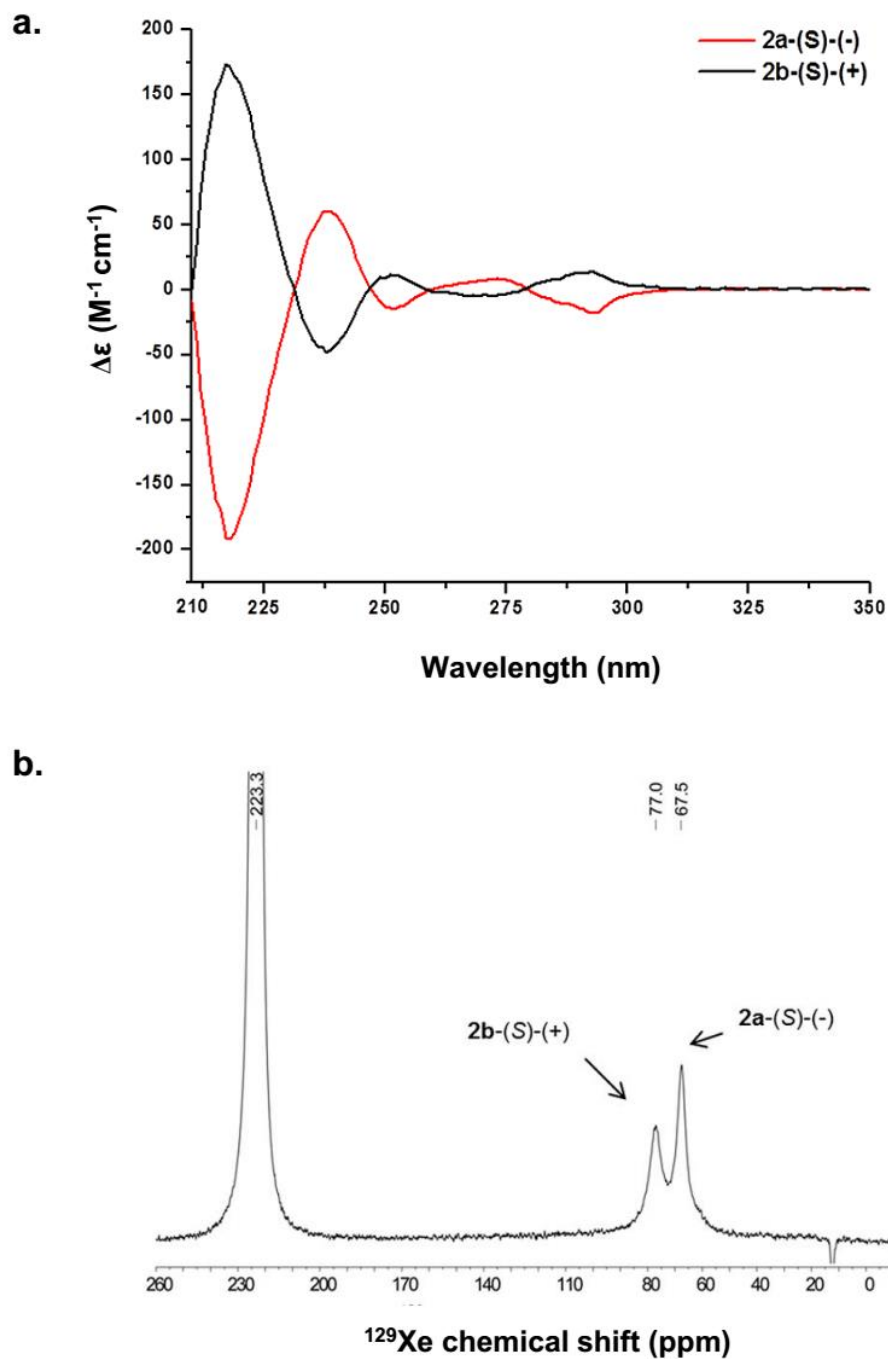


Figure 2. 6. ECD spectra of diastereomers **2a** and **2b** (~0.5 mM) in 1,4-dioxane (a) and ^{129}Xe NMR spectrum of diastereomers **2a** and **2b** ~10 mM in $\text{C}_2\text{D}_2\text{Cl}_4$ at 299 ± 2 K (b). Reprinted with permission from O. Taratula; M.P. Kim; Y. Bai; J.P. Philbin; B.A. Riggle; D.N. Haase; and I.J. Dmochowski. Synthesis of Enantiopure, Trisubstituted Cryptophane-A Derivatives. *Org Lett.* **2012**, 14, (14) 3580-3583. Copyright 2012 American Chemical Society.

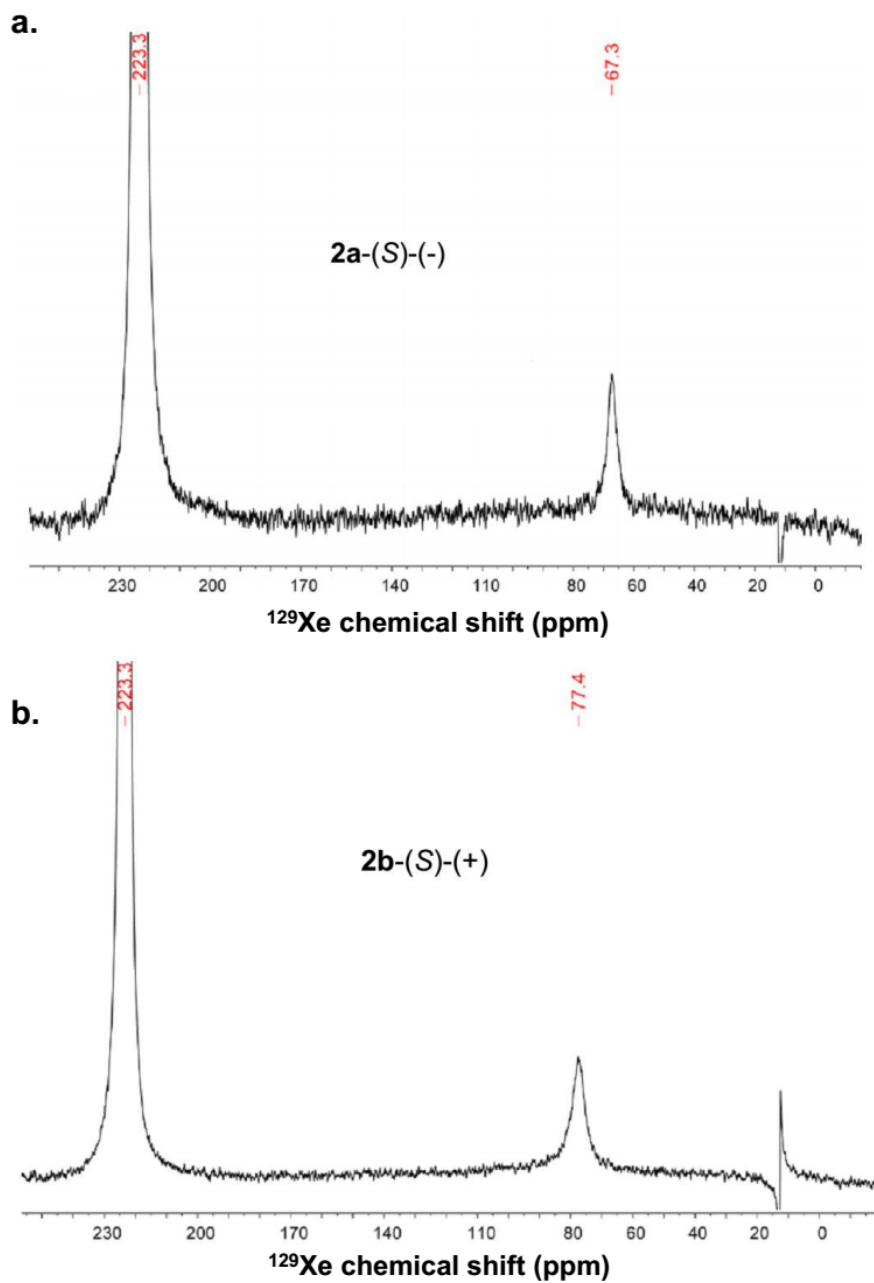


Figure 2. 7. ^{129}Xe NMR of a) **2a-(S)-(-)**; and b) **2a-(S)-(+)**. Reprinted with permission from O. Taratula; M.P. Kim; Y. Bai; J.P. Philbin; B.A. Riggle; D.N. Haase; and I.J. Dmochowski. Synthesis of Enantiopure, Trisubstituted Cryptophane-A Derivatives. *Org Lett.* **2012**, *14*, (14) 3580-3583. Copyright 2012 American Chemical Society.

The interaction between xenon and the trisubstituted cryptophane diastereomers **2a** and **2b** was investigated by hp ^{129}Xe NMR spectroscopy in a nonintercalating organic solvent, 1,1,2,2-tetrachloroethane- d_2 ($\text{C}_2\text{D}_2\text{Cl}_4$). Hyperpolarized ^{129}Xe was mixed with a sample solution in an airtight NMR tube, and spectra were taken quickly with four transients (Figure 2. 6b). Standardized by the signal from dissolved hp ^{129}Xe in $\text{C}_2\text{D}_2\text{Cl}_4$,⁴⁶ hp ^{129}Xe NMR chemical shifts for the mixture of diastereomers **2a**-(-) (67.5 ppm) and **2b**-(+) (77.0 ppm) in $\text{C}_2\text{D}_2\text{Cl}_4$ at 299 K were recorded 9.5 ppm apart (Figure 2. 6b), which is the largest chemical shift difference reported for cryptophane diastereomers. Peaks were assigned by collecting the hp ^{129}Xe NMR spectrum for both of the individual diastereomers (Figure 2. 7). Previously, for the mono-(-)-camphanic acid cryptophane diastereomers, a chemical shift difference of ~ 7 ppm was observed for the two diastereomers.¹⁰⁹ Notably, for the camphanic acid derivative, the more downfield peak arose from the cryptophane-(-) diastereomer, whereas with three Mosher acids it was the cryptophane-(+) diastereomer. With a 1:1 mixture of diastereomers **2a** and **2b**, the two resonances are clearly resolvable (Figure 2. 6b) by hp ^{129}Xe NMR spectroscopy. The isolated cryptophane diastereomers are useful precursors for preparing various enantiopure functionalized cryptophanes. Removal of the Mosher moieties occurs without loss of optical activity. Diastereomers **2a** and **2b** were deprotected via basic hydrolysis at 70 °C, affording enantiopure trihydroxy cryptophanes **3a**-(-) and **3b**-(+) (Figure 2. 8). The recorded ECD spectra were mirror images (within experimental error) of each other, as expected for a pair of enantiomers (Figure 2. 9a). In the absence of an X-ray crystal structure for the isolated enantiomers, the structural assignment for the two

enantiomers was made by reacting cryptophane **3b**-(+) with methyl iodide to yield (+)-cryptophane-A, **Figure 2. 10**. The recorded ECD spectrum (Figure 2. 9c) was found to be opposite of the previously reported spectrum for (-)-cryptophane-A.¹¹⁰

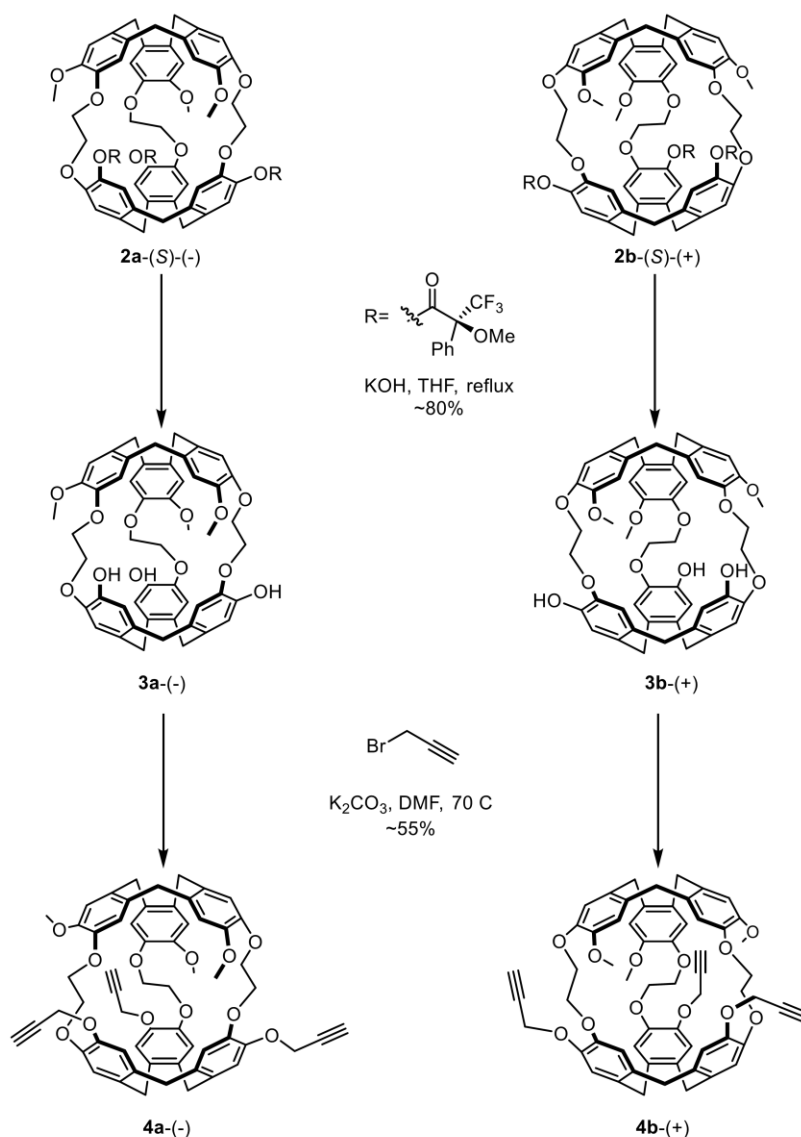


Figure 2. 8. Synthesis of enantiopure, trifunctionalized cryptophanes **3**-($-$), **3**-($+$), **4**-($-$), and **4**-($+$). Reprinted with permission from O. Taratula; M.P. Kim; Y. Bai; J.P. Philbin; B.A. Riggle; D.N. Haase; and I.J. Dmochowski. Synthesis of Enantiopure, Trisubstituted Cryptophane-A Derivatives. *Org Lett.* **2012**, 14, (14) 3580-3583. Copyright 2012 American Chemical Society.

Similarly to (+)-cryptophane-A, various trisubstituted enantiopure cryptophane derivatives could be easily synthesized from trihydroxy cryptophane enantiomers **3a**(-) and **3b**(+). For example, reaction with excess propargyl bromide gave the enantiomerically pure tripropargyl cryptophanes **4a**(-) and **4b**(+) (Figure 2. 8; Figure 2. 9b).^{111, 112} We previously showed that alkyl azides can react with tripropargyl cryptophane in nearly quantitative yields via the Cu(I)-catalyzed Huisgen [3 + 2] cycloaddition reaction. This route gave enantiopure tripropargyl cryptophanes **4a**(-) and **4b**(+), each in 15% overall yield starting from racemic trihydroxy cryptophane **1**(±).

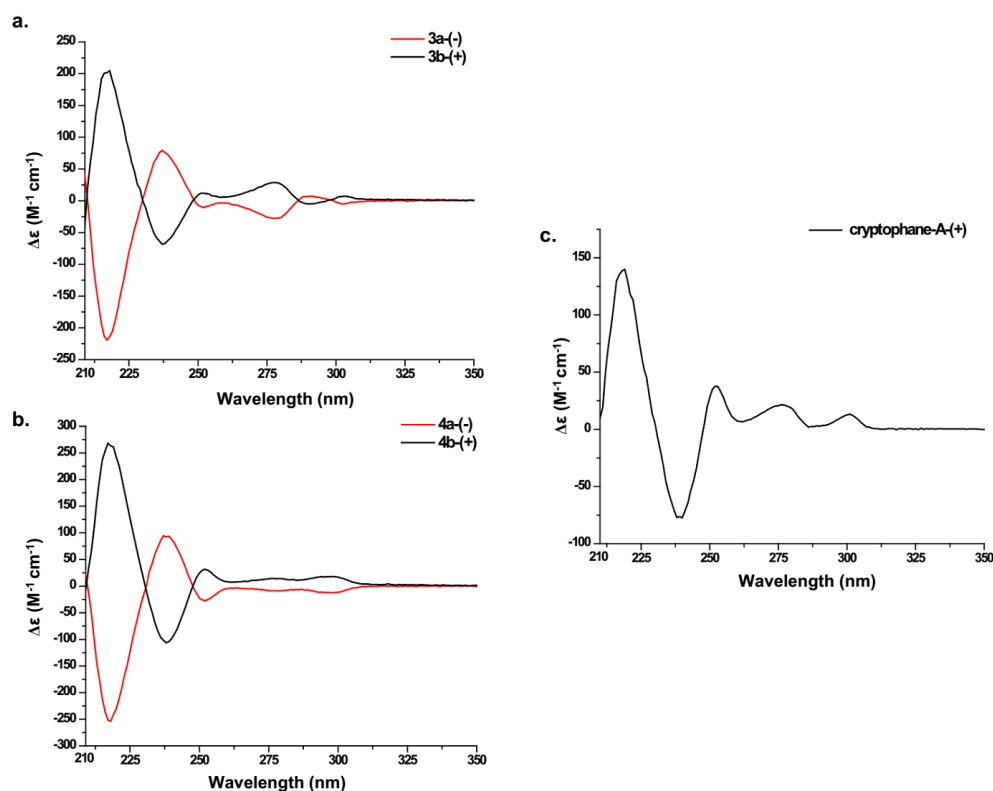


Figure 2. 9. Electronic circular dichroism for a) **3a**(-) and **3a**(+); b) **4a**(-) and **4a**(+); c) (+)-cryptophane-A. Reprinted with permission from O. Taratula; M.P. Kim; Y. Bai; J.P. Philbin; B.A. Riggle; D.N. Haase; and I.J. Dmochowski. Synthesis of Enantiopure, Trisubstituted Cryptophane-A Derivatives. *Org Lett.* **2012**, 14, (14) 3580-3583. Copyright 2012 American Chemical Society.

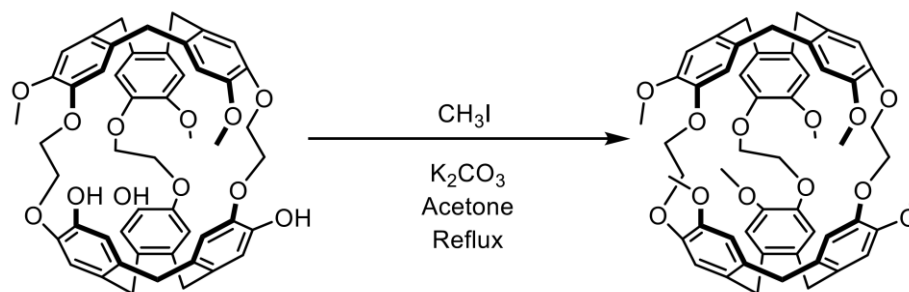


Figure 2. 10. Synthesis of Cryptophane-A (+)

In conclusion, an efficient synthesis of enantiopure trifunctionalized cryptophanes was developed using chromatographically resolved trisubstituted cryptophane diastereomers. ECD spectroscopy confirmed the expected chiroptical properties of the isolated diastereomeric and enantiomeric pairs. Hyperpolarized ^{129}Xe NMR chemical shifts were recorded at 9.5 ppm apart for the cryptophane diastereomers. The potential for synthesizing gram-scale quantities of enantiomerically pure cryptophane would provide access to the various functionalized cryptophanes, precursors for many cryptophane-based enantiopure biosensors. Particularly, enantiopure Xe biosensors are desired to facilitate high-resolution X-ray crystallographic studies^{53, 65, 70, 71, 113} and to simplify the assignment of peaks in ^{129}Xe NMR spectra. These methods are currently being applied to synthesizing enantiopure **C8B**.

Materials and Methods

General Methods. ^1H NMR (360 and 500 MHz) and ^{13}C NMR (90 and 125 MHz) spectra were obtained on Bruker DMX 360 and AMX 500 spectrometers at the University of Pennsylvania NMR facility and were recorded at room temperature in deuterated chloroform (CDCl_3) or dimethyl sulfoxide ($\text{DMSO}-d_6$) unless otherwise noted. The ^1H and

^{13}C NMR spectra were referenced to the central line of the residual solvent. ^1H NMR and ^{13}C NMR chemical shifts (δ) are given in parts per million (ppm) and reported to a precision of ± 0.01 and ± 0.1 ppm, respectively. Proton coupling constants (J) are given in Hz and reported to a precision of ± 0.1 Hz. Column chromatography was performed using silica gel (60 Å pore size, 40-75 μm particle size) from Sorbent Technologies. Thin layer chromatography (TLC) was performed using silica gel plates (60 Å pore size, Whatman) with UV light at 254 nm as the detection method. High resolution mass spectrometry (HRMS) data were obtained using electrospray ionization (ESI) mass spectrometry on a Micromass Autospec at the Mass Spectrometry Center in the Chemistry Department at the University of Pennsylvania. Electronic circular dichroism (ECD) spectra were recorded at room temperature on a ChirascanTM Circular Dichroism Spectrometer using cells with a pathlength of 0.1 cm. UV-visible spectra were measured using a diode-array Agilent 89090A spectrophotometer.

Hyperpolarized ^{129}Xe NMR spectroscopy. We utilized an in-house ^{129}Xe hyperpolarizer based on the IGI.Xe.2000 system made by the former Nycomed-Amersham (now GE). Hyperpolarized gas supply (Concorde Gases) was a mixture of 89% N_2 , 10% He, and 1% natural abundance Xe (29.4% ^{129}Xe). ^{129}Xe nuclei were hyperpolarized to 10-15% after being cryogenically separated, accumulated, thawed, and collected in degassed airtight NMR tubes (CAV5, New Era).⁷⁰ This traps ~ 2 atm of hyperpolarized Xe in the tube. After hyperpolarized Xe was retrieved, NMR tubes were shaken vigorously to mix Xe with cryptophane solutions. All ^{129}Xe NMR measurements were made on a 500 MHz Bruker BioDRX NMR spectrometer at the University of Pennsylvania NMR Facility. RF pulse

frequency for ^{129}Xe was 138.12 MHz. Samples were observed using a Bruker 5 mm PABBO NMR probe. ^{129}Xe spectra were processed using standard protocols, and ^{129}Xe NMR spectral calibration was performed as previously reported for cryptophane solutions in $\text{C}_2\text{D}_2\text{Cl}_4$.⁵⁴ All air- and moisture-sensitive reactions were performed under inert atmosphere in glassware flamed under vacuum, and using anhydrous solvents. Standard workup procedures involved multiple (~3) extractions with the indicated organic solvent, followed by washing of the combined organic extracts with water or brine, drying over Na_2SO_4 and removal of solvents *in vacuo*. All yields are reported after purification by column chromatography or crystallization.

Materials. Organic reagents and solvents were used as purchased from the following commercial sources: *Sigma-Aldrich*: dimethyl sulfoxide (DMSO, anhydrous, 99.9%), (S)-(+)- α -methoxy- α -trifluoromethylphenylacetyl chloride (Mosher's acid chloride, 98%, Aldrich); *Fisher*: acetone (HPLC grade), sodium hydroxide, potassium hydroxide, hydrochloric acid, sodium sulfate (anhydrous), sodium chloride, sea sand (washed), potassium carbonate (K_2CO_3 , anhydrous), methyl iodide, toluene (HPLC grade), ethyl acetate (EtOAc, HPLC grade), hexanes (HPLC grade), chloroform (CHCl_3 , HPLC grade), dichloromethane (CH_2Cl_2 , HPLC grade), methyl alcohol (MeOH, HPLC grade), ethyl ether (Et_2O , anhydrous); *Acros Organics*: 4-dimethylaminopyridine (DMAP), *N,N*-dimethylformamide (DMF, anhydrous, 99.8%), sodium hydride (NaH, 60% dispersion in mineral oil), allyl bromide (99%), benzyl bromide (98%), propargyl bromide (80% solution in toluene), 3,4-dihydroxybenzaldehyde (97%), fluorobenzene (99%), dichloromethane (99.8%, extra dry, over molecular sieves), cesium carbonate (Cs_2CO_3 , 99.5%), anhydrous

dimethylsulfoxide (DMSO), anhydrous dimethylformamide (DMF), methyl sulfoxide-*d*6, chloroform-*d* (CDCl₃), acetone-*d*6, 4-hydroxy-3-methoxybenzyl alcohol (99%), 1,2-dibromoethane, sodium borohydride (NaBH₄, powder, 98%), scandium(III) trifluoromethanesulfonate (Sc(OTf)₃, 95%); methyl alcohol (MeOH, extra dry, over molecular sieves), tetrahydrofuran (THF, extra dry, over molecular sieves), acetonitrile (CH₃CN, anhydrous); *Concord Specialty Gases*: xenon gas (scientific grade). Triethylamine (Et₃N, Acros) was distilled from KOH under nitrogen prior to use.

Synthetic Procedures and Analytical Data

Trihydroxy cryptophane (**1**) was obtained in six steps with an overall yield of 9.5%.⁵⁴

Cryptophane (**2**, diastereomeric mixture): An oven-dried flask was charged with trihydroxy cryptophane **1** (0.201 g, 0.236 mmol, 1.0 equiv), DMAP (0.010 g, 0.078 mmol, 0.3 equiv), and triethylamine (2 mL) in DMF (12 mL). Finally, Mosher's acid chloride (0.197 g, 0.778 mmol, 3.3 equiv) was added and the reaction mixture was stirred at 70 °C for 2 days. The reaction mixture was cooled to rt followed by standard workup procedure using dichloromethane for extraction. The diastereomers were purified and separated by silica gel column chromatography (Et₂O:CH₂Cl₂, 0.5:99.5, v/v) to yield 0.12 g (0.081 mmol, yield: 34%) **2a** and of 0.12 g (0.081 mmol, yield: 34%) **2b** as white solids. Typically 20-30% of the trihydroxy cryptophane starting material was recovered from the chromatography column.

Diastereomer 2a-(S)-(-): mp >130 °C dec; TLC (silica gel, Et₂O: CH₂Cl₂, 1:99, v/v): R_f(**2a**) = 0.63; ¹H NMR (CDCl₃) δ (ppm): 7.73-7.55 (m, 15H), 6.78 (s, 3H), 6.67 (s, 3H), 6.65 (s, 3H), 6.59 (s, 3H), 4.67 (d, *J* = 13.8 Hz, 3H), 4.54 (d, *J* = 13.7 Hz, 3H), 4.37-3.91 (m, 12H),

3.82 (s, 9H), 3.50 (d, $J = 12.6$ Hz, 3H), 3.43 (s, 9H), 3.37 (d, $J = 13.9$ Hz, 3H); ^{13}C NMR (CDCl_3) δ (ppm): 164.6, 149.0, 148.9, 144.6, 138.8, 138.2, 133.2, 132.3, 132.0, 131.2, 130.0, 128.5, 127.6, 122.6, 122.4, 117.0, 114.9, 68.8, 68.5, 56.0, 55.4, 36.2, 36.0; Shown in Figure 2. 11. HRMS (m/z): $[\text{M}+\text{Na}]^+$ calculated for $\text{C}_{81}\text{H}_{69}\text{F}_9\text{O}_{18}\text{Na}$, 1523.4238; found, 1523.4221. NMR spectra are shown in Appendix B.

Diastereomer 2b-(S)-(+): mp >220 °C dec; TLC (silica gel, Et_2O : CH_2Cl_2 , 1:99, v/v): $R_f(2b) = 0.60$; ^1H NMR (CDCl_3) δ (ppm): 7.75-7.55 (m, 15H), 6.87 (s, 3H), 6.76 (s, 3H), 6.55 (s, 3H), 6.44 (s, 3H), 4.68 (d, $J = 13.9$ Hz, 3H), 4.51 (d, $J = 13.7$ Hz, 3H), 4.26-3.88 (m, 12H), 3.77 (s, 9H), 3.53 (d, $J = 13.9$ Hz, 3H), 3.48 (s, 9H), 3.31 (d, $J = 13.9$ Hz, 3H); ^{13}C NMR (CDCl_3) δ (ppm): 164.5, 149.8, 149.5, 146.0, 139.1, 139.0, 133.9, 133.2, 132.2, 131.3, 130.0, 128.7, 128.1, 123.3, 122.4, 119.2, 114.7, 69.3, 69.2, 55.9, 36.6, 36.3; shown in Figure 2. 12. HRMS (m/z): $[\text{M}+\text{Na}]^+$ calculated for $\text{C}_{81}\text{H}_{69}\text{F}_9\text{O}_{18}\text{Na}$, 1523.4238; found, 1523.4202. NMR spectra are shown in Appendix B.

Trihydroxy cryptophane 3a-(-): A solution of 2 M KOH (4 mL) was added to the solution of cryptophane **2a**-(S)-(-) (0.051 g, 0.034 mmol) in THF (6 mL). The solution was stirred overnight at 70 °C. THF was removed under vacuum. Water was then added and the resulting solution was acidified with concentrated HCl and extracted with CH_2Cl_2 . The solution was washed with water and the organic layer was dried over Na_2SO_4 . The solvent was removed *in vacuo* and the crude mixture was purified by silica gel column chromatography (MeOH : CH_2Cl_2 , 1:99, v/v) to yield 0.022 g (0.026 mmol, yield: 77%). TLC (silica gel, $\text{MeOH}/\text{CH}_2\text{Cl}_2$, 5:95, v/v): $R_f(3a) = 0.33$; mp >200 °C dec; ^1H NMR and ^{13}C NMR spectra for **3a** are identical to the spectra of the racemic ($\pm$) trihydroxy

cryptophane previously reported in our lab;^{54, 56} HRMS (m/z): [M+Na]⁺ calcd for C₅₁H₄₈O₁₂Na, 875.3043; found, 875.3047.

Trihydroxy cryptophane 3b-(+): Following the procedure for the synthesis of **3a**, compound **2b**-(S)-(+) (0.049 g, 0.033 mmol) in the presence of 2 M KOH (4 mL) in THF (6 mL) afforded 0.023 g (0.027 mmol, 81 % yield) of **3b** as a white solid. TLC (silica gel, MeOH/CH₂Cl₂, 5:95, v/v): R_f(**3b**) = 0.33. mp >200 °C dec; ¹H NMR and ¹³C NMR spectra for **3b** are identical to the spectra of **3a** and the racemic (±) trihydroxy cryptophane previously reported in our lab,^{54, 56} for confirmation, HRMS (m/z): [M+Na]⁺ calculated for C₅₁H₄₈O₁₂Na 875.3043; found, 875.3041.

Tripropargyl cryptophane 4a-(-): Compound **3a** (0.041 g, 0.048 mmol, 1 equiv) and K₂CO₃ (0.033 g, 0.24 mmol, 5 equiv) were added into dry acetone (10 mL) under nitrogen. The mixture was stirred at rt for 30 min. The reaction mixture was cooled to 0°C and propargyl bromide (0.05 mL, 0.48 mmol, 10 equiv) was then added dropwise followed by stirring for 30 min at rt. Finally, the reaction mixture was refluxed for 2 days with stirring. The solvent was removed *in vacuo* and the crude mixture was purified by silica gel column chromatography (CH₂Cl₂→Acetone:CH₂Cl₂ 5:95, v/v) to yield 0.024 g (0.025 mmol, 52% yield) of **4a** as a white powder. mp >200 °C dec; TLC (silica gel, acetone: CH₂Cl₂, 1:9, v/v): R_f(**4a**) = 0.73; ¹H NMR and ¹³C NMR spectra for **4a** are identical to the spectra of the racemic (±) tripropargyl cryptophane previously reported in our lab;⁵⁴ HRMS (m/z): [M+Na]⁺ calculated for C₆₀H₅₄O₁₂Na, 989.3513; found, 989.3514.

Tripropargyl cryptophane 4b-(+): Following the procedure for the synthesis of **4a**, compound **3b** (0.045 g, 0.053 mmol, 1 equiv) in the presence of K₂CO₃ (0.037 g, 0.27

mmol, 5 equiv) and propargyl bromide (0.06 mL, 0.53 mmol, 10 equiv) in dry acetone (10 mL) afforded 0.028 g (0.029 mmol, 55% yield) of **4b** as a white solid, mp >200 °C dec; TLC (silica gel, acetone: CH₂Cl₂, 1:9, v/v): R_f(**4b**) = 0.73. ¹H NMR and ¹³C NMR spectra for **4b** are identical to the spectra of **4a** and the racemic (±) tripropargyl cryptophane previously reported in our lab; HRMS (*m/z*): [M+Na]⁺ calculated for C₆₀H₅₄O₁₂Na, 989.3513; found, 989.3533.

UVvis Spectroscopy. A stock solution of 20 mgs tripropargyl cryptophane in 1,4-dioxane was prepared in a volumetric flask. Dilutions were made to generate a range of sample of known concentration. The absorbance of these samples were measured and used to calculate an extinction coefficient of 10,000 M⁻¹cm⁻¹, shown in Figure 2. 11.

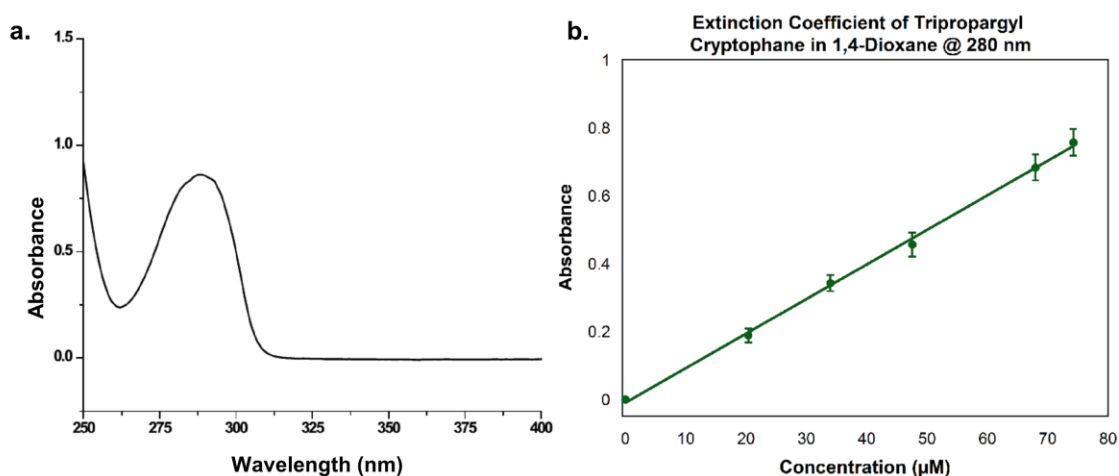


Figure 2. 11. Representative UV-vis spectrum of tripropargyl cryptophane (a); and Beer's Law plot determining the extinction coefficient of tripropargyl cryptophane (b). Adapted with permission from O. Taratula; M.P. Kim; Y. Bai; J.P. Philbin; B.A. Riggle; D.N. Haase; and I.J. Dmochowski. Synthesis of Enantiopure, Trisubstituted Cryptophane-A Derivatives. *Org Lett.* **2012**, 14, (14) 3580-3583. Copyright 2012 American Chemical Society.

Cryptophane-A-(+): An excess of methyl iodide (0.035 g, 0.25 mmol, 10 equiv) was added to **3b-(+)** (0.021 g, 0.025 mmol, 1 equiv) and K₂CO₃ (0.017 g, 0.13 mmol, 5 equiv) in dry acetone (5 mL) under nitrogen. The reaction mixture was refluxed for 2 days with stirring. The solvent was removed *in vacuo* and the crude was purified by silica gel column chromatography (CH₂Cl₂→Acetone:CH₂Cl₂ 5:95, v/v) to yield 0.018 g (0.021 mmol, 85% yield) of **5** as a white powder. TLC (silica gel, acetone:CH₂Cl₂, 1:9, v/v): R_f(**5**) = 0.82; ¹H NMR (CDCl₃) δ (ppm): 6.77 (s, 6H), 6.68 (s, 6H), 4.61 (d, *J* = 13. Hz, 6H), 4.17 (m, 12H), 3.81 (s, 9H), 3.42 (d, *J* = 13.8 Hz, 3H); ¹³C NMR (CDCl₃) δ (ppm): 149.9, 146.9, 134.4, 131.8, 121.0, 113.9, 69.6, 55.9, 36.4. HRMS (*m/z*): [M+Na]⁺ calculated for C₅₄H₅₄O₁₂Na, 917.3513; found, 917.3517.

Acknowledgment

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§ 2.4 Enantiopure Cryptophane-¹²⁹Xe NMR Biosensors Targeting Carbonic Anhydrase^{**}

Utilizing the synthetic strategies discussed in Section 2.3, we endeavored to determine if the + and – enantiomers of the carbonic anhydrase targeting cryptophane, **C7B**, bound CA in different ways and thus resulted in the observed multiple bound peaks. As shown in Figure 1. 20, **C7B** demonstrated the largest chemical shift from free biosensor ($\Delta\delta=7.5$ ppm) and from the other bound peak ($\Delta\delta=4.2$ ppm) and thus was selected for initial attempts to elucidated how diastereomers formation affects biosensor binding. Enantiopure **C7B** was achieved in two steps from enantiopure tripropargyl cryptophane as shown in Figure 2. 12. Enantiopurity was confirmed by circular dichroism spectroscopy as shown in Figure 2. 13.

^{**} This section summarizes work published by Taratula *et al.* and the data figures are directly used from the paper in reference ⁸⁶

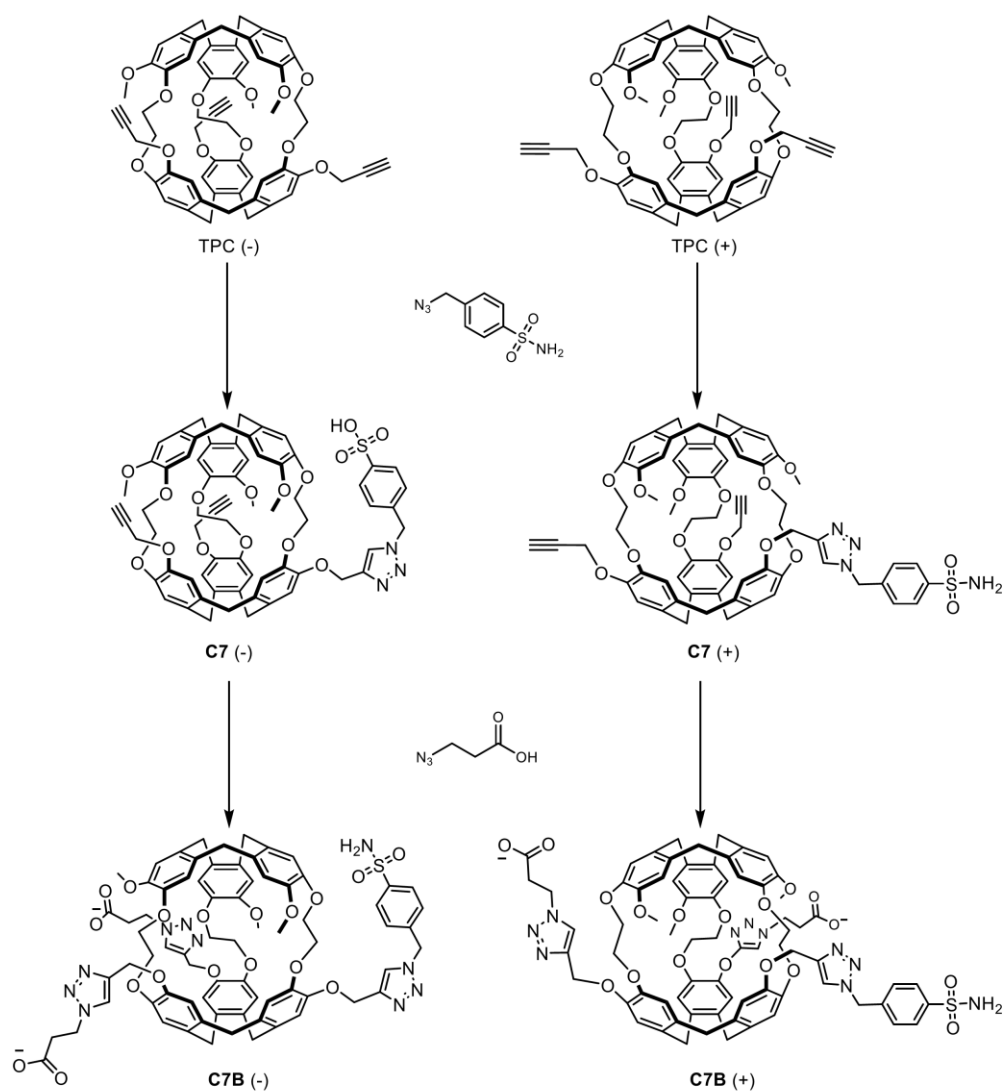


Figure 2. 12. Synthesis of enantiopure cryptophane biosensors (-) and (+) **C7B** from enantiopure tripropargyl cryptophane.

We had demonstrated with hp ¹²⁹Xe NMR that **C7B** in 50 mM Tris at pH 8, has a chemical shift of 63.9 ppm. Upon addition of stoichiometric amounts of WT CAII, there are three observable peaks. The first is at 63.7 ppm which we attribute to unbound biosensor. The second and third are at 67.0 and 71.2 ppm, respectively. We attributed those two peaks to biosensor binding or otherwise interacting with the protein.

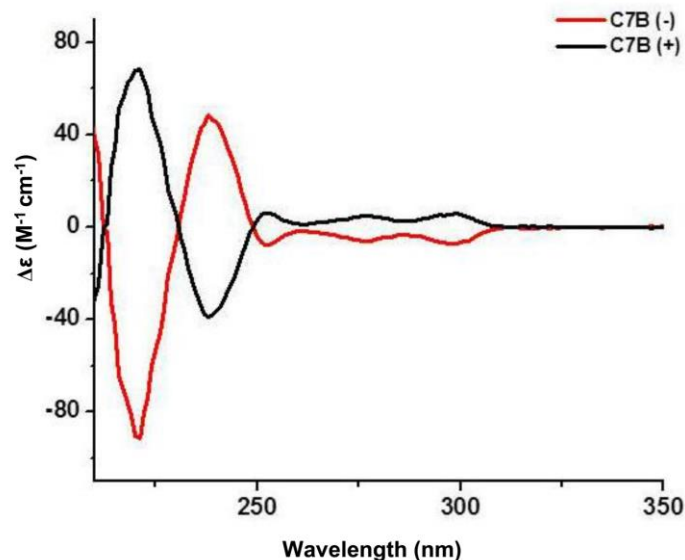


Figure 2. 13. Electronic Circular Dichroism for **C7B**-(−) and **C7B**-(+). Reproduced with permission from Taratula *et al.*⁸⁶

It is well documented in the literature that benzene sulfonamide binds CAII in 1:1 stoichiometry.⁸⁵ Thus we hypothesized that the different diastereomers formed upon active site binding was the most probable cause for the observed multiple peaks. We performed ¹²⁹Xe NMR with both the (+) and (−) **C7B** compounds at 0.5 and 1.0 equivalents. We endeavored to both assign each bound peak to a given diastereomers and to ascertain if they bound with equal affinity. The results, however, depicted a more convoluted binding interaction, see Figure 2. 14. The (+) enantiomer appears to bind CAII with higher affinity, at both 0.5 and 1.0 equivalents we observe no unbound peak. The (−) enantiomer, on the other hand, seems to bind more weakly as there is observable unbound biosensor even with 1.0 equivalent of CAII but the (−) enantiomer gives the desired large (~7 ppm) chemical shift upon binding. Reexamining racemic **C7B** binding to WT CAII (Figure 2. 15)

definitively demonstrates that diastereomer formation does contribute to the presence of multiple bound peaks but that it isn't the only contributing factor.

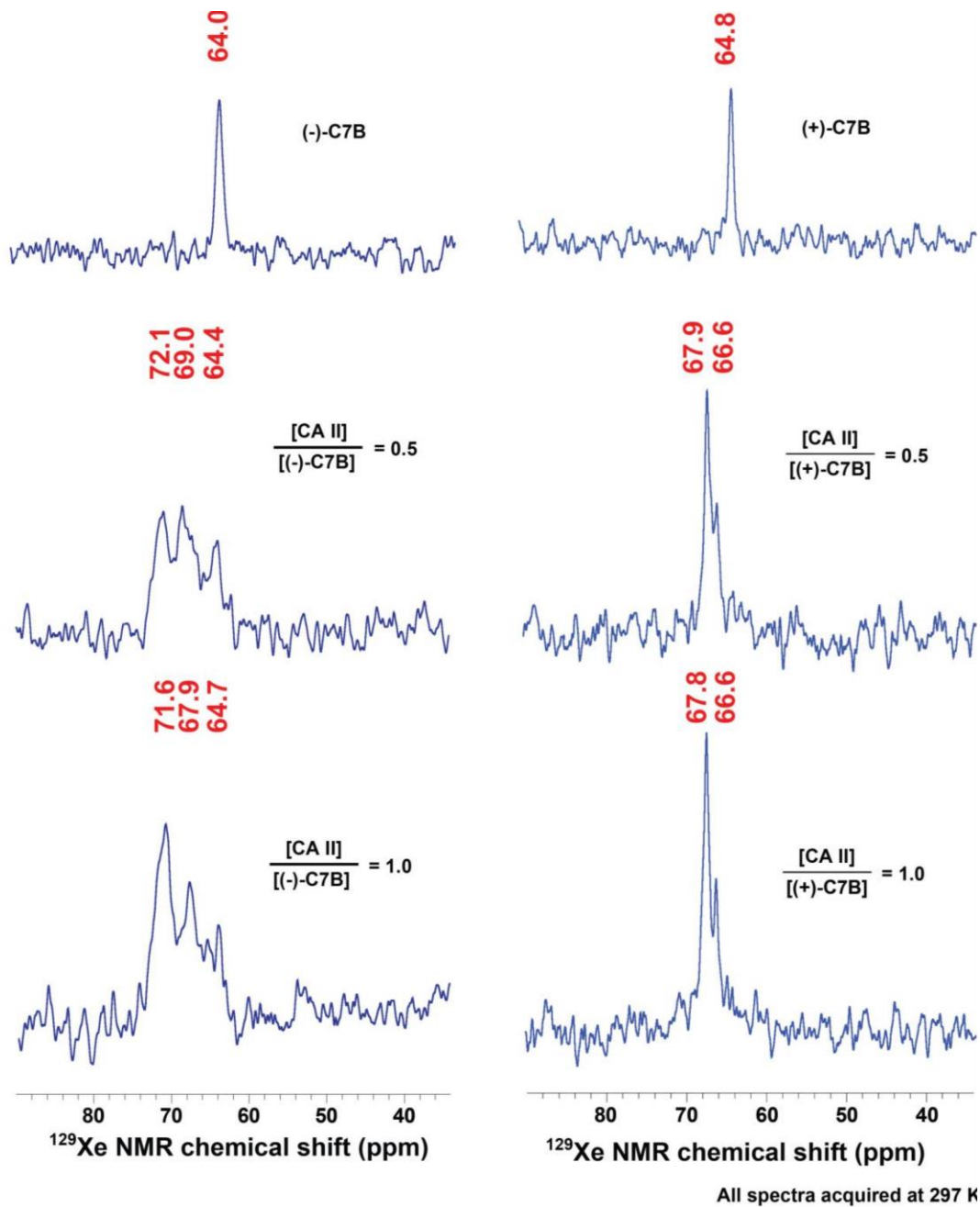


Figure 2. 14. ^{129}Xe NMR spectra of **C7B**-(**-**) and **C7B**-(**+**) in the before and after binding WT CAII. Reproduced with permission from Taratula *et al.*⁸⁶

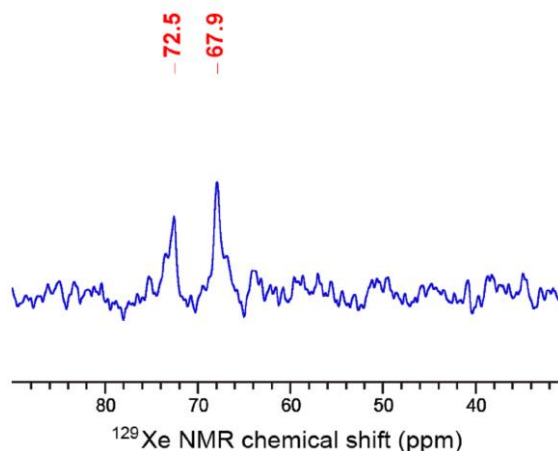


Figure 2. 15. ^{129}Xe NMR spectrum of racemic **C7B** and WT CAII. Reproduced with permission from Taratula *et al.*⁸⁶

§ 2.5 Two-Site Hypothesis

The need to understand how we generate specific chemical shifts was not completely resolved by the enantiopure **C7B** studies, thus we returned to the literature to find anomalies in benzene sulfonamide binding to CA. One paper mentioned, as an aside, that in crystallographic studies a benzenesulfonamide derivative was observed to bind to the external protein surface which they termed a “b” site shown in Figure 2. 16.

The cause of this binding was attributed to the “relatively high concentrations of inhibitors employed in the crystal soaking protocol...”¹¹⁴ Nonetheless, we wondered if the cryptophane itself could be strengthening and thus promoting binding to this “b” site even in stoichiometric concentrations of biosensor. To determine if this was the case we produced two initial mutants, H94R--to abolish active-site binding and D19L--to abolish “b” site binding, see Figure 2. 17. Following on the initial enantiopure work with **C7B**, we decided to employ **C8B** for these studies as it is the only CA biosensor with which we obtained a bound crystal structure.⁸⁹ Initial ^{129}Xe NMR and ITC experiments (Figure 2.

17b, ITC data not shown) demonstrated single site binding between the biosensor and D19L CAII. However, attempts with H94R showed no signal by ^{129}Xe NMR (data not shown) and the protein foamed extensively precluding any ITC measurements. Although these preliminary data do not prove whether the biosensors can bind to the “b” site they do show that removing the charged residue in the 19 position results in a single bound peak. We made further attempts to determine whether our CA biosensors bind to the purported “b” site. Benjamin Roose next expressed an H94A CAII mutant which was documented as being more stable in the literature. ITC data showed no binding (data not shown) and ^{129}Xe NMR also showed no bound signal, while indicating biosensor free in solution, Figure 2. 17c. Thus, these experiments with H94A succeeded in negating the “b site hypothesis.” All evidence for multi-site binding must result from biosensor binding in different conformations and in different regions of the active-site (‘a site’) channel.

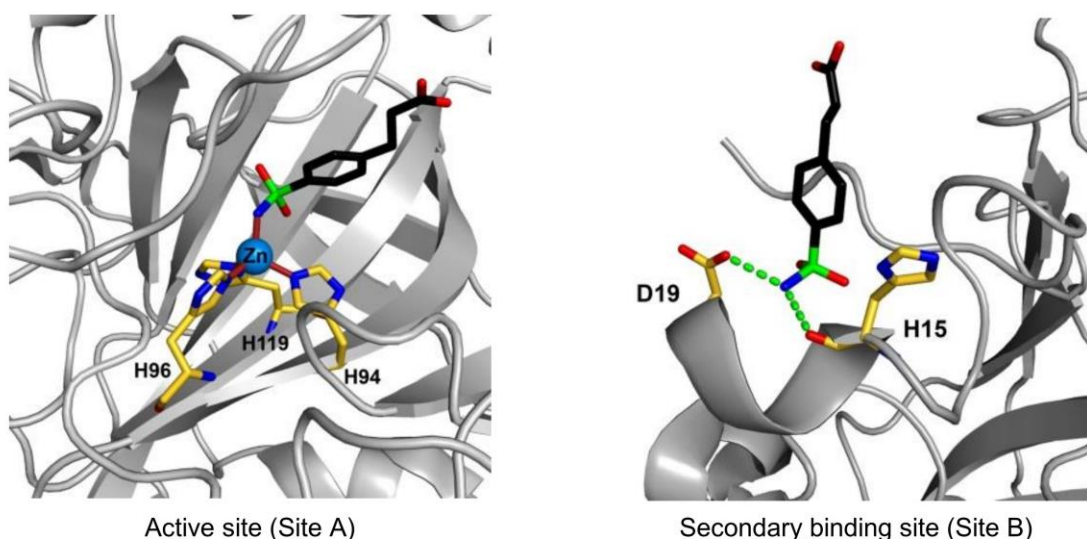


Figure 2. 16. Crystal structures of a benzenesulfonamide derivative bound to CAII i) enzyme active site or Site A; and ii) an external binding site or Site B.

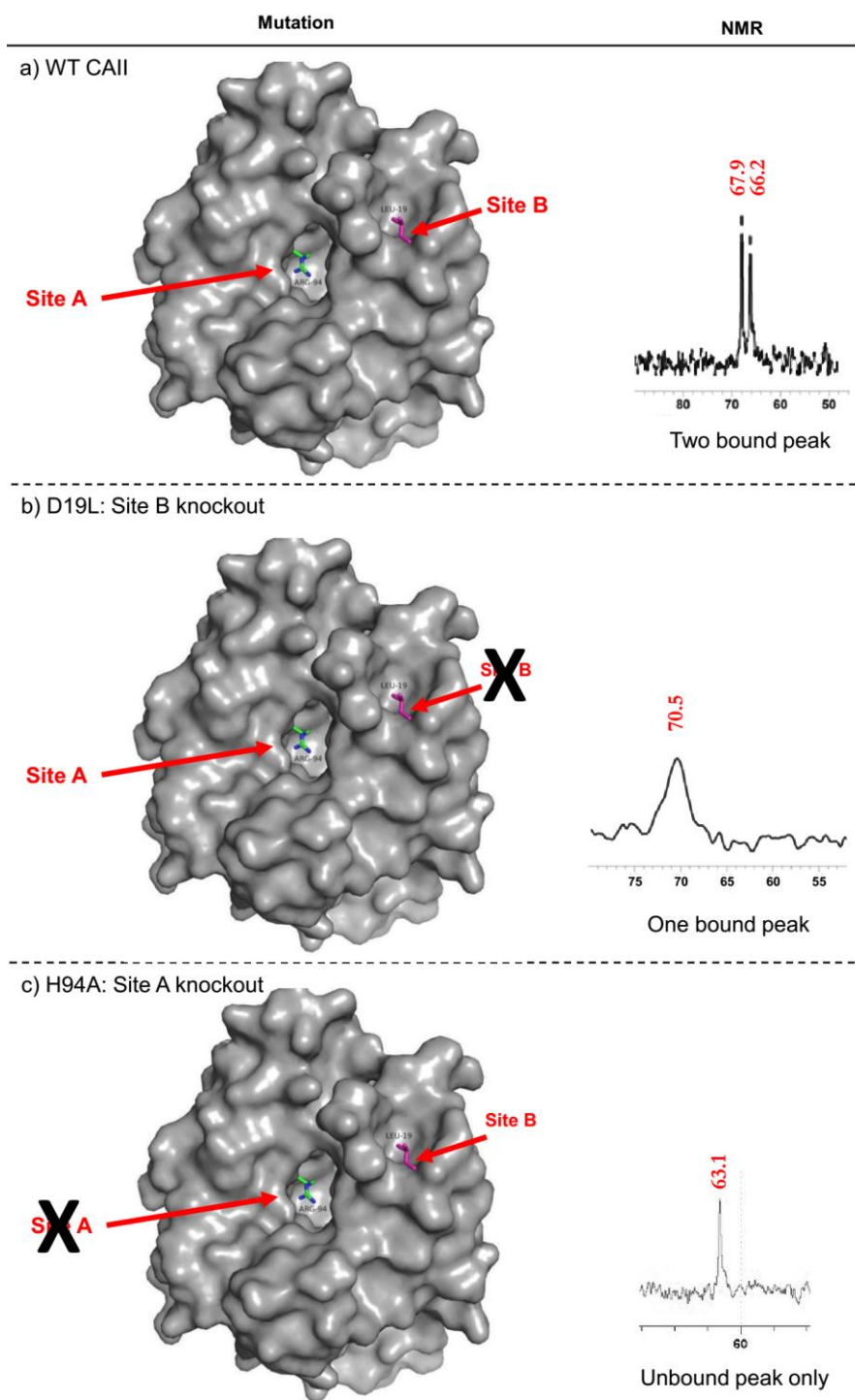


Figure 2. 17. Depiction of the two purported benzene sulfonamide binding sites on CA II and the proposed mutations, H94A in green and blue and D19L in magenta. ^{129}Xe NMR shows A) two bound peaks for **C8B** with WT CAII; B) one bound peak for **C8B** with D19L CAII; and C) only an unbound peak for **C8B** with H94R CAII.

Utilizing **C8B** and a variety of protein double and triple mutants we were able to observe that the charged 19 residue is on a flexible portion of the protein and is in close enough proximity with the biosensor as to interact with it, possibly via the cryptophane propionates, and influence biosensor positioning relative to residues at the mouth of the active site channel. These perturbations then result in changes in the ^{129}Xe chemical shift. Figure 2. 18 demonstrates the proximity of the two sites and demonstrates the conformational flexibility of the tail containing D19. A manuscript detailing these results is in preparation thus the data have been omitted from this dissertation.

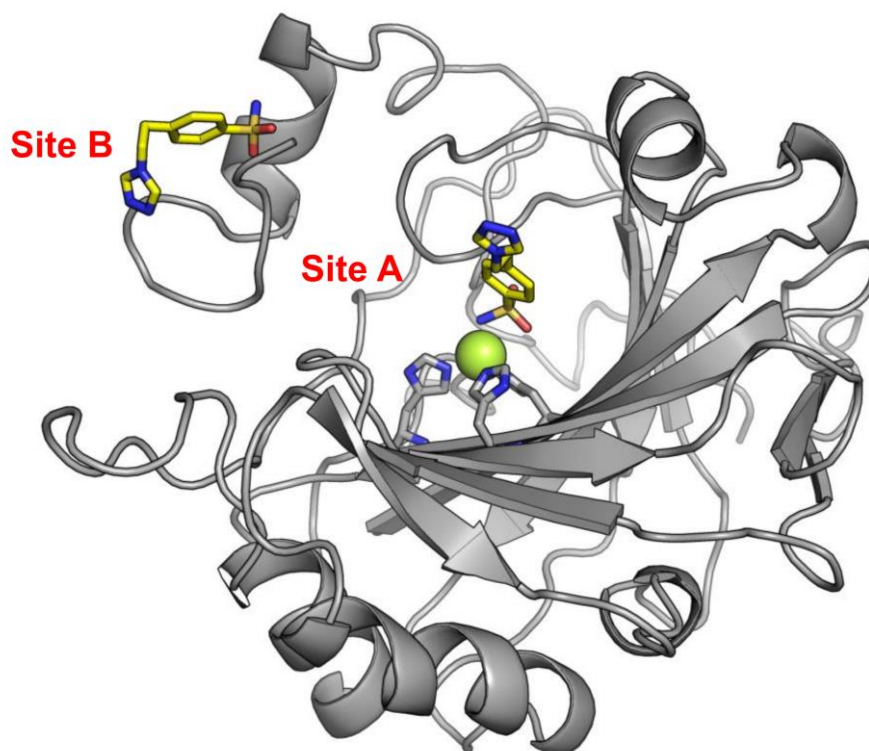


Figure 2. 18. WT CAII bound to benzene-sulfonamide substrates. The “b site” is found on a flexible loop of CA II which may facilitate D19 interaction with biosensor bound to the protein active site.

§ 2.6 Moving Forward

The final component to that manuscript will be to repeat some of the double and triple protein mutant studies with enantiopure **C8B**. Although these specific compounds have not been previously prepared, I am utilizing our previously published routes to **C7B**, discussed in Sections 2-4 of this chapter. Studies comparing the results of racemic **C8B** to (+) and (–) enantiopure **C8B** with the variety of prepared CAII mutants should resolve many components that result in the observed multiple bound peaks. The synthesis will be completed by Mara Greenberg, a talented undergraduate student who I trained.

Chapter 3: Smart ^{129}Xe NMR Biosensor for pH-Dependent Cancer Cell Labeling

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§ 3.1 Introduction

Magnetic resonance imaging (MRI) and spectroscopy (MRS) are versatile and commonly employed techniques for the diagnosis and staging of disease.²⁰ The development of targeted and stimuli-responsive (i.e., “smart”) contrast agents improves the capabilities of MRI/MRS for molecular imaging.¹¹⁵ Targeted therapeutic and diagnostic imaging techniques are typically directed to one or more receptors associated with a disease state. However, in cancer, as a result of large natural variations between cells and the heterogeneous nature of tissue within a tumor, there is also need for more general biomarkers.^{116, 117} For example, hypoxia and acidification occur in 90% of tumors and are key microenvironmental factors in progression and treatment resistance in solid tumors.^{118, 119} The tumor microenvironment is acidified to levels approaching pH 6.0 from a normal pH of 7.4, which increases metastasis, mutation rate, and cell viability.^{117, 120-122} Therefore, being able to identify cells in acidic environments has practical importance in the design of cancer therapies and controlled-release drug delivery mechanisms.¹²¹ Additionally, acidic environments can mitigate the efficacy of weakly basic chemotherapeutics such as doxorubicin, further necessitating methodologies to probe extracellular pH (pH_e).¹²³ Here, we present an ultrasensitive xenon-based MR contrast agent that can identify and label cell populations based on their acidic pH_e.

A variety of pH-responsive MR contrast agents have been designed previously, including Gd complexes,^{124, 125} tunable micelle-encapsulated polymers and ¹⁹F compounds,^{126, 127} and CEST agents,¹²⁸⁻¹³⁴ among others.^{135, 136} These probes enable measurements of solution pH but do not selectively label cells in acidic environments, e.g.,

as needed for identifying small populations of cancer cells or performing cell tracking experiments. In parallel efforts over the past two decades, many strategies have been developed for labeling cells with MRI contrast agents such as membrane-targeting Gd chelates,²³ monocrystalline iron-oxide particles (MIONs),¹³⁷ micron-sized iron-oxide particles (MPIOs),¹³⁸ ultrasmall dextran-coated iron oxide particles (USPIOs),¹³⁹ and superparamagnetic iron oxide (SPIO) glycol chitosan.^{122, 140} Pioneering studies by the Tsourkas lab and others have explored pH-dependent cell labeling with these reagents, but there remain significant limitations, e.g., pH-responsive SPIO typically requires significant incubation time (~24 h) between administration and imaging.¹²² More generally, applications with conventional MRI contrast agents are limited by low detection sensitivity on a per-monomer basis (i.e., low mM). One strategy for improving NMR detection sensitivity involves the use of exogenously supplied “hyperpolarized” (hp) nuclei, e.g., ¹²⁹Xe, ¹³C, and ³He, with magnetic spin reservoirs that exceed the normal Boltzmann distribution by several orders of magnitude. Xe binds void spaces in materials,¹⁴¹ proteins,¹⁴² and spores¹⁴³ but shows highest affinity and useful exchange kinetics for a class of host molecules known as cryptophane.^{48, 50, 55-57, 86, 144, 145} Perturbation of the large (~42 Å³ volume) ¹²⁹Xe electron cloud can produce significant nuclear magnetic chemical shift changes and results in a nearly 300 ppm chemical shift window when bound to different cryptophanes in aqueous solution.^{50, 60, 61}

Based on these principles, we and others have developed ¹²⁹Xe-cryptophane NMR biosensors⁶⁶ for the sensitive detection of protein receptors,^{66-69, 73} enzymes,⁷⁰ DNA,⁷¹ and metal ions in solution.¹¹³ In one proof-of-concept experiment, Berthault *et al.* decorated

cryptophane with six carboxylic acids to create a pH reporter: unique chemical shifts were measured over the pH 3.5-5.5 range with a total $\Delta\delta$ of 3.55 ppm.¹⁴⁶ However, solubility issues precluded work near neutral pH.

Recent studies have moved xenon biosensing from buffer solutions to lipid membrane suspensions and living cells. The Pines lab developed ultrasensitive methods for detecting cryptophane in solution using hp ^{129}Xe chemical exchange saturation transfer (Hyper-CEST) NMR spectroscopy.⁹⁵ They also discovered that cryptophane in association with a dilute suspension of sub-micron Intralipid vesicles yielded a ^{129}Xe NMR peak that was shifted ~ 10 ppm downfield from the aqueous ^{129}Xe -cryptophane peak;⁹⁰ similar results were later obtained with different lipid compositions.¹⁴⁷ The Schröder lab subsequently performed Hyper-CEST NMR spectroscopy and imaging studies in cells loaded with lipophilic cryptophane and found a similar 9-11 ppm downfield chemical shift change, likely due to membrane association.^{75, 97, 148} These studies highlight the large ^{129}Xe NMR chemical shift changes that can be achieved by engineering cryptophane-lipid membrane interactions.

Building on these examples, we set out to develop an ultrasensitive ^{129}Xe NMR contrast agent for labeling cells in acidic microenvironments. Recent work from our laboratory⁹⁶ and elsewhere^{149, 150} has demonstrated nM-to-pM detection of water-soluble cryptophane using Hyper-CEST NMR spectroscopy. Most recently, Witte *et al.* demonstrated effective contrast via Hyper-CEST by site-specific labeling of cell-surface glycans with nM concentrations of cryptophane.⁹¹ Thus, Hyper-CEST NMR should enable ultrasensitive detection of cryptophane-labeled cells that reside in acidic environments and

differentiation from “normal” cells residing in neutral pH environments, provided that cryptophane-cell interactions can be modulated over the pH range 5.5-7.5.

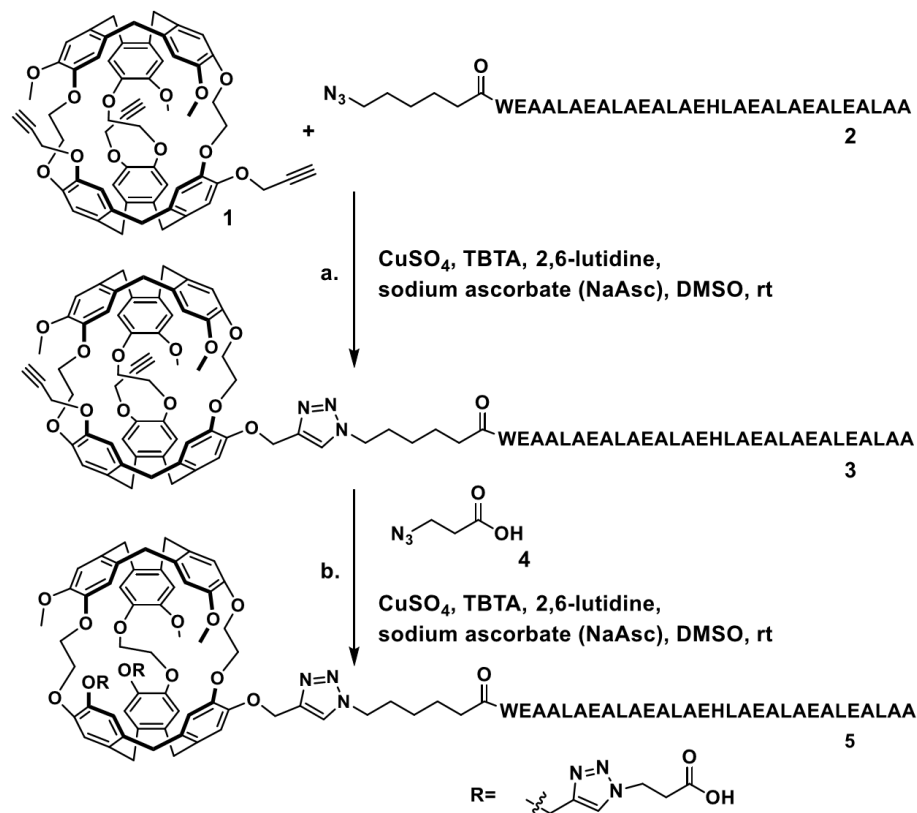


Figure 3. 1. Reaction scheme for the formation of water soluble EALA-cryptophane (WEC). a. **1** (1 eq), **2** (1 eq), CuSO_4 (1 eq), TBTA (5 eq), 2,6-lutidine (1 eq), NaAsc (10 eq), 12 h; b. **3** (crude), **4** (10 eq), CuSO_4 (1 eq), TBTA (5 eq), 2,6-lutidine (1 eq), NaAsc (10 eq), 12 h. Adapted with permission from B.A. Riggle; Y. Wang; I.J. Dmochowski. A “Smart” ^{129}Xe NMR Biosensor for pH-Dependent Cell Labeling. *J. Am. Chem. Soc.* **2015**, 137, (16) 5542-5548. Copyright 2015 American Chemical Society.

We hypothesized that modifying cryptophane with an EALA-repeat peptide, WEAALAEALAEALAEHLAEALAEALAEALAA,¹⁵¹ should modulate ^{129}Xe NMR chemical shift in response to physiologic pH changes. By our strategy, ^{129}Xe NMR chemical shift should vary both from pH-dependent peptide conformational changes as well as pH-dependent peptido-cryptophane-cell membrane association. The synthetic

EALA-repeat peptide was inspired originally by hemagglutinin (HA), which membrane inserts in low-pH environments.¹⁵¹ The poly-glutamic acid nature of the EALA-repeat peptide elevates the pK_a to around 6, resulting in a conformational change from random coil (pH 7.5) to mostly alpha helical (pH 5.5), over this biologically relevant pH range.¹⁵² As the glutamates are protonated, the EALA-repeat peptide becomes more helical and hydrophobic, and it inserts into lipophilic membranes.¹⁵³ This pH-dependent membrane insertion has been used in living cells to facilitate endosomal escape of both nanocapsule and gene payloads.¹⁵⁴⁻¹⁵⁶ Thus, by appending cryptophane to a membrane-inserting EALA peptide, we endeavored to generate a xenon contrast agent capable of being “activated” in acidic cell environments to label cell membranes and give large ^{129}Xe NMR chemical shift changes. We based the design on our previously reported tripropargyl cryptophane-A derivative (with two cyclotrimeratrylene units tethered by three ethylene linkers),⁴⁵ which should allow facile attachment of a pH-responsive peptide and also two water-solubilizing moieties (Figure 3.1) to mitigate the potential for cryptophane aggregation.⁹⁷

§ 3.2 Results and Discussion

Synthetic Procedures

Figure 3.1 shows the synthesis of the water-soluble EALA-cryptophane (WEC) pH-responsive biosensor **5**, the details of which are provided in the Materials and Methods Section of this chapter. Briefly, the synthesis of tripropargyl cryptophane **1** was performed in six non-linear steps with modifications to previously published methods,⁵⁴ with an overall yield of 6.4%. The yield for the five linear steps was 9.9%. The azido-EALA-repeat peptide **2** was prepared with standard Fmoc synthetic methods. The peptide was attached

to the cryptophane via copper(I)-catalyzed [3+2] azide-alkyne cycloaddition (CuAAC) to form **3**.^{111, 112} The mono-peptide cryptophane was achieved in preference by controlling reaction stoichiometry. The resulting triazole-hexyl spacer kept the peptide in close proximity to the ¹²⁹Xe nucleus while minimizing steric clashes with cryptophane during conjugation. Formation of compound **3** was confirmed by MALDI-MS and the yield quantified by analytical reverse-phase HPLC to be 60-80%. A solubilizing linker, 3-azidopropionic acid **4**, was synthesized in one step from the commercially available β -propiolactone (see Material and Methods Section)^{70, 74} and reacted with crude **3** via a second CuAAC. Starting from tripropargyl cryptophane **1**, WEC **5** was isolated in ~40% yield after sequential CuAAC reactions with **2** and **4** and HPLC purification to remove unreacted EALA peptide and unreacted cryptophane.

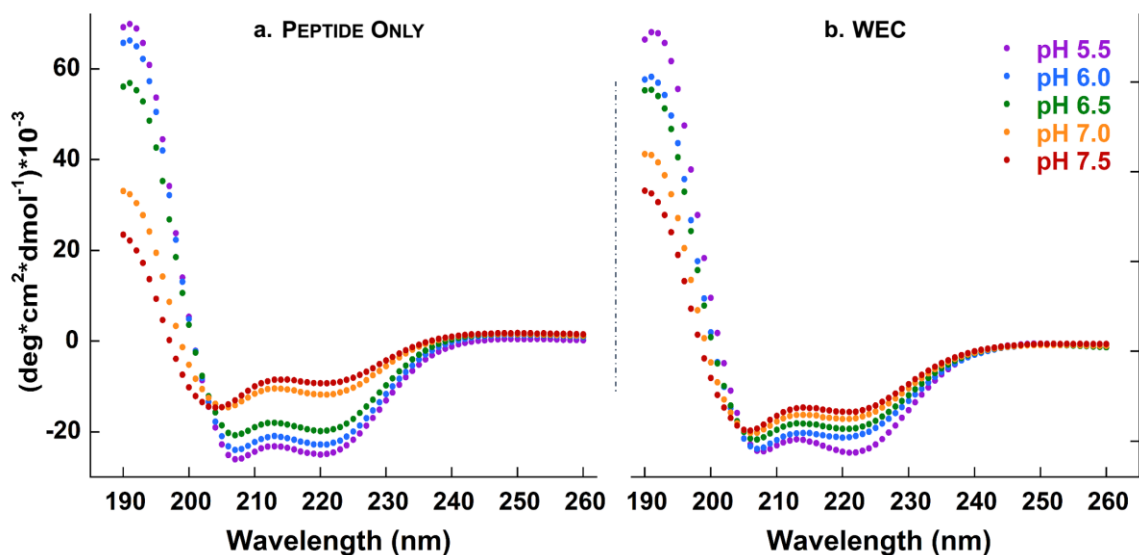


Figure 3. 2. pH titrations monitored by ECD spectroscopy for a. azido-EALA peptide and b. water-soluble EALA-cryptophane (WEC). Samples (30 μM) were in 10 mM sodium phosphate buffer over the pH range 5.5-7.5 at 298 K. Reprinted with permission from B.A. Riggle; Y. Wang; I.J. Dmochowski. A “Smart” ¹²⁹Xe NMR Biosensor for pH-Dependent Cell Labeling. *J. Am. Chem. Soc.* **2015**, 137, (16) 5542-5548. Copyright 2015 American Chemical Society.

Electronic Circular Dichroism (ECD) Spectroscopy

For ECD studies, all samples of azido-peptide **2** or WEC were prepared at 30 μM concentration in 10 mM sodium phosphate buffer, as confirmed by UV-vis spectroscopy (peptide: $\epsilon_{280} = 5,700 \text{ M}^{-1}\text{cm}^{-1}$, WEC: $\epsilon_{280} = 17,700 \text{ M}^{-1}\text{cm}^{-1}$) and pH adjusted with 1 M HCl or 1 M NaOH. We used CD spectroscopy to confirm that azido-peptide **2** maintained pH sensitivity (Figure 3.2a):^{151, 157, 158} indeed, percent helicity increased from 25% to 67% as the pH was decreased from 7.5 to 5.5 (Table 3.1). The CD signal at pH 5.5 had pronounced local minima at 208 and 222 nm, indicative of an alpha-helical secondary structure. At pH 7.5, the spectrum approached a minimum at 204 nm while subsequently decreasing in negative ellipticity at 222 nm, characteristic of a more disordered state. For WEC (Figure 3.2b and Figure 3.3), we observed a similar increase in EALA helicity from 36% (pH 7.5) to 61% (pH 5.5) see Table 3.2. These data established that the peptide still undergoes a significant conformational change when conjugated to the cryptophane. Samples showed reproducible and reversible secondary structure changes between pH 5.5 and 7.5 (Figure 3.4).

Table 3.1: α -helical content of EALA peptide at various pH values in 10 mM Na phosphate buffer			Table 3.2: α -helical content of WEC biosensor at various pH values in 10 mM Na phosphate buffer		
pH	$[-\theta]_{222} \text{ (deg}^{\circ}\text{cm}^2\text{dmol}^{-1}\text{)}$	Helicity (%)	pH	$[-\theta]_{222} \text{ (deg}^{\circ}\text{cm}^2\text{dmol}^{-1}\text{)}$	Helicity (%)
5.5	24,579	67	5.5	22,530	61
6.0	22,414	61	6.0	18,825	51
6.5	19,415	53	6.5	17,081	46
7.0	11,670	32	7.0	14,854	40
7.5	9,142	25	7.5	13,390	36

Interestingly, WEC was more ordered at pH 7.5 than peptide alone, suggesting that the cryptophane elevated the conjugated peptide pK_a . Similar pK_a elevation was previously

observed for the analogous tris-propionic acid cryptophane, due to the bulky, hydrophobic cryptophane disfavoring ionization of the nearby propionates.⁵⁵

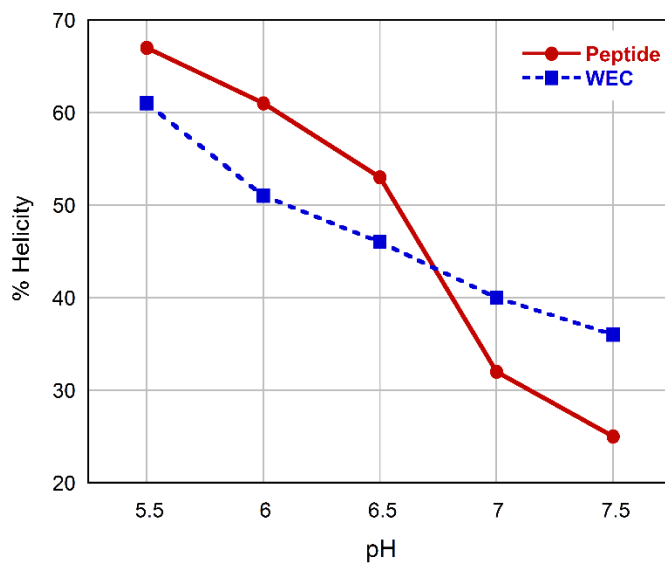


Figure 3. 3. Change in helicity as a function of pH of the azido-peptide (red) and WEC (blue). Reprinted with permission from B.A. Riggle; Y. Wang; I.J. Dmochowski. A “Smart” ^{129}Xe NMR Biosensor for pH-Dependent Cell Labeling. *J. Am. Chem. Soc.* **2015**, 137, (16) 5542-5548. Copyright 2015 American Chemical Society.

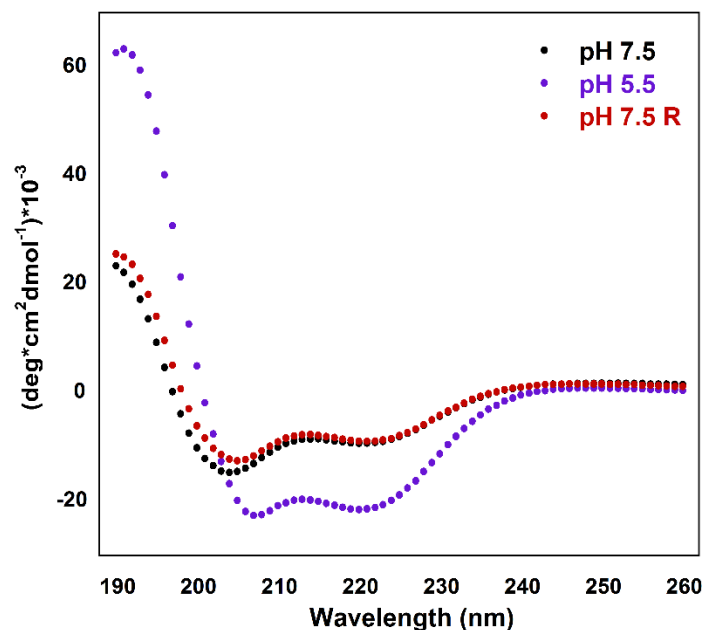


Figure 3. 4. pH reversibility experiment shows the peptide can transition from disordered (pH 7.5, black) to alpha-helical (pH 5.5, blue), back to disordered structure (pH 7.5, red). Reprinted with permission from B.A. Riggle; Y. Wang; I.J. Dmochowski. A “Smart” ^{129}Xe NMR Biosensor for pH-Dependent Cell Labeling. *J. Am. Chem. Soc.* **2015**, 137, (16) 5542-5548. Copyright 2015 American Chemical Society.

Tryptophan Fluorescence

The EALA-repeat peptide contains a single N-terminal tryptophan that we hypothesized should provide a useful local probe of peptide conformation, as well as peptide-cryptophane interaction. Fluorescence studies ($\lambda_{\text{ex}} = 280 \text{ nm}$) with peptide **2** demonstrated blue-shifted and somewhat quenched Trp emission with decreasing pH: 352 nm (pH 7.5) to 343 nm (pH 5.5), Figure 3.5a. Trp maximum emission wavelength for the WEC decreased from 336 nm to 322 nm over the same pH range (Figure 3.5b), which was considerably blue-shifted relative to peptide **2** alone, consistent with the Trp experiencing a less solvated environment near cryptophane. We note that the fluorescence signal for the amino acid tryptophan is typically not perturbed by pH changes in the range of 4-8,¹⁵⁹

whereas Trp incorporated within peptides can exhibit emission that is very sensitive to the peptide folded state. Cryptophane fluoresces ($\lambda_{\text{max}} = 313 \text{ nm}$) with comparable intensity to Trp, which further blue-shifts the observed emission spectrum.^{55, 67} At all pH values, cryptophane quenched Trp emission, as compared to the free peptide (Figure 3.5c). Plots of F/F_0 vs. pH (Figure 3.5c, where F is the fluorescence emission at a given pH and F_0 is the maximal fluorescence emission at pH 7.5) confirmed that cryptophane quenching increased from pH 7.5 down to pH 5.5, where cryptophane-Trp interactions were presumably more prevalent with the relatively uncharged, alpha-helical peptide. This analysis is in agreement to an earlier work with a peptide-cryptophane conjugate where we examined the interaction between Trp-containing peptide and cryptophane with a temperature-dependent quenching assay and Stern-Volmer analysis.⁶⁷ These experiments revealed that Trp(peptide)-cryptophane complex formation resulted in loss of Trp fluorescence. Previous studies identified high-affinity interactions between C_{60} (an aromatic molecule with similar dimensions and spherical shape to cryptophane) and Trp-containing proteins, which also resulted in Trp fluorescence quenching and blue-shifted emission.¹⁶⁰⁻¹⁶² These results support a mechanism by which the EALA peptide can mediate Trp-cryptophane complex formation in WEC (Figure 3.5d) and result in pH-dependent Trp fluorescence quenching. Importantly, Trp-cryptophane pi-stacking interactions have the potential to deshield ^{129}Xe within the cryptophane cavity, and produce a downfield chemical shift.¹⁶³⁻¹⁶⁵

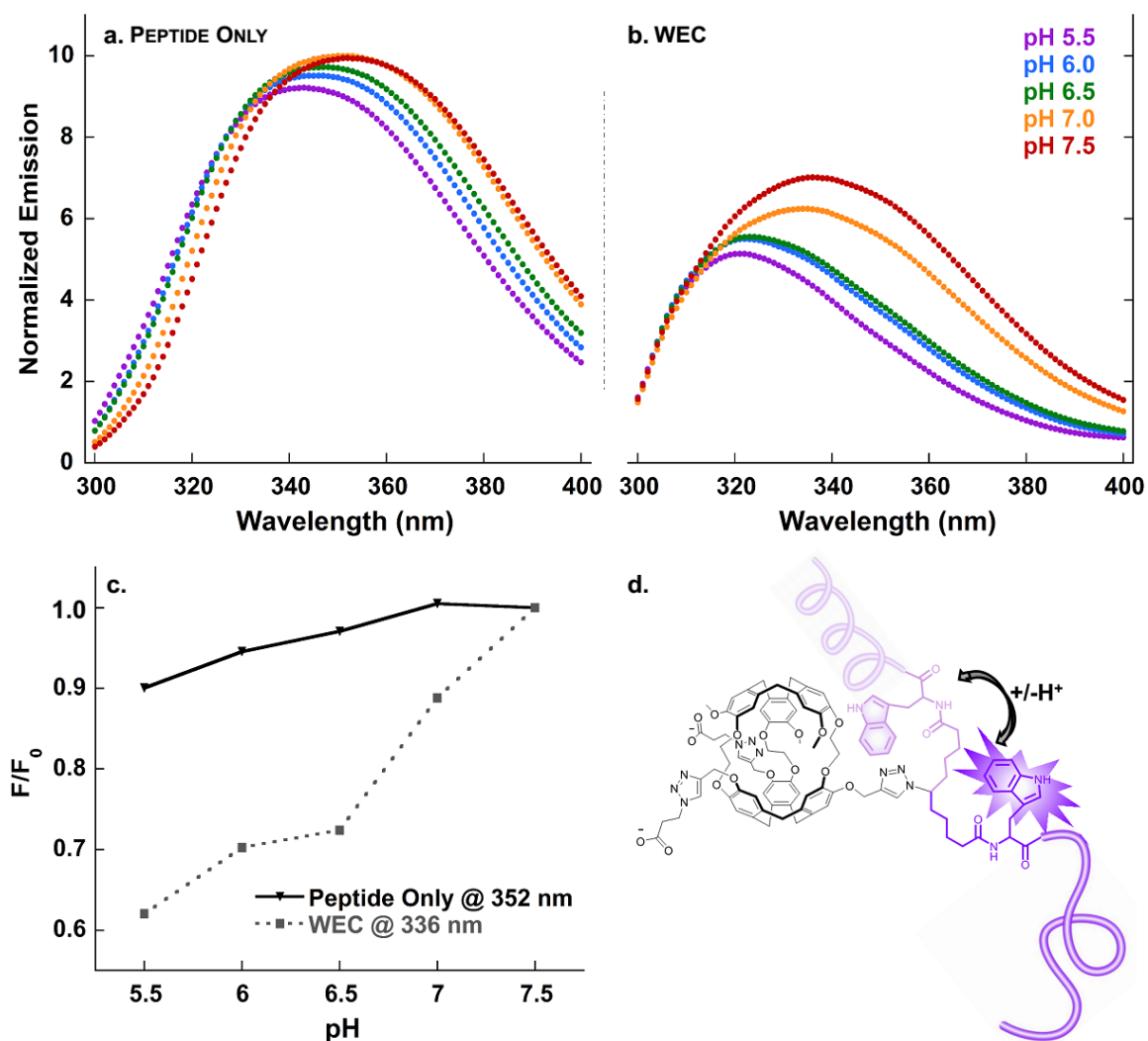


Figure 3. 5. pH titration monitored by Trp fluorescence for a. azido-EALA peptide; b. water-soluble EALA-cryptophane (WEC); c. plot of F/F_0 for the $\lambda_{\max}$ of peptide only (352 nm) and WEC (336 nm) as a function of pH; and, d. representation of alpha-helical and disordered peptide(Trp)-cryptophane interaction. Samples (30 μ M) were in 10 mM sodium phosphate buffer over the pH range 5.5-7.5 at 298 K. Reprinted with permission from B.A. Riggle; Y. Wang; I.J. Dmochowski. A "Smart" ^{129}Xe NMR Biosensor for pH-Dependent Cell Labeling. *J. Am. Chem. Soc.* **2015**, 137, (16) 5542-5548. Copyright 2015 American Chemical Society.

^{129}Xe NMR Spectroscopy

We initially performed hp ^{129}Xe NMR studies to examine the sensitivity of the cryptophane-encapsulated ^{129}Xe chemical shift to the nearby peptide conformational state. NMR samples were identically prepared at 30 μ M concentrations in 10 mM sodium

phosphate buffer. Repeated trials at 300 ± 1 K ($[\text{Xe}] = 6.2$ mM)²⁶ with the WEC at pH 7.5, 6.5, and 5.5 gave reproducible chemical shifts (Figure 3.6). A single peak was observed at both pH 5.5 (67.6 ± 0.5 ppm) and pH 7.5 (64.2 ± 0.5 ppm), with a chemical shift difference of 3.4 ppm. Interestingly, although the cryptophane itself is a racemic mixture of stereoisomers and the EALA-repeat peptide is chiral, we did not observe a pair of diastereomeric peaks at pH 7.5 or pH 5.5 as we reported for previous peptide-cryptophane xenon biosensors^{67, 69} and has been seen for various racemic xenon biosensors complexed to protein active sites.¹¹³ We hypothesize that the two diastereomers provide a very similar environment for the bound xenon atom, and produce what appears to be a single ^{129}Xe NMR peak at both pH values. In this case, the inclusion of solubilizing propionates promotes open, xenon-binding conformations of the cryptophane, regardless of peptide conformation. Conversely, the equal-intensity peaks observed at pH 6.5 ($\delta = 67.0$ and 64.4 ppm) may result from hp ^{129}Xe experiencing very different environments within the two WEC diastereomers.

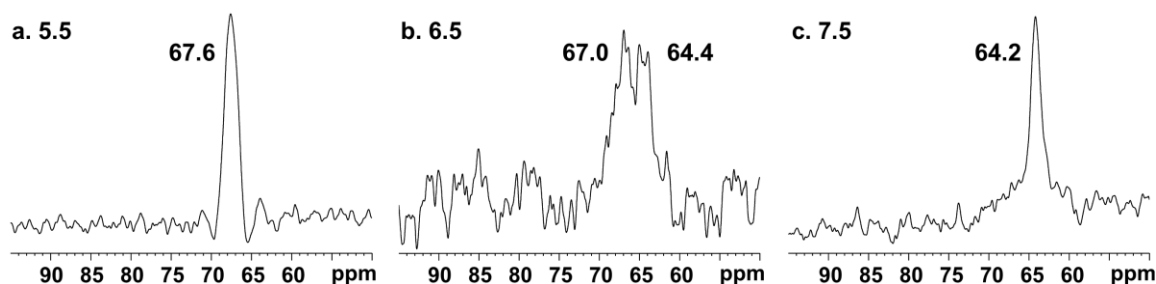


Figure 3. 6. HP ^{129}Xe NMR spectra (average of 16 scans, line-broadening = 60 Hz) of WEC (30 μM) in 10 mM sodium phosphate buffer at 300 ± 1 K, with peak widths (FWHM) indicated in Hz: a. pH 5.5, 211 Hz; b. pH 6.5, 317 and 214 Hz; c. pH 7.5, 154 Hz. Reprinted with permission from B.A. Riggle; Y. Wang; I.J. Dmochowski. A “Smart” ^{129}Xe NMR Biosensor for pH-Dependent Cell Labeling. *J. Am. Chem. Soc.* **2015**, 137, (16) 5542-5548. Copyright 2015 American Chemical Society.

Hyper-CEST ^{129}Xe NMR

To improve detection sensitivity of WEC over direct detection by nearly six orders of magnitude, we employed Hyper-CEST NMR spectroscopy. This indirect detection method took advantage of the exchanging ^{129}Xe population between bulk aqueous solution and the xenon host molecule (Figure 3.7) by selectively saturating the bound signal (Figure 3.7b). Because of xenon exchange, the selective depolarization resulted in a concomitant signal loss from the $^{129}\text{Xe}@$ water peak, which was readily monitored (Figure 3.7a). This signal was compared with a reference measurement where an “off resonance” saturation was applied to account for the natural self-relaxation of $^{129}\text{Xe}@$ water over time.

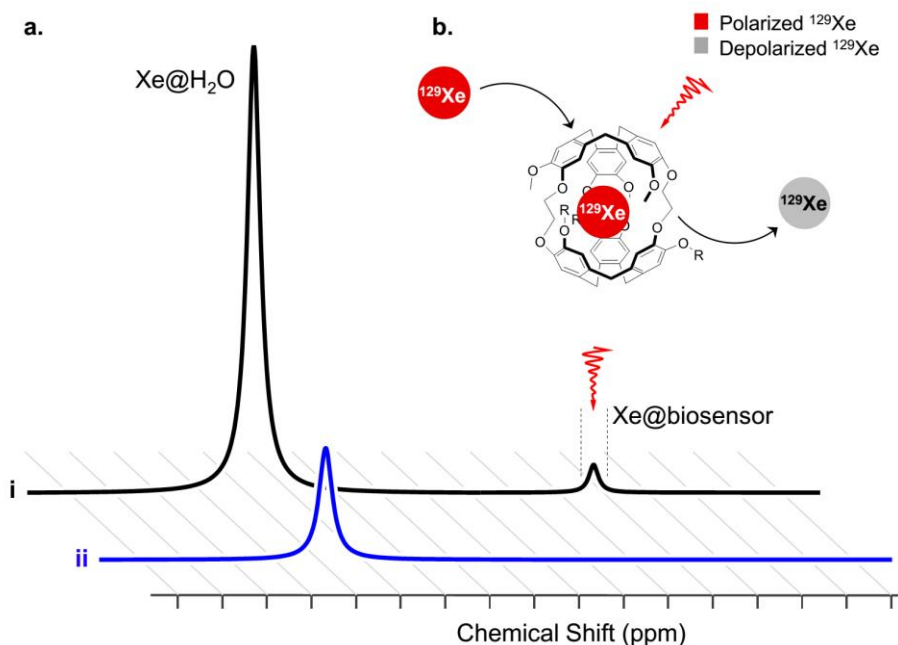


Figure 3. 7. Hyper-CEST detection scheme for WEC-encapsulated ^{129}Xe . a. Representative spectra are shown for i. the initial spectrum and ii. the resulting spectrum from selective “on resonance” saturation of the WEC-encapsulated ^{129}Xe and commensurate bulk $^{129}\text{Xe}@H_2O$ depolarization; b. selective radio frequency depolarization of WEC-encapsulated ^{129}Xe . Reprinted with permission from B.A. Riggle; Y. Wang; I.J. Dmochowski. A “Smart” ^{129}Xe NMR Biosensor for pH-Dependent Cell Labeling. *J. Am. Chem. Soc.* **2015**, 137, (16) 5542-5548. Copyright 2015 American Chemical Society.

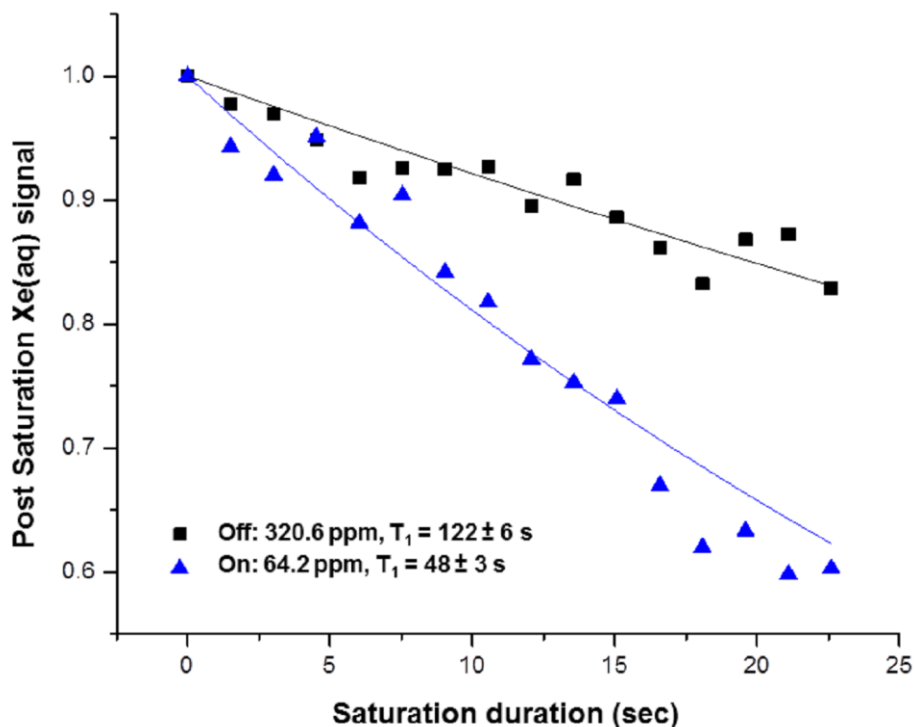


Figure 3. 8. Hyper-CEST signal decay of 34 μM WEC at pH 7.5, 310 K. Depolarization rates were measured with radiofrequency pulses either on-resonance (64.2 ppm) or off-resonance (320.6 ppm) with hp $^{129}\text{Xe}@WEC$. Reprinted with permission from B.A. Riggle; Y. Wang; I.J. Dmochowski. A “Smart” ^{129}Xe NMR Biosensor for pH-Dependent Cell Labeling. *J. Am. Chem. Soc.* **2015**, 137, (16) 5542-5548. Copyright 2015 American Chemical Society.

Using 34 μM WEC (pH 7.5, 310 K, $[\text{Xe}] = 0.15 \text{ mM}$) indirect detection via Hyper-CEST was performed by applying shaped radiofrequency saturation pulses at the $^{129}\text{Xe}@WEC$ resonant frequency and measuring the residual aqueous ^{129}Xe signal for different saturation duration (Figure 3.8). WEC was observed to “catalyze” this depolarization process through on-resonance (64.2 ppm) saturation rf pulses with $^{129}\text{Xe}@WEC$ in pH 7.5 buffer. In contrast, saturation pulses applied off-resonance (320.6 ppm) gave a depolarization time that approximated the natural T_1 of hp ^{129}Xe in water.

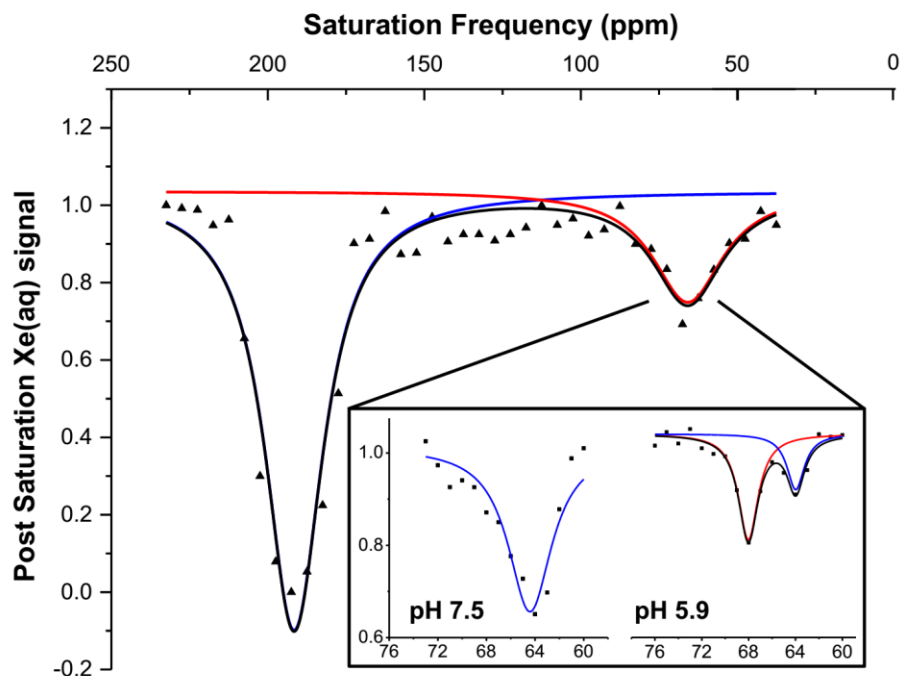


Figure 3. 9. Hyper-CEST scan of WEC (1 μ M) at 300 K. Full image was collected with 5-ppm step and individual peaks with 1-ppm step at pH 7.5 and pH 5.9 (inset). Reprinted with permission from B.A. Riggle; Y. Wang; I.J. Dmochowski. A “Smart” ^{129}Xe NMR Biosensor for pH-Dependent Cell Labeling. *J. Am. Chem. Soc.* **2015**, 137, (16) 5542-5548. Copyright 2015 American Chemical Society.

We also investigated the pH sensitivity of WEC-hp ^{129}Xe NMR chemical shift to look at “normal” (pH 7.5) and acidic (pH 5.9) buffer solutions, Figure 3.9. Because depolarization efficiency is decreased with a narrower saturation pulse, WEC was employed at 1 μ M concentrations, which is still at least 10^3 -fold more dilute than demonstrated for ^1H CEST pH reporters.¹⁶⁶ Prior to detecting a free xenon signal, a loop of selective Dsnob-shaped saturation pulses was scanned over the chemical shift range of 40-230 ppm in 5-ppm (700 Hz) steps, which corresponded to pulse length of 3748.6 μ s and power of 77 μ T. Two saturation responses centered at 195 ppm ($^{129}\text{Xe}@H_2O$) and 65 ppm ($^{129}\text{Xe}@WEC$) were observed (Figure 3.9 full image). By decreasing the frequency

scanning step size to 1 ppm (138.2 Hz), which corresponded to shaped pulse length of 19014 μ s and power of 15 μ T, we were able to distinguish the WEC-encapsulated ^{129}Xe peak for pH 7.5 and pH 5.9 samples at 300 K (Figure 3.9, inset). The total time to record the Hyper-CEST NMR spectra was composed of xenon delivery time (20 s) and data collection time. For the latter, each data point required time T :

$$T = (sp6 + d12) * L6 + d1 + p1$$

In the 5-ppm step scanning experiments, $sp6$ (saturation pulse length) = 3.748 ms, $d12$ (delay between saturation pulses) = 20 μ s, $L6$ (number of saturation cycles) = 400, $d1$ (delay before acquisition pulse) = 0.5 s, $p1$ (acquisition pulse) = 22 μ s. Thus, the total time needed to acquire the whole spectrum was 860 s. In the 1-ppm step scanning experiments, $sp6$ = 19.014 ms, $L6$ = 600, and the total time needed was 478 s. The observed pair of peaks at pH 5.9 was similar to hp ^{129}Xe NMR data collected for 30 μ M WEC by direct detection at pH 6.5 (Figure 3.6). As illustrated by these data, the Hyper-CEST ^{129}Xe NMR spectrum readily distinguished between physiologically normal and acidic pH values.

Cellular Hyper-CEST ^{129}Xe NMR

Finally, we investigated the utility of WEC in a biological setting through ^{129}Xe NMR cell studies. Human cervical carcinoma (HeLa) cells were grown in a flask to confluency. Cells were washed and suspended in either pH 7.5 or 5.5 sodium phosphate buffer containing 5-10 μ M WEC to give 1×10^7 cells/mL concentrations. Pluronic L-81 (0.1% final conc.) was added to reduce foaming that can result from Xe bubbling.⁷⁵ Cells were incubated in these conditions for 45-60 min and then transferred to an NMR tube. Spectra were acquired at both pH values with frequency scanning step size of 1 ppm (138.2

Hz), 400 cycles, which corresponded to shaped pulse length of 19014 μ s and power of 15 μ T. Figure 3.10a shows xenon in cells (196.3 ppm, red trace) and xenon dissolved in aqueous solution of HeLa cells suspended in pH 7.5 buffer with WEC (192.3 ppm, blue trace). Figure 3.10b shows WEC-encapsulated xenon in the same sample. $^{129}\text{Xe}@WEC_{aq}$, pH 7.5 gave a chemical shift of 65.0 ppm, which corresponds to free biosensor in buffer at pH 7.5.

In pH 5.5 experiments, Figure 3.10c shows two peaks, one for $\text{Xe}@cells$ (198.0 ppm, red trace) and one for $\text{Xe}@aq$ (192.0 ppm, blue trace). Figure 3.10d shows the biosensor region of the same sample and exhibits two peaks, one at 68.0 ppm corresponding to free alpha-helical WEC in buffer (blue trace) and one at 78.4 ppm that we assign to WEC inserted in cell membrane (red trace). Notably, upon biosensor-membrane insertion at pH 5.5, we observed a 13.4 ppm downfield chemical shift compared to biosensor-cell solutions at pH 7.5. Contrary to previous ^{129}Xe NMR cell studies performed with a more lipophilic cryptophane, we did not observe cryptophane-membrane association at pH 7.5.⁹⁰ This result is also consistent with previous studies with the EALA peptide that showed no membrane association at pH 7.5.¹⁶⁷ By targeting acidic pHe as a general cancer biomarker, along with membrane association, we increased the chemical shift difference between $\text{Xe}@biosensor_{cells}$ and $\text{Xe}@biosensor_{aq}$ as compared to earlier studies.^{75, 97, 147} We note that in living organisms there will be additional factors (beyond pHe) that impact the $^{129}\text{Xe}@biosensor_{cells}$ chemical shift, including cell type and membrane composition. Follow-up studies will be required to assess this variability.

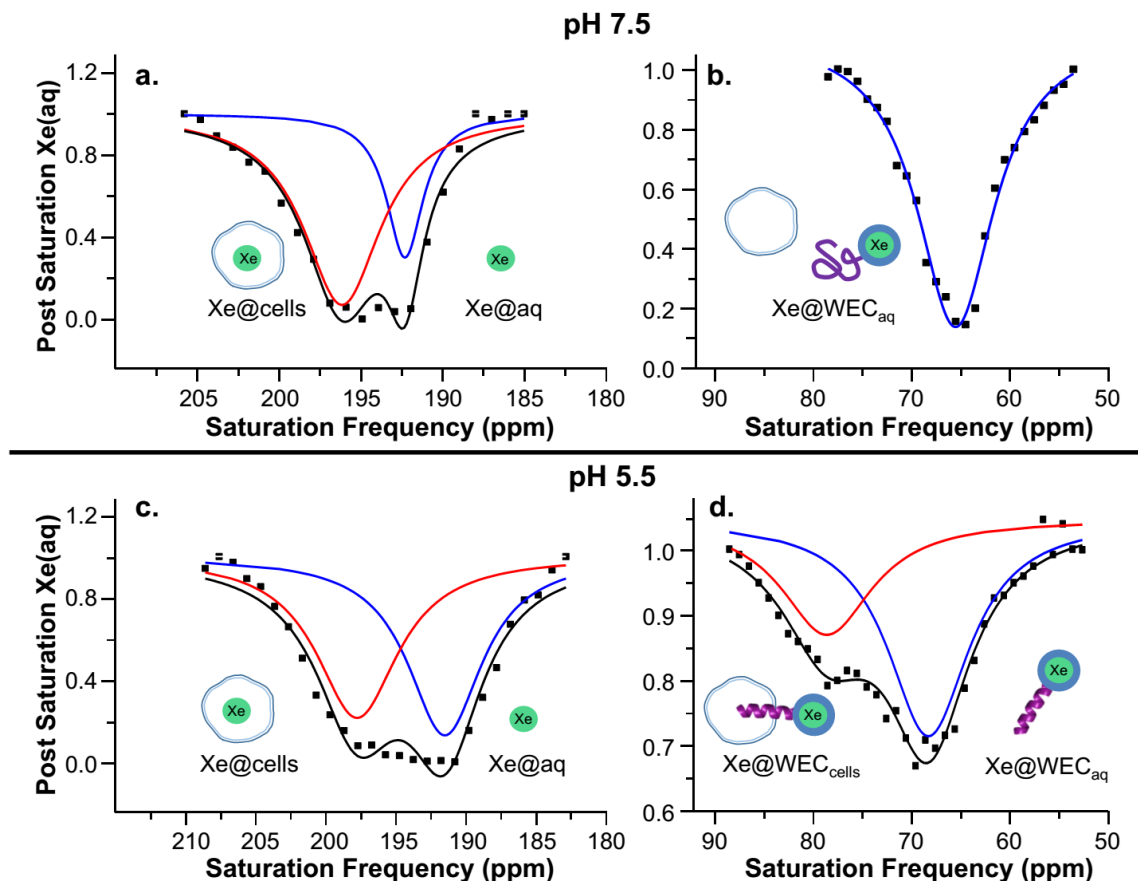


Figure 3. 10. Hyper-CEST ^{129}Xe NMR spectra for 5-10 μM WEC in 10 mM sodium phosphate buffer with 0.1% Pluronic L-81 in a suspension of 1×10^7 cells/mL. Data were collected at pH 7.5, a. Xe@cells-red trace (196.3 ppm), Xe@aq-blue trace (192.3 ppm); b. Xe@WEC_{aq} (65.0 ppm); and at pH 5.5, c. Xe@cells-red trace (198.0 ppm), Xe@aq-blue trace (192.0 ppm); d. Xe@WEC_{cells}-red trace (78.4 ppm) and Xe@WEC_{aq}-blue trace (68.0 ppm). Exponential Lorentzian fits are shown as colored, solid lines and the corresponding sums are shown as solid black lines. Reprinted with permission from B.A. Riggle; Y. Wang; I.J. Dmochowski. A “Smart” ^{129}Xe NMR Biosensor for pH-Dependent Cell Labeling. *J. Am. Chem. Soc.* **2015**, *137*, (16) 5542-5548. Copyright 2015 American Chemical Society.

§ 3.3 Conclusions

In summary, by attaching a pH-responsive, membrane-inserting peptide and two water-solubilizing moieties to a tripropargyl cryptophane host, we were able to generate an ultrasensitive ^{129}Xe NMR biosensor capable of labeling cells in acidic microenvironments. This xenon biosensor is unique for undergoing a rapid and reversible conformational

change (over a range of physiologic pH values) as well as functional changes: at pH 5.5, the pendant EALA-repeat peptide was mostly alpha-helical and gained membrane-insertion capabilities. This represents a “smart” ^{129}Xe MR contrast agent, and builds on previous examples of xenon biosensors that bind specific targets (e.g., protein receptors, DNA, cell-surface glycans) or undergo a modification event (i.e., enzyme-mediated peptide cleavage).

Significantly, this study demonstrated that appending the peptide to the ~1 nm diameter, hydrophobic cryptophane did not significantly reduce its ability to undergo a conformational change. Circular dichroism, Trp fluorescence, and hp ^{129}Xe NMR spectroscopies were employed to measure the change in helical character of the peptide in the pH range 5.5-7.5. EALA peptide helix formation resulted in a ^{129}Xe NMR downfield chemical shift change of 3.4 ppm, which was likely enhanced by significant cryptophane interactions with the nearby, N-terminal Trp residue. This suggests a general strategy for engineering larger chemical shift changes with xenon biosensors, particularly to monitor molecular events occurring nanometers away from the xenon-cryptophane reporter. These data represent a significant advance over the previous example of a peptido-cryptophane biosensor, which monitored MMP-7 activity: only a 0.5 ppm chemical shift change was observed upon enzyme-mediated peptide cleavage, perhaps because the Trp was positioned much farther from the cryptophane.⁶⁷ For some in vivo applications, it may be useful to maintain the full range of pH-dependent conformational changes of the EALA-repeat peptide, and it will be interesting to explore different peptides and conjugation strategies that work to achieve this goal.

The design of a cryptophane-EALA peptide conjugate capable of membrane insertion at acidic pH advances our long-range goal of developing ultrasensitive ^{129}Xe MR contrast agents to aid in cancer diagnosis and treatment.^{153, 158} Picomolar (10^{-11} M) concentrations of WEC were detected by Hyper-CEST NMR, making this approach 8-9 orders of magnitude more sensitive than commonly employed MR contrast agents. We demonstrated a 13.4 ppm downfield chemical shift change from disordered-peptide biosensor at pH 7.5 to the helical, membrane-inserted biosensor at pH 5.5. This represents the largest chemical shift change that has been engineered to date for a ^{129}Xe @cryptophane-biomolecule interaction, the magnitude of which should facilitate multiplexed detection in many experimental formats. The development and cellular implementation of this “smart” xenon biosensor are important steps towards future biomedical applications.

§ 3.4 Materials and Methods

General Information

Instrumentation and Methods. ^1H NMR (500 MHz) data were obtained in deuterated chloroform (CDCl_3) or dimethyl sulfoxide ($\text{DMSO}-d_6$) using a Bruker DMX 500 NMR spectrometer. Column chromatography was performed using silica gel (60 Å pore size, 40-75 µm particle size) from Sorbent Technologies. Thin layer chromatography (TLC) was performed using silica gel plates (60 Å pore size, Silicycle) with UV light at 254 nm as the detection method. MALDI-MS data were collected using a Bruker Ultraflex III TOF/TOF mass spectrometer. All HPLC purifications were performed on a Varian Prostar 210 system equipped with a quaternary pump and diode array detector. All air- and moisture-sensitive

reactions were performed under inert atmosphere in glassware flamed under vacuum, using anhydrous dry solvents. Standard workup procedures involved multiple (~3) extractions with the indicated organic solvent, followed by washing of the combined organic extracts with water or brine, drying over Na₂SO₄ and removal of solvents *in vacuo*. All yields reported were determined after purification by column chromatography or reverse phase HPLC, unless otherwise noted. All data were collected using instruments in the Chemistry Department at the University of Pennsylvania.

Materials. Organic reagents and solvents were used as purchased from the following commercial sources: Sigma-Aldrich: N,N-diisopropylethylamine (DIPEA); dimethyl sulfoxide (DMSO, anhydrous, 99.9%); Sigmacote®. Fisher: acetone (HPLC grade); chloroform (CH₂Cl₃, HPLC grade); dichloromethane (CH₂Cl₂, HPLC grade); ethyl acetate (EtOAc, HPLC grade); hexanes (HPLC grade); hydrochloric acid; methyl alcohol (MeOH, HPLC grade), perchloric acid (60%); Pluronic L-81; potassium carbonate (anhydrous); sea sand (washed); sodium chloride (NaCl); sodium hydroxide (NaOH); sodium sulfate (anhydrous). Novabiochem (currently EMD Millipore; Billerica, MA, USA): 6-azidohexanoic acid; 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU). MarCor: deionized (DI) water filtered (18 MΩ). Acros Organics: β-propiolactone (90%); cesium carbonate (Cs₂CO₃, 99.5%); chloroform-*d* (CDCl₃); 1,2-dibromoethane; 3,4-dihydroxybenzaldehyde (97%); N,N-dimethylformamide (DMF, 99.8%, anhydrous, acrosseal); dimethylsulfoxide-*d*₆, 4-hydroxy-3-methoxybenzyl alcohol (99%); propargyl bromide (80% solution in toluene); scandium(III) trifluoromethanesulfonate (Sc(OTf)₃, 95%); sodium borohydride (NaBH₄, 98%); sodium hydride

(NaH, 60% dispersion in mineral oil); tetrahydrofuran (THF, extra dry, over sieves); triisopropylsilane (TIS). MG Industries (Linde Group, NJ): xenon gas (scientific grade).

Synthetic Procedures

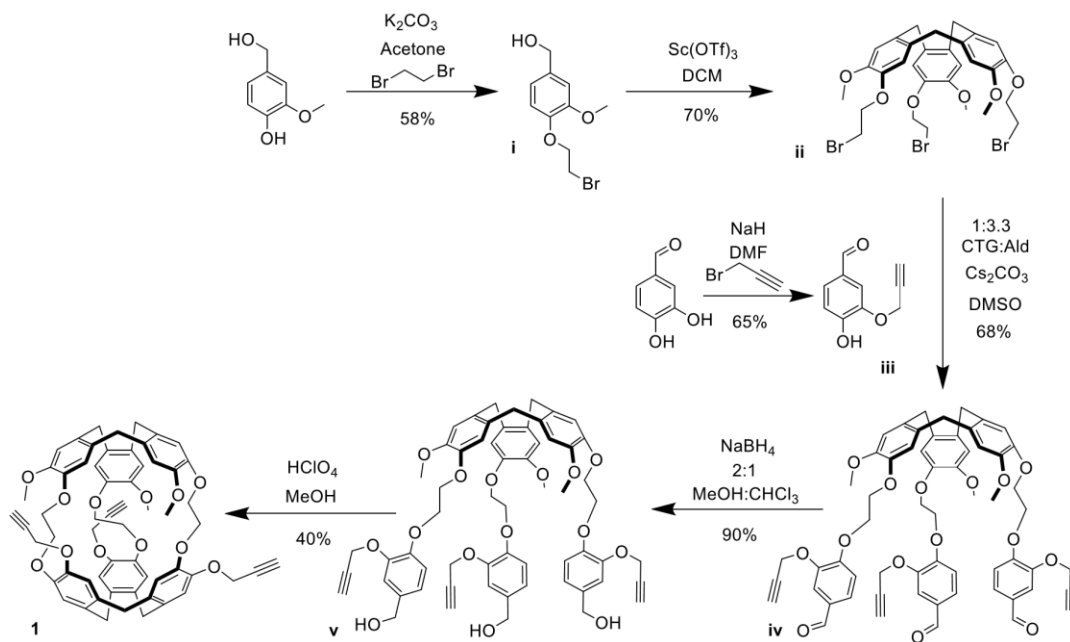


Figure 3. 11. 6-step, non-linear synthesis of tripropargyl cryptophane (**1**). Yield = 9.9% for the five linear steps. Reprinted with permission from B.A. Riggle; Y. Wang; I.J. Dmochowski. A “Smart” ^{129}Xe NMR Biosensor for pH-Dependent Cell Labeling. *J. Am. Chem. Soc.* **2015**, *137*, (16) 5542-5548. Copyright 2015 American Chemical Society.

Cryptophane Synthesis. Tripropargyl cryptophane was achieved in a 6-step synthesis with a 6.4% overall yield from two commercially available compounds, 3,4-dihydroxybenzaldehyde and vanillyl alcohol shown in Figure 3.11.

4-((2-bromoethoxy)-3-methoxyphenol) methanol (i): In a dry two-necked flask with a nitrogen inlet, vanillyl alcohol (10.0 g, 64.9 mmol, 1 eq) was combined with potassium carbonate (44.8 g, 324 mmol, 5 eq) in acetone (100 mL) and stirred at rt for 30 min. 1,2-dibromoethane (56.3 mL, 650 mmol, 10 eq) was then added to the reaction via syringe.

The reaction flask was then transferred to a pre-heated oil bath at 57 °C and refluxed overnight. The organic components were isolated with EtOAc on aqueous workup and the final product was purified by column chromatography (1:1 to 7:3 hexanes:EtOAc, gradient method; TLC 1:1 hexanes:EtOAc $R_f(\mathbf{i}) = 0.43$) to yield 10.2 g (39.1 mmol, 60% yield). The spectroscopic data match those reported in the literature.⁵⁴ ^1H NMR CDCl_3 : 6.96 (s, 1H, aryl); 6.91 (d, 1H, aryl); 6.89 (d, 1H, aryl), 4.64 (s, 2H, CH_2OH); 4.34 (t, 2H, $\text{OCH}_2\text{CH}_2\text{Br}$) 3.89 (s, 3H, OCH_3); 3.66 (t, 2H, $\text{OCH}_2\text{CH}_2\text{Br}$).

2,7,12-Tris-(2-bromoethoxy)-3,8,13-trimethoxy-10,15-dihydro-2H-

*tribenzo[*a,d,g*]cyclononene* (**ii**, also known as cyclotrimeratrylene) In a dry two-necked flask 8 g of 4-((2-bromoethoxy)-3-methoxyphenol methanol was combined with 0.50 g of scandium (III) trifluoromethanesulfonate and the reaction flask was put *in vacuo* for 1 h to remove moisture. An oil bath was preheated to 40 °C. Dry dichloromethane (200 mL) was added to the reaction flask and the reaction was put to reflux overnight. Reaction progress was monitored by TLC (98% DCM/2% Et_2O , $R_f(\mathbf{ii}) = 0.67$). The reaction was quenched on completion with 1 M NaOH and extracted with DCM. The final product was purified by flash column chromatography (isocratic method 1% Et_2O in DCM) to a yield of 70% (5.19 g, 7.12 mmol). The spectroscopic data match those previously described.⁵⁴ ^1H NMR CDCl_3 : 6.94 (s, 3H, cap aryl); 6.86 (s, 3H, cap aryl); 4.72 (d, 3H, H_{ax}); 4.31 (t, 6H, $\text{OCH}_2\text{CH}_2\text{Br}$); 3.86 (s, 9, OCH_3) 3.59 (t, 6H, $\text{OCH}_2\text{CH}_2\text{Br}$); 3.55 (d, 3H, H_{eq})

3-propargyloxy-4-hydroxybenzaldehyde (**iii**) To a flame or oven-dried round bottom flask cooled under nitrogen, 7.24 g of 60% sodium hydride (2.5 eq, 302 mmol) was added under argon. Dry DMF (50 mL) was added via syringe and the reaction was put to stirring at rt.

In a separate dry flask, 10 g (1 eq, 72.4 mmol) of 3, 4-dihydroxybenzaldehyde was dissolved in 40 mL dry DMF under argon and stirred until a homogenous solution was achieved. The reaction flask containing NaH was put on ice and chilled to 0 °C. The solution of 3, 4-dihydroxybenzaldehyde was added to the NaH dropwise over a period of ~10 min. The flask was subsequently rinsed with an additional 10 mL of dry DMF and added dropwise to the reaction flask while still on ice. The reaction was then allowed to stir for 45 min or until gas ceased to evolve while slowly warming to rt. Subsequently, 6.44 mL of propargyl bromide, 80% in toluene (0.8 eq, $\rho = 1.34$ g/mL, 57.9 mmol) was added dropwise to the reaction flask. The reaction was stirred for 4 h at rt and then placed on ice to quench with the addition of 1 M HCl. The reaction was extracted with EtOAc and purified by flash column chromatography with an isocratic method of 15% ethyl acetate in hexanes. Note, the desired meta-substituted product ($R_f(\text{iii}) = 0.48$) is not well resolved from the para substituted side product ($R_f = 0.46$). The final product was prepared in 65% yield (8.29 g, 47.1 mmol) with the spectroscopic data following our published protocols.⁶⁷

Product (meta): ¹H NMR δ (ppm) CDCl₃ (7.27): 9.84 (s, 1H, aldehyde); 7.53 (d, 1H, aryl); 7.49 (dd, 1H, aryl), 7.09 (d, 1H, aryl); 6.28 (bs, 1H, -OH); 4.84 (d, 3H, OCH₂CCH); 2.60 (t, 1H, OCH₂CCH). Side product (para): ¹H NMR CDCl₃ (7.27): 9.86 (s, 1H, aldehyde); 7.47 (d, 1H, aryl); 7.45 (dd, 1H, aryl), 7.12 (d, 1H, aryl); 5.84 (bs, 1H, -OH); 4.86 (d, 3H, OCH₂CCH); 2.62 (t, 1H, OCH₂CCH).

2,7,12-Tris-[2-[4-formyl-2-propargyloxyphenoxy]ethoxy]-3,8,13-trimethoxy-10,15-dihydro-2H-tribenzo[a,d,g]cyclononene (iv): An oil bath was preheated to 55 °C. In a two-necked round bottom flask 2.72 g of propargyl linker (iii), (3.3 eq, 15.48 mmol) and 5.05

g cesium carbonate (3.3 eq, 15.48 mmol) were combined and dried *in vacuo* for 1 h. Dry DMF (50 mL) was added to the reaction flask and stirred at rt for 30 min. Dry CTV (**ii**), (1 eq, 4.69 mmol) was added to the reaction and the reaction flask was submerged in the heated oil bath and stirred overnight. The reaction was diluted with brine and extracted with DCM. Purified product was achieved from flash column chromatography, isocratic method 3% acetone in 97% DCM, R_f (**iv**) = 0.63. Final product was obtained in 68% yield (1.08 g, 1.06 mmol) and confirmed to agree with our previously published data.⁵⁴ ^1H NMR δ (ppm) CDCl_3 (7.27): 9.87 (s, 3H, Ph-CHO), 7.56 (s, 3H, aryl), 7.50 (d, J = 8.2 Hz, 3H, aryl), 7.05 (d, J = 8.2 Hz, 3H, aryl), 7.01 (s, 3H, aryl), 6.86 (s, 3H, aryl), 4.77 (d, 3H, H_{ax}), 4.74 (q, 6H, -O-CH₂-CCH), 4.40-4.44 (m, 12H, Ph-CH₂-OH, -O-CH₂-CCH), 3.75 (s, 9H, -O-CH₃), 3.57 (d, J = 13.8 Hz, 3H, H_{eq}), 2.52 (t, J = 2.3 Hz, 3H, -O-CH₂CCH).

*2,7,12-Tris-[2-[4-(hydroxymethyl)-2-propargyloxyphenoxy]ethoxy]-3,8,13-trimethoxy-10,15-dihydro-2H-tribenzo[*a,d,g*]cyclononene* (**v**) Compound **iv** was dissolved in a 1:1 mixture of chloroform and THF. Next, MeOH was added slowly to ensure **iv** remained in solution. The reaction as then chilled to -10 °C with a salted ice bath. NaBH₄ was then added and the reaction stirred at 0 C for 20 min. The reaction was allowed to warm to rt and stir for 4 h. Reaction progress was monitored by TLC (5% MeOH in CH₂Cl₂), R_f (**v**) = 0.25. Additional equivalents of NaHB₄ were added as necessary, until quantitative conversion was observed. No further purification was necessary after aqueous work-up. In this manner, >90% yield (0.971 g, 0.951 mmol) was achieved. Spectroscopic data agreed with our previously published work.⁵⁴ ^1H NMR δ (ppm) CDCl_3 (7.27): 7.03 (s, 3H, aryl), 6.98 (s, 3H, aryl), 6.89 (m, 6H, aryl), 6.82 (m, 3H, aryl), 4.74 (d, J = 13.6, 3H, H_{ax}), 4.60

(m, 12H, Ph-CH₂-OH, -O-CH₂-CCH), 4.34 (m, 12H, -O-CH₂-CH₂-O-), 3.71 (s, 9H, -O-CH₃), 3.52 (d, J = 13.8 Hz, 3H, **H_{eq}**), 2.45 (t, J = 2.2 Hz, 3H, -O-CH₂CCH).

Tripropargyl Cryptophane (1): Methanol (200 mL) was added to a 1 L reaction flask containing compound **v** (50.0 mg, 0.0490 mmol, 1 eq). The reaction was put to stirring in a salted ice bath (-10 °C) and fitted with an addition funnel. Perchloric acid (60%) (150 mL) was added drop-wise over several hours. Once addition was complete, the reaction was allowed to slowly warm to rt and stir overnight. Afterwards, the reaction was again placed on a salted ice bath and more perchloric acid (50 mL) was added. The reaction was monitored by TLC (5% acetone in CH₂Cl₂) and subsequent 25 mL perchloric acid additions were performed as necessary to drive the reaction to completion. The reaction was quenched with the addition of dH₂O and brine (200 mL each) and then extracted 3x with CH₂Cl₂ (100 mL). The combined organics were then washed with dH₂O, sat. sodium bicarbonate, and brine (100 mL), dried over sodium sulfate and concentrated *in vacuo*. The crude material was purified by silica gel flash column chromatography (5% acetone in CH₂Cl₂, v/v) to yield 20.0 mg (0.0207 mmol, 40% yield) of **1** as a white powder. TLC (silica gel, 5% acetone in CH₂Cl₂, v/v); R_f(**1**)= 0.47. The spectroscopic data match those reported in the literature.^{54, 55} ¹H NMR δ(ppm) CDCl₃ (7.27) : 6.91 (s, 3H, aryl), 6.80 (s, 3H, aryl), 6.78 (s, 3H, aryl), 6.71 (s, 3H, aryl), 4.71 (d, 3H, **H_{ax}**), 4.70 (d, 3H, **H_{ax}**), 4.62 (q, 9H, -O-CH₂-CCH), 4.17 (m, 12H, -O-CH₂-CH₂-O), 3.83 (s, 9H, -O-CH₃), 3.43 (d, J = 13.8 Hz, 3H, **H_{eq}**), 3.42 (d, J = 13.9 Hz, 3H, **H_{eq}**), 2.71 (t, J = 2.3 Hz, 3H, -O-CH₂-CCH).

Peptide Synthesis and Purification. The EALA-repeat peptide, sequence: WEAALAEALAEALAEHLAEALAEALEALAA, was synthesized (100 micromole scale) by

solid-phase peptide synthesis, using Fmoc chemistry, on a Liberty 1 Automated Microwave Peptide Synthesizer located in the UPenn Biological Chemistry Resource Center. Piperidine (20%) in DMF was used as the deprotection agent, 0.5 M HBTU in DMF was used as the activator, and 2 M DIPEA in N-methyl-2-pyrrolidone (NMP) was used as the activator base. Five molar equivalents of the amino acid were used for each coupling on Rink Amide MBHA resin (0.59 mmol/g substitution, Novabiochem). Residue Ala₃₀ used method 1 and His₁₆ used method 3 and all other residues used method 2. Method 1: Initial 30 s microwave deprotection (35 W, 75 °C), followed by 3-min microwave deprotection (35 W, 75 °C). Two consecutive coupling cycles were used. Both couplings were for 5 min under microwave power (25 W, 75 °C). The instrument was then paused and taken off-line while the unreacted resin was acetylated with a mixture of 2 mL acetic anhydride, 1.2 mL of *N*-methylmorpholine (NMM), and 16.8 mL of DMF. Method 2: Initial 30-s microwave deprotection (35 W, 75 °C), followed by a 3-min microwave deprotection (35 W, 75 °C). Two consecutive coupling cycles were used. Both couplings were for 5 min under microwave power (25 W, 75 °C). Method 3: Initial 30-s microwave deprotection (35 W, 75 °C), followed by a 3-min microwave deprotection (35 W, 75 °C). Two consecutive coupling cycles were used. Both couplings were for 2 min at rt followed by 3 min under microwave power (25 W, 50 °C). The peptide then underwent a final deprotection (initial 30 s microwave deprotection (35 W, 75 °C), followed by a 3 min microwave deprotection (35 W, 75 °C) leaving the peptide on resin with a free N-terminus.

Azido-EALA peptide (2): The EALA peptide on resin in a sigmacoted vessel (0.1 mmol, 1 eq) was incubated in an excess (~10 mL) of DMF with magnetic stirring for 30 min to

allow for resin swelling. DMF was subsequently removed by vacuum suction. The 6-azidohexanoic acid (78.6 μ L, 0.5 mmol, 5 eq) was combined with DMF (6 mL), HBTU (190 mgs, 0.5 mmol, 5 eq) and DIPEA (175 μ L, 1.0 mmol, 10 eq) and added to the resin under magnetic stirring for 1 h. The reagent mixture was drained with vacuum suction and the resin was iteratively washed with MeOH, DCM, and DMF and then drained with vacuum. The resin was subjected to a second round of coupling with the same reagent mixture. After rinsing extensively, the resin was dried over DCM for 30 min on vacuum. The azido peptide was then dosed with a cleavage cocktail of 95% trifluoroacetic acid (TFA), 2.5% triisopropylsilane (TIS), and 2.5% dH₂O and stirred for 2.25 h. The vessel was drained and the resulting solution was collected and reduced to approximately 1 mL on a rotary evaporator. The peptide was then precipitated from solution with cold diethyl ether and the resulting solid was isolated by centrifugation. The peptide was resuspended in HPLC solvents (1:1 acetonitrile : dH₂O with 0.1% TFA). The azido-EALA peptide was purified using reverse-phase HPLC and monitored at 215 and 280 nm using a Zorbax RxC8 semi-preparative column (9.4×250 mm, 5 μ m beads). The elution gradient was composed of two solvents: 0.1% aqueous TFA (solvent A) and a 0.1% solution of TFA in CH₃CN (solvent B). The purification method went from 65% A to 40% A over 5 min, then from 40% A to 25% A over 5 min, and then from 25% A to 0% A over 35 min at a flow rate of 4 mL/min and with a 1 mL injection volume. The peptide eluted at 21.04 min, Figure 3.12. MALDI MS m/z calculated for N₃-EALA C₁₄₂H₂₂₅N₃₇O₄₅ (M+H⁺) 3169.65; found 3169.51, Figure 3.13.

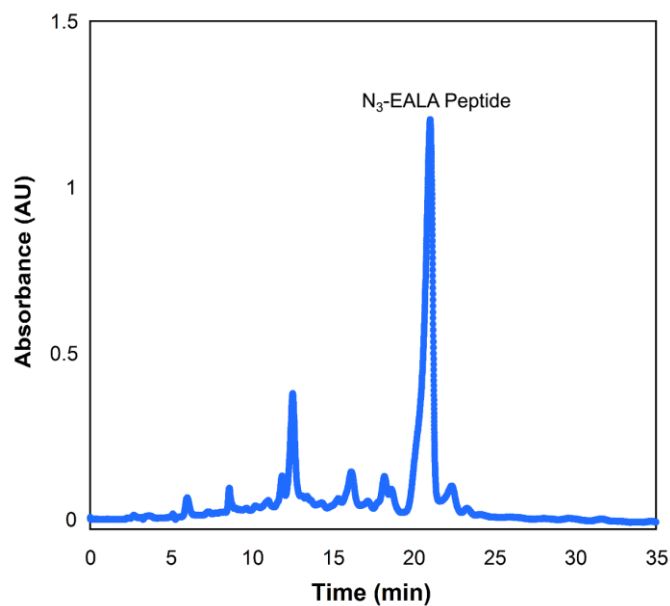


Figure 3. 12. HPLC chromatogram of N₃-EALA (**2**). UV absorbance monitored at 280 nm. Peak assignment based on MALDI-MS. Reprinted with permission from B.A. Riggle; Y. Wang; I.J. Dmochowski. A “Smart” ¹²⁹Xe NMR Biosensor for pH-Dependent Cell Labeling. *J. Am. Chem. Soc.* **2015**, 137, (16) 5542-5548. Copyright 2015 American Chemical Society.

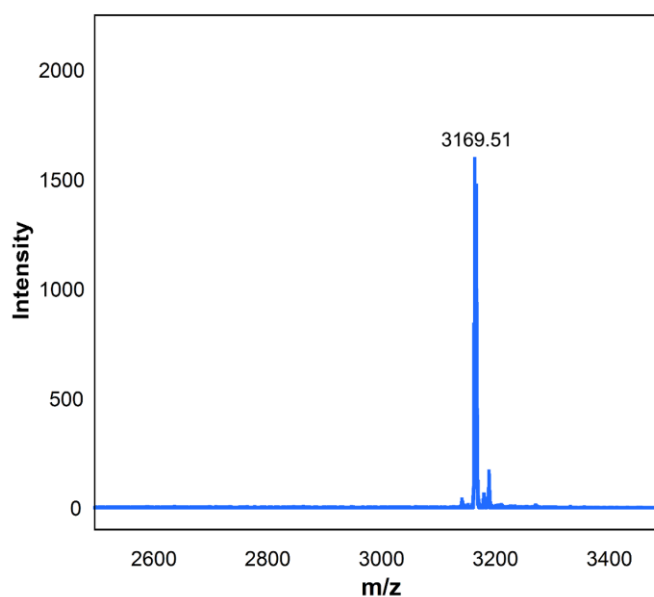


Figure 3. 13. MALDI-MS spectrum of N₃-EALA peptide (**2**). Expected mass [M+H⁺] 3169.65; found 3169.51. Reprinted with permission from B.A. Riggle; Y. Wang; I.J. Dmochowski. A “Smart” ¹²⁹Xe NMR Biosensor for pH-Dependent Cell Labeling. *J. Am. Chem. Soc.* **2015**, 137, (16) 5542-5548. Copyright 2015 American Chemical Society.

Solubilizing Linker Synthesis. 3-Azidopropionic acid was prepared from β -propiolactone by literature procedure and matched the reported ^1H NMR spectrum.¹⁶⁸

3-azidopropionic acid (**4**). Briefly, sodium azide (4.5 g, 0.69 mmol, 1 eq) was dissolved in ddH₂O water. β -propiolactone (4.4 mL, 0.069 mmol, 1 eq) was added dropwise and the reaction was allowed to stir at rt for 6 h. The reaction was neutralized with 1 M HCl and then extracted 3 times with diethyl ether. The organic layer was then dried over sodium sulfate and filtered through cotton. A clear oil in a 15% yield was recovered, requiring no further purification.

WEC Biosensor Synthesis. The copper(I)-catalyzed [3+2] azide-alkyne cycloaddition (CuAAC) between N₃-EALA peptide and cryptophane and the subsequent cycloaddition reaction between the cryptophane and 3-azidopropionic acid yielded the WEC biosensor.

EALA-cryptophane (**3**). To conjugate the azido-EALA peptide to the tripropargyl cryptophane (**1**) CuAAC was utilized with modified conditions of those previously employed.^{67, 68, 70, 73} Firstly, 4 mg (1.0 eq) of **1** and 13 mg of **2** (1 eq) were dissolved in 1 mL of dry methyl sulfoxide (DMSO) in a conical reaction vessel along with 1 cm of 18 g copper wire. The reaction mixture was put to stirring and degassed. In a separate vial, 10 mg (5 eq) of tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methylamine (TBTA) copper ligand was dissolved in 60 μL of DMSO. Separate solutions of 0.6 M copper (II) sulfate (CuSO₄) and 4 M sodium ascorbate (NaAsc) in water were also prepared. To the TBTA solution, 6 μL of CuSO₄ (1 eq), 0.5 μL of 2,6-lutidine (1 eq), and 9 μL (+)-sodium-L-ascorbate (10 eq) were added sequentially, vortexing between additions. After addition of NaAsc, the mixture turned clear indicative of Cu(I) formation. The entire reagent mixture was then

added to the reaction vessel. The reaction vessel was again degassed and then covered with foil and allowed to stir overnight under nitrogen at rt. A small aliquot of reaction was removed (10 μ L) and diluted in an HPLC solvent mixture, 50:50 mixture of acetonitrile and water with 0.1% trifluoroacetic acid. To verify product formation, analytical reverse-phase HPLC was performed using a Zorbax RxC8 analytical column (4.6 $\times$ 150 mm, 5 μ m beads) and monitored at 215 and 280 nm. The elution gradient was composed of two solvents: 0.1% aqueous TFA (solvent A) and a 0.1% solution of TFA in CH₃CN (solvent B). The purification method went from 65% A to 40% A over 15 min, then from 40% A to 18% A over 25 min, and then from 18% A to 0% A over 3 min at a flow rate of 1 mL/min with a 1 mL injection volume. The EALA-cryptophane eluted at 36.50 min, Figure 3.14. MALDI MS m/z calculated for EALA-cryptophane C₂₀₂H₂₇₉N₃₇O₅₇ (M+H⁺) 4136.01; found 4136.84, Figure 3.15.

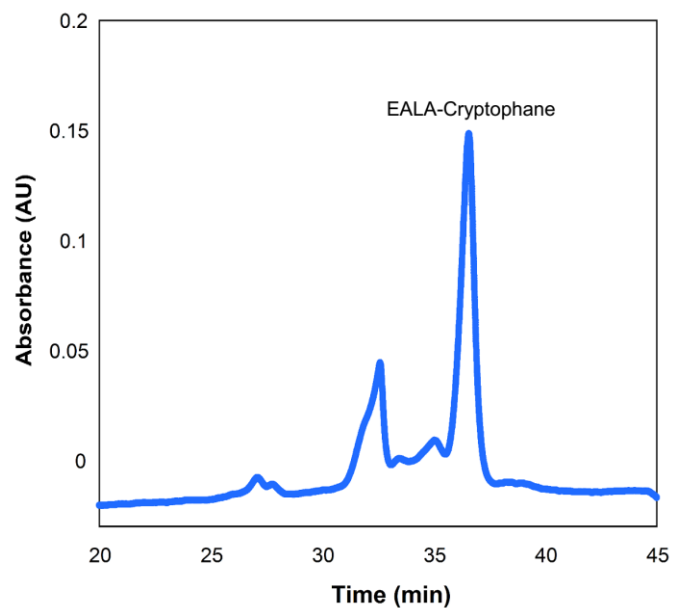


Figure 3. 14. HPLC chromatogram of EALA-cryptophane (**3**). UV absorbance monitored at 280 nm. Peak assignment based on MALDI-MS. Reprinted with permission from B.A. Riggle; Y. Wang; I.J. Dmochowski. A “Smart” ^{129}Xe NMR Biosensor for pH-Dependent Cell Labeling. *J. Am. Chem. Soc.* **2015**, *137*, (16) 5542-5548. Copyright 2015 American Chemical Society.

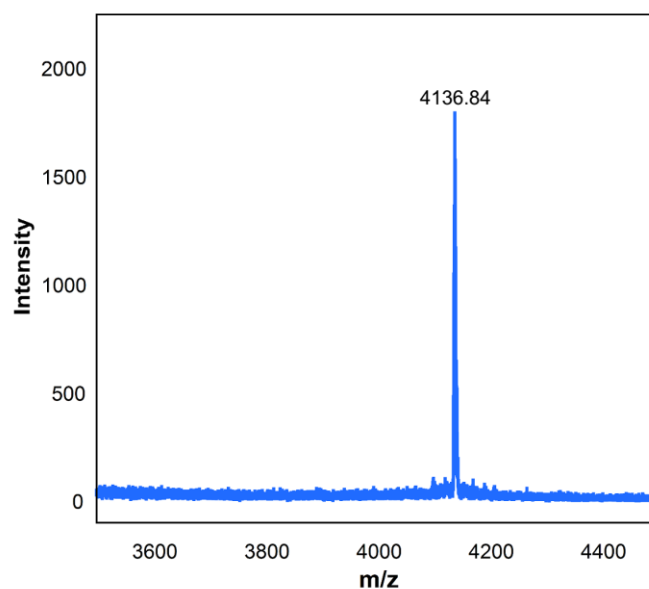


Figure 3. 15. MALDI-MS spectrum of EALA-cryptophane (**3**). Expected mass $[\text{M}+\text{H}^+]$ 4136.01; found 4136.84. Reprinted with permission from B.A. Riggle; Y. Wang; I.J. Dmochowski. A “Smart” ^{129}Xe NMR Biosensor for pH-Dependent Cell Labeling. *J. Am. Chem. Soc.* **2015**, *137*, (16) 5542-5548. Copyright 2015 American Chemical Society.

Water-soluble EALA-cryptophane (WEC) (5). The crude reaction (**3**) was then dosed with 4.6 μ L 3-azidopropionic acid (**4**) (10 eq), and the same reagent mixture as in the previous reaction and allowed to stir overnight while covered, to yield the water-soluble EALA-cryptophane (WEC) biosensor. The reaction was diluted 10-fold in 50:50 ACN:H₂O and purified. Purification was achieved through reverse-phase HPLC employing the semi-preparative version of the previous method by using a Zorbax RxC8 semi-preparative column (9.4 $\times$ 250 mm, 5 μ m beads) with 4 mL/min flow rate. The pH sensor eluted at 33.87 min, Figure 3.16. MALDI MS m/z calculated for WEC C₂₀₈H₂₈₉N₄₃O₆₁ (M+H⁺) 4366.08; found 4366.36, Figure 3.17. The pure fractions were then collected and diluted with 0.1 % ammonium hydroxide to 10% acetonitrile. Amicon-ultra-4mL 3K NMWL Centricon tubes were used to concentrate and buffer exchange the final product **5** into 10 mM sodium phosphate buffer at pH 7.5.

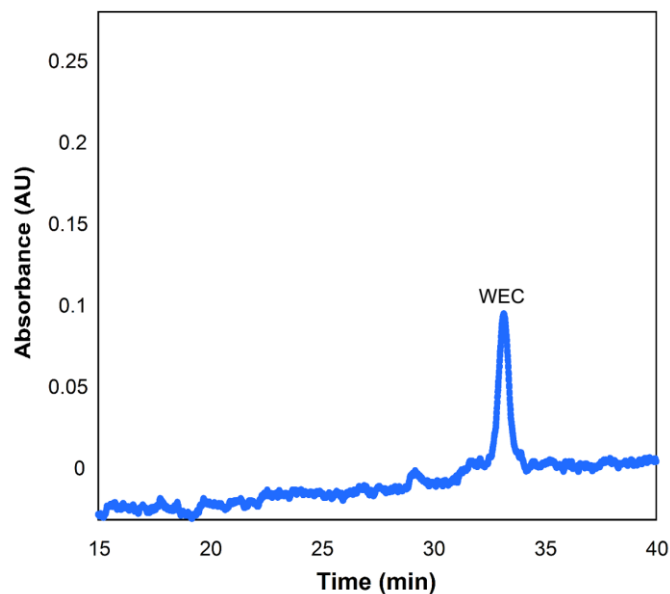


Figure 3. 16. HPLC chromatogram of WEC (**5**). UV absorbance monitored at 280 nm. Peak assignment based on MALDI-MS. Reprinted with permission from B.A. Riggle; Y. Wang; I.J. Dmochowski. A “Smart” ^{129}Xe NMR Biosensor for pH-Dependent Cell Labeling. *J. Am. Chem. Soc.* **2015**, 137, (16) 5542-5548. Copyright 2015 American Chemical Society.

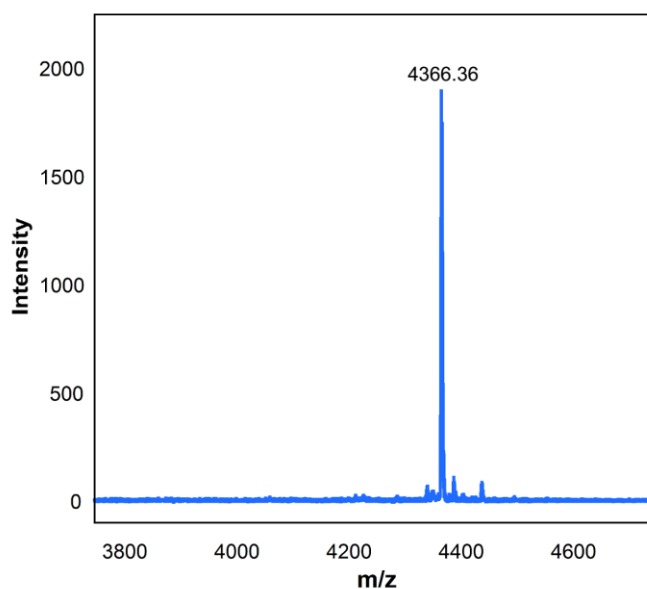


Figure 3. 17. MALDI-MS spectrum of WEC (**5**). Expected mass $[\text{M}+\text{H}^+]$ 4366.08; found 4366.36. Reprinted with permission from B.A. Riggle; Y. Wang; I.J. Dmochowski. A “Smart” ^{129}Xe NMR Biosensor for pH-Dependent Cell Labeling. *J. Am. Chem. Soc.* **2015**, 137, (16) 5542-5548. Copyright 2015 American Chemical Society.

Characterization Methods

Electronic Circular Dichroism (ECD) Spectroscopy. All spectroscopy experiments were performed on the Aviv 410 CD spectrometer. Data were collected at 25 °C from 260-190 nm, with a 30 s averaging time, 1 nm wavelength step, 1 s averaging time, and 1 nm bandwidth. The samples were prepared by concentrating the purified WEC to 30 μ M in 10 mM sodium phosphate at pH 7.5 and aliquoting the stock into five Eppendorf tubes and adjusting the pH to 5.5, 6.0, 6.5, 7.0, and 7.5 with a few microliters of 1 M HCl. The concentration was confirmed by measuring the absorbance at 280 nm, $\epsilon_{280} = 17,700 \text{ M}^{-1} \text{ cm}^{-1}$ and using an Agilent 89090A UV-visible spectrophotometer.

Data Analysis. The molar ellipticity was calculated from the observed ellipticity (mdeg) and has the units of $\text{deg cm}^2 \text{ dmol}^{-1}$. The molar ellipticity is given by equation 3.1 where C is the concentration of the peptide or biosensor, ℓ is the path length of the cuvette-0.1 cm, and n_r is the number of residues-31.¹⁶⁹

$$[\theta] = \frac{\theta_{\lambda}}{(C * \ell * n_r * 10)} \quad (3.1)$$

The helical content of the peptide and biosensor was determined from circular dichroism studies at 30 μ M concentrations and in accordance with literature precedent.¹⁷⁰ Helicity was calculated using the formulas 3.2 and 3.3 where n_r is the number of amide bonds in the peptide, in this case 31. Racemic cryptophane was employed for these experiments, and thus did not contribute to the measured CD signal. Data are shown in Tables 3.1 and 3.2 and graphically in Figure 3.3.

$$\%helicity = 100 * \frac{[\theta]_{222}}{\max[\theta]_{222}} \quad (3.2)$$

$$\max[\theta]_{222} = -40000 * [1 - \frac{2.5}{n_r}] \quad (3.3)$$

pH Reversibility. Repeated trials with the azido-peptide alone and with WEC showed that the peptide conformational change was reversible. Whether the sample was first prepared at pH 5.5 or at pH 7.5 and titrated to higher or lower pH in this range, the ECD signal confirmed the conformation aligned with what is shown in Figure 3.2. One example of this reversibility is shown in Figure 3.4. In this experiment the peptide sample at pH 7.5 shown in Figure 1a was titrated to pH 5.5 and the CD signal was measured. The same sample was then titrated back to pH 7.5 (labeled pH 7.5R) and the CD signal was measured and found to align with the original spectrum at pH 7.5.

Tryptophan Fluorescence Studies. All fluorescence studies were carried out on Photon Technology International (PTI) QuantaMaster™ 40 fluorescence spectrometer (Birmingham, NJ, USA). The 30 µM samples at pH 5.5, 6.0, 6.5, 7.0, and 7.5 were removed from the CD cuvettes and placed in the fluorimeter. Fluorescence spectra were collected at 25 °C in quartz cuvettes with a 1-cm path length. The samples were excited at 280 nm and emission data were collected from 300-400 nm. For all spectra the slit widths were 5 nm, scan rate was 60 nm/min, averaging time was 1 s, and the data interval was 1 nm.

Hyperpolarized ^{129}Xe NMR Spectroscopy. HP ^{129}Xe was generated using spin-exchange optical pumping (SEOP) method with a home-built version of the previously commercially available Nycomed-Amersham (now GE) model IGI.Xe.2000 ^{129}Xe hyperpolarizer. A gas mixture of 89% helium, 10% nitrogen, and 1% natural abundance xenon (Linde Group,

NJ) was used as the hyperpolarizer input. 795 nm circularly polarized diode laser was used for optical pumping of Rb vapor. ^{129}Xe was hyperpolarized to 10–15% after being cryogenically separated, accumulated, thawed, and collected in controlled atmosphere valve NMR tubes (New Era). After hp ^{129}Xe collection, NMR tubes were shaken vigorously to mix cryptophane solutions with hp Xe. All ^{129}Xe NMR measurements were carried out on a Bruker BioDRX 500 MHz NMR spectrometer (138.12 MHz frequency for ^{129}Xe), using a 10-mm BBO NMR probe. Sample temperature was controlled by VT unit on NMR spectrometer to 300 ± 1 K. Eburp2 shaped pulse was used to selectively excite Xe@WEC biosensor peak. Spectra were averaged over 16 scans. A delay of 0.5 s was given between scans to allow for xenon exchange. All acquired NMR spectra were processed with 60 Hz Lorentz broadening. Chemical shifts were referenced to free xenon gas of 0 atm at 0 ppm, shown in Figure 3.6.

Hyper-CEST Frequency Scan Spectroscopy. Prior to applying saturation pulse, gas mixture including hp ^{129}Xe was bubbled into a 10-mm NMR tube containing 2.5 mL sample solution by a home-built continuous-flow gas delivery setup. For each data point in the Hyper-CEST spectrum, the gas mixture was bubbled for 20 s, followed by a 3-s delay to allow bubbles to collapse. All Hyper-CEST experiments were carried out using a Bruker 500 MHz NMR spectrometer, with 10-mm PABBO probe. A 90° hard pulse of this probe has pulse length of 22 μs .

Hyperpolarized ^{129}Xe Chemical Exchange Saturation Transfer Depolarization Curve. Saturation frequencies of Dsnob shaped pulse were positioned at $(192.4 - 128.2) = 64.2$ ppm and $(192.4 + 128.2) = 320.6$ ppm, for “on” and “off” resonance, respectively. In each

experiment, pulse sequences of the following parameters were used: Pulse length $t_{\text{pulse}} = 3.748$ ms, field strength $B_{1,\text{max}} = 77$ μT , delay between pulse = 20 μs , maximum number of saturation cycles = 6000. Data are shown in Figure 5. Sample temperature was controlled by VT unit on NMR spectrometer to 310 ± 1 K. Xenon was introduced by continuous flow.

Cell Culture. Human cervical carcinoma (HeLa) cells in T-25 cell culture flasks in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum and 1% penicillin/streptomycin were grown to 80% confluency. The medium was then removed and the cells were washed 3x with Dulbecco's Phosphate Buffered Saline (DPBS). Cells were suspended with 0.25% trypsin incubation for 5 min. The trypsin was quenched with a 10-fold excess of DPBS. Cell suspension (10 μL) was removed and combined with 10 μL of Trypan Blue. Cells were counted with a hemocytometer after 5-min incubation. 1×10^7 cells/mL were used in all experiments. The cell suspension was then centrifuged for 7 min at 2 krpm and the cell pellet was isolated. Cells were resuspended in 10 mM sodium phosphate buffer with 0.1% Pluronic L-81⁷⁵ at either pH 5.5 or pH 7.5 and also 5-10 μM WEC. The cell/biosensor solution was gently vortexed to mix. After < 1-h incubation, the cell suspension was transferred to an NMR tube.

§ 3.5 Acknowledgments

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Chapter 4: ^{129}Xe as a Biophysical Probe of Protein Conformational Change

The content of this chapter has been submitted for publication. It has been adapted here:

Reprinted with permission from B.A. Riggle; M.L. Greenberg; Y. Wang; R.F. Wissner; E.J. Petersson; and I.J. Dmochowski. A “Turn-On” xenon-129 Biosensor for Calmodulin.

§ 4.1 Introduction

Calmodulin (CaM) is a small (17 kDa) calcium-binding messenger protein that is expressed in all eukaryotic cells. CaM transduces calcium signals to mediate a wide variety of processes from muscle contraction and metabolism to memory formation, inflammation and immune response. Because of the diverse roles CaM plays it is ubiquitously expressed throughout the body. CaM binds four Ca^{2+} ions, which activates binding to a variety of helix-forming peptide sequences with μM to nM K_D values.¹⁷¹ A solution structure of apo-CaM was solved and revealed Ca^{2+} binding induces necessary conformational changes for peptide binding.¹⁷² When CaM binds to cognate peptide it undergoes a dramatic conformational change (Figure 4. 1), which has been probed using many spectroscopic techniques.^{161, 171, 173-177} Here we investigate the potential for using sensitive ^{129}Xe NMR spectroscopy to detect active CaM in solution.

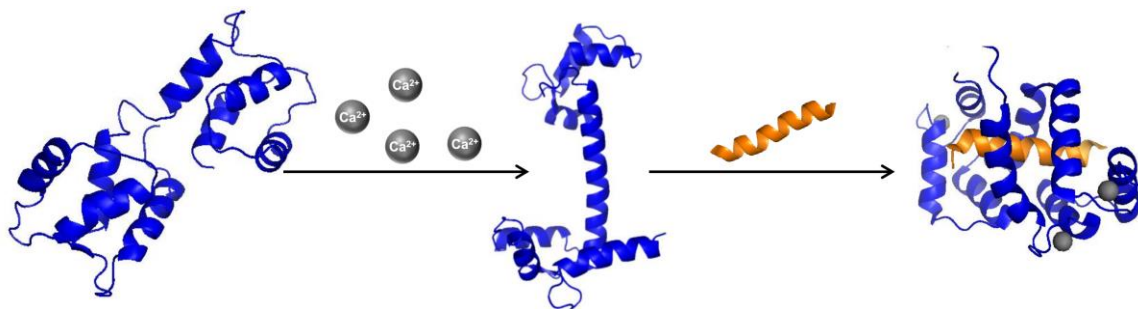


Figure 4. 1. Conformational change of calmodulin with the addition of 4 Ca^{2+} ions and subsequent folding in the presence of helical-peptide substrate.

Early work investigated the role of calcium-calmodulin and ion channel modulation. Specifically, researchers looked at the interaction of the CaM complex with cyclic nucleotide-activated cation channels that function to mediate olfactory and visual signal transduction.¹⁷⁸ They determined that CaM binds to a 26-amino acid N-terminal

portion of the transmembrane channel for rat (r), bovine (b), and fish (f) olfactory cyclic nucleotide-activated channel (OCNC) but that Ca^{2+} is necessary for substrate binding.¹⁷⁸ ¹⁷⁹ The CaM binding region, despite a high degree of sequence flexibility, is characterized by several attributes, namely, a basic amphiphilic structure, the ability to form helix, and two aromatic or long chain aliphatic “anchor residues” 12 amino acids apart, shown boxed in blue in Figure 4. 2. ^{178, 180, 181} From the original 26 amino acid sequence, later work determined a 17-mer truncated peptide that also bound CaM, termed FRRIAR.¹⁷³ The CaM-FRIARR interaction is a model for many protein-protein interactions, based on the burial of hydrophobic surface area and key electrostatic interactions, etc. CaM binds and regulates more than 30 target enzymes.¹⁷²

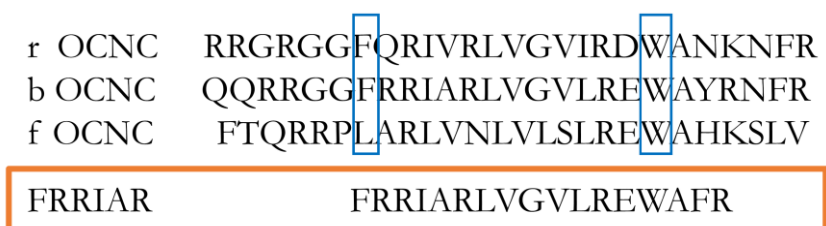


Figure 4. 2. Sequences of three in vivo substrates of CaM and the FRRIAR truncated substrate.

More recent work presented the NMR structure of calmodulin bound to α -synuclein.¹⁸² The role of Ca^{2+} -CaM in the function of α -synuclein is of particular interest due to the role of α -synuclein in Parkinson’s disease.

Cryptophane xenon host-guest chemistry for use as targeted contrast agents in MRI is an area of considerable research.¹⁸³ Our lab and others have developed ¹²⁹Xe NMR biosensors for detecting proteins and other biomolecules and metal ions in solution.^{113, 184} Less investigated is the potential for studying protein-protein interactions using ¹²⁹Xe

NMR spectroscopy.^{68, 73} Xenon-129 has a spin- $\frac{1}{2}$ nucleus and high isotopic abundance of 26.4%. Non-Boltzmann distributions of spins can be achieved by spin-exchange optical pumping (SEOP) in a processes termed hyperpolarization (hp). This characteristic coupled with the lack of endogenous xenon *in vivo* results in the potential for very large signal-to-noise ratios, compared to traditional proton MRI. Due to xenon's polarizable electron cloud, xenon exhibits appreciable affinity for small-molecule cryptophane hosts, and to a lesser extent, cucurbit[6]uril.^{44, 185} Cryptophanes can be made water-soluble and provide a hydrophobic cavity with internal volume well matched to that of Xe ($D = 4.3 \text{ \AA}$, $V \sim 40 \text{ \AA}^3$).^{55-57, 65} Our lab^{68-70, 73, 77} and others^{49, 66, 75, 95, 97, 147, 149} have designed a wide variety of cryptophane-xenon biosensors targeted to specific biomarkers indicated in cancer, including integrin and folate receptors, MMP-7, carbonic anhydrase. . . In these previous examples, cryptophane biosensor produced a specific hp ^{129}Xe NMR chemical shift, which then shifted downfield upon introduction of protein analyte.

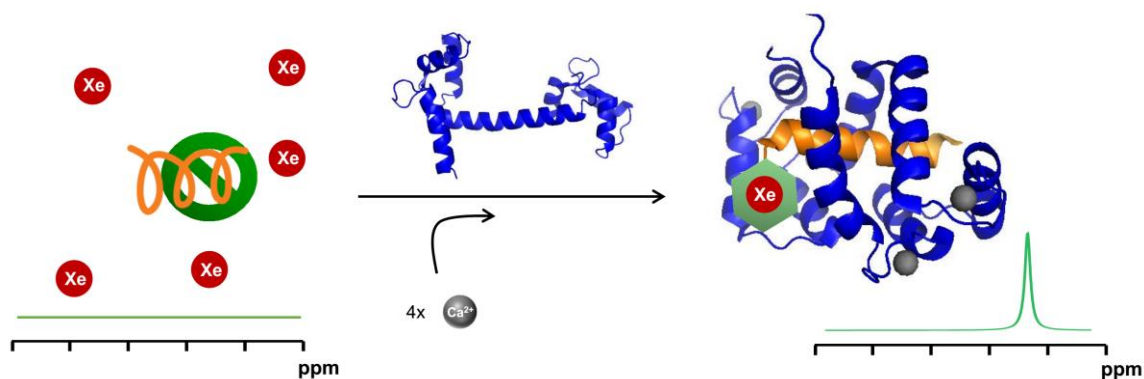


Figure 4. 3. ^{129}Xe "turn-on" NMR signal resulting from FRRIAR-TUC binding holo-calmodulin.

Cryptophane-based biosensors have been used to detect Zn^{2+} with xenon NMR¹¹³ as well as Pd^{2+} and Cd^{2+} .¹⁸⁴ In both of these cases the authors tethered cryptophane with a

nitrilotriacetic acid group and relied on changes in the chemical shift to differentiate between the three ions. We have chosen a different approach whereby we indirectly detect the presence of Ca^{2+} ions through a protein conformational change and protein-peptide interaction. Herein, we demonstrate a novel turn-on detection approach whereby we detect biosensor binding to calmodulin protein through hp ^{129}Xe -cryptophane NMR signal is absent until protein-peptide interaction occurs. We demonstrate a binary (ON/OFF) ^{129}Xe signal in the presence and absence of Ca^{2+} -activated CaM, Figure 4. 3. Building on our earlier work conjugating peptides to cryptophane, we present a synthetic route to conjugate the CaM-binding peptide, FRRIAR, to our tripropargyl cryptophane host molecule, Figure 4. 4.

§ 4.2 Results and Discussion

Synthetic Procedures

Figure 4. 4 shows the synthesis of the FRRIAR peptide-trifunctionalized, ultrasensitive cryptophane conjugate (FRRIAR-TUC) **5**, the details of which are provided in the Materials and Methods Section. Briefly, the synthesis of tripropargyl cryptophane **1** was performed in six non-linear steps with a yield for the five linear steps of 9.9%.⁷⁷ Azido-FRRIAR peptide **2** was prepared with standard Fmoc synthetic methods. The peptide was attached to the cryptophane via copper(I)-catalyzed [3+2] azide-alkyne cycloaddition (CuAAC) to form **3** after a 3 h reaction.^{111, 112} Mono-peptide cryptophane conjugates were achieved in preference by controlling reaction stoichiometry. The resulting triazole-hexyl spacer kept the peptide in close proximity to the ^{129}Xe nucleus while minimizing steric clashes with calmodulin during protein binding. Formation of compound **3** was confirmed

by MALDI-MS and the yield quantified by analytical reverse-phase HPLC to be quantitative as all the azido-peptide was consumed (these data are provided in the Materials and Methods section). A solubilizing linker, 3-azidopropionic acid **4**, was synthesized in one step from the commercially available β -propiolactone (see Material and Methods Section)^{70, 74} and reacted with crude **3** without the addition of more copper catalyst. Starting from tripropargyl cryptophane **1**, FRRIAR-TUC **5** was isolated in ~70% yield after sequential CuAAC reactions with **2** and **4** and HPLC purification to remove TBTA and tris-triazole propionic acid cryptophane.

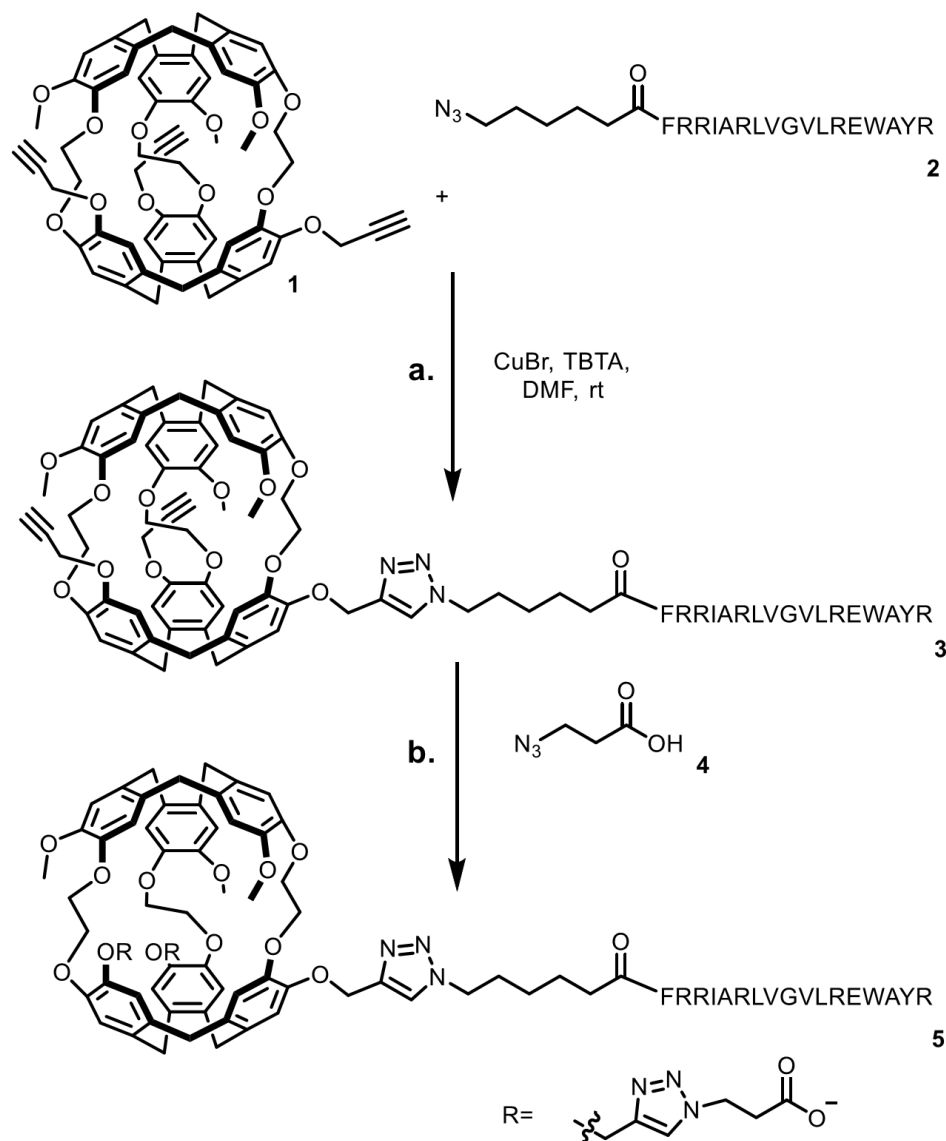


Figure 4. 4. Reaction scheme for the formation of FRRIAR peptide-trisubstituted ultrasensitive cryptophane (FRRIAR-TUC). a. **1** (1 eq), **2** (0.8 eq), CuBr (2 eq), TBTA (3 eq), 3 h; b. **3** (crude), **4** (10 eq), 12 h.

Initial attempts at synthesis were met with difficulty. [Note: all of the following attempts, unless otherwise noted, utilized the copper ligand TBTA]. Building off the work with EALA (see Chapter 3), 1.1 eq of FRRIAR were used. The reaction proceeded rapidly and resulted in the formation of di- and tri- substituted peptido-cryptophane which wasn't

readily separable by HPLC. Subsequent attempts utilized reduced equivalents of FRRIAR peptide; 0.8:1 peptide to cryptophane. This resulted in preferential formation of only the mono-substituted product, however the reaction did not seem to proceed to completion rather it stalled after 1 h. As time passed the product seemed to disappear, see Figure 4. 5. Control studies in which FRRIAR peptide and cryptophane in DMSO but without reagents and FRRIAR peptide in DMSO alone were monitored by HPLC over time. These time course studies demonstrated that FRRIAR formed some sort of irreversible aggregate that could not be resolubilized in mixtures of acetonitrile and water or in buffer after DMSO was removed by lyophilization. Other reaction solvents were investigated. The requirements were a solvent that could readily dissolve both the hydrophobic tripropargyl cryptophane and hydrophilic FRRIAR peptide. Additionally, a copper source was needed that would be compatible with the chosen solvent. The solubility of cryptophane and FRRIAR was tested in trifluoroethanol (TFE) and both were found to be readily soluble. Unfortunately, both copper sulfate and sodium ascorbate precipitated upon addition though minimal product formation was observed. Next, TFE was employed as a co-solvent with DMSO but aggregation was still observed. Copper(I) bromide was substituted for copper sulfate with some success but the reaction precipitated over the timescale of the required multitude of semi-preparative HPLC runs. Attempts were made in mixtures of acetonitrile and water but afforded no product, possibly because acetonitrile coordinated the copper. Finally, the stability of FRRIAR was investigated in N,N-dimethylformamide (DMF). The peptide appeared to be stable long term in DMF but sodium ascorbate wasn't soluble in DMF. Following literature precedent copper (II) bromide was used in conjunction with

triphenylphosphine to make a copper catalyst complex (in the absence of TBTA) which has been seen to efficiently result in triazole formation. Alas, this complex resulted in no product formation. Thus copper (I) bromide was again employed but this time in 100% DMF and found to work with strong efficacy.

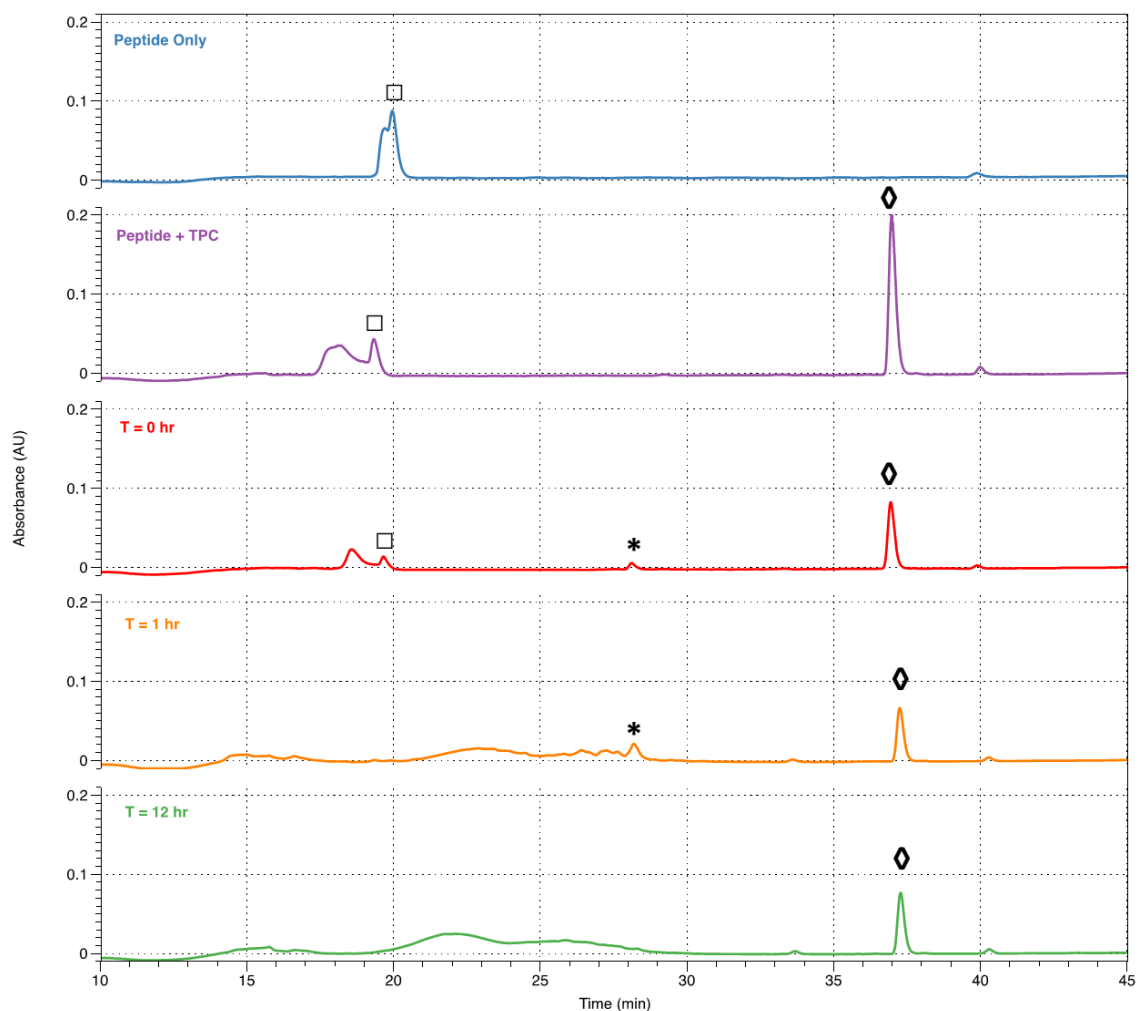


Figure 4. 5. Time-course HPLC of FRRIAR peptide degradation over time. Where the * denotes peptide-cryptophane conjugate formation, the ◊ indicates tripropargyl cryptophane, and the □ is FRRIAR peptide as confirmed by HPLC. No mass could be obtained on the increasing shoulder that elutes right before FRRIAR nor on the broad humps between 20 and 30 min at 12 h.

Gel Shift Assay

The binding of calmodulin protein to the peptide is readily observable by native gel shift assay as shown by Liu *et al.*¹⁷⁸ This assay was performed using polyacrylamide gel electrophoresis (PAGE) and through it we are able to determine that binding is still achieved and the binding stoichiometry of FRRIAR-TUC biosensor. This assay supports the idea that the biosensor, FRRIAR-TUC binds in 1:1 stoichiometry with CaM in agreement with the peptide alone.¹⁷⁸ These data are depicted in Figure 4. 18. At 0.25, 0.50, and 0.75 equivalents of FRRIAR-TUC biosensor two bands were observed--one band from CaM-Ca²⁺ and a second that migrated to a lesser degree, representing a complex of biosensor and CaM-Ca²⁺. This higher band increased in intensity with increasing equivalents of FRRIAR-TUC until it was a single band at 1:1 CaM-Ca²⁺:FRRIAR-TUC. This band migrated similarly to CaM-Ca²⁺:FRRIAR peptide.

Moving forward, we wanted to verify that FRRIAR-TUC would not bind to calmodulin in the absence of Ca²⁺. Apo CaM was prepared and the native gel was repeated with FRRIAR and FRRIAR-TUC. These data indicate FRRIAR-TUC induces CaM protein folding only in the presence of Ca²⁺ ions, Figure 4. 6. Indeed, FRRIAR-TUC seems to discriminate between apo and holo CaM more readily than FRRIAR alone.

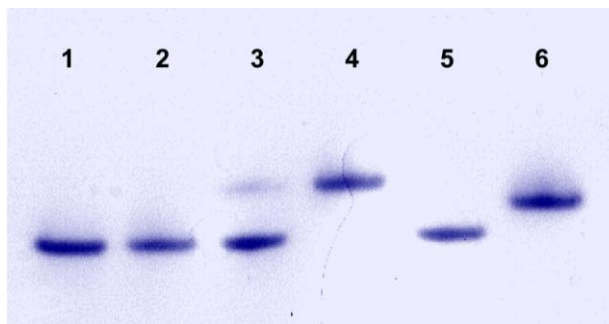


Figure 4. 6. Native gel shift assay, where sample are all 1:1 concentration of 10 μM CaM only or 10 μM CaM plus either 10 μM FRRIAR or 10 μM FRRIAR-TUC. 1) Apo CaM only; 2) Holo CaM only; 3) Apo CaM + FRRIAR peptide; 4) Holo CaM + FRRIAR peptide; 5) Apo CaM + FRRIAR-TUC; and 6) Holo CaM + FRRIAR-TUC.

Tryptophan Fluorescence

The FRRIAR peptide contains a single tryptophan residue towards the C-terminus that we hypothesized should provide a useful local probe of peptide and protein binding, as well as changes in peptide-cryptophane interaction.¹⁸⁶⁻¹⁸⁸ Fluorescence studies ($\lambda_{\text{ex}} = 295 \text{ nm}$) with peptide **2** demonstrated a hypsochromic shift and increased Trp emission intensity upon binding of calmodulin protein, Figure 4. 7a. Trp emission maximum wavelength for the FRRIAR peptide decreased from 352 nm to 332 nm with increasing concentrations of CaM protein. This quenching phenomenon was also observed in a much narrower, and considerably blue shifted range with FRRIAR-TUC and CaM from 328 nm to 325 nm (Figure 4. 7b), consistent with the Trp experiencing a less solvated environment near cryptophane. Cryptophane fluoresces ($\lambda_{\text{max}} = 313 \text{ nm}$) with comparable intensity to Trp, which further contributes significantly to the observed emission spectrum.^{55, 67}

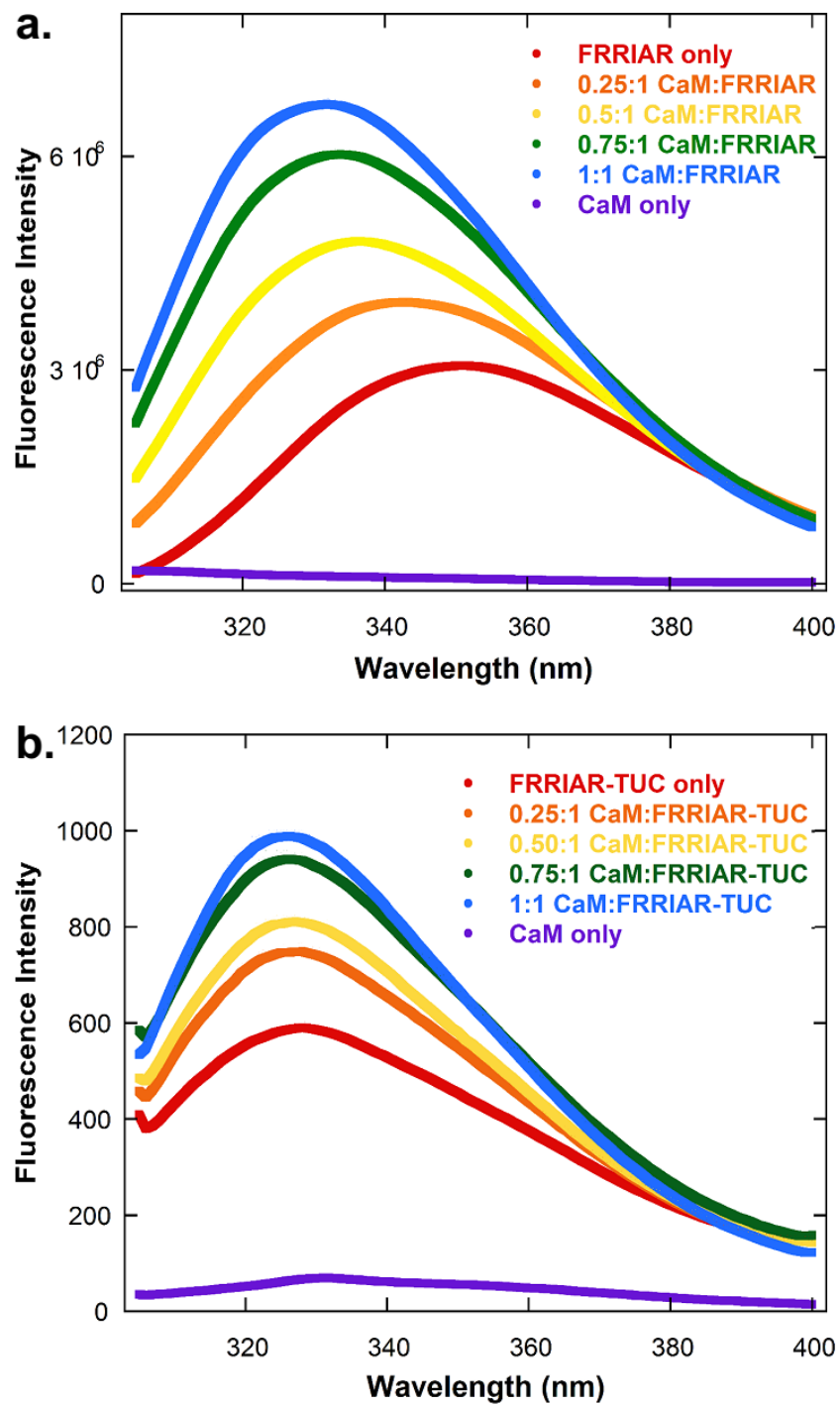


Figure 4. 7. Calmodulin binding monitored by Trp fluorescence for a. FRRIR peptide (30 μM) with varying concentrations of CaM protein (0, 7.5, 15, 22.5, or 30 μM) and; b. FRRIR-TUC (30 μM) with varying concentrations of CaM protein (0, 7.5, 15, 22.5, or 30 μM).

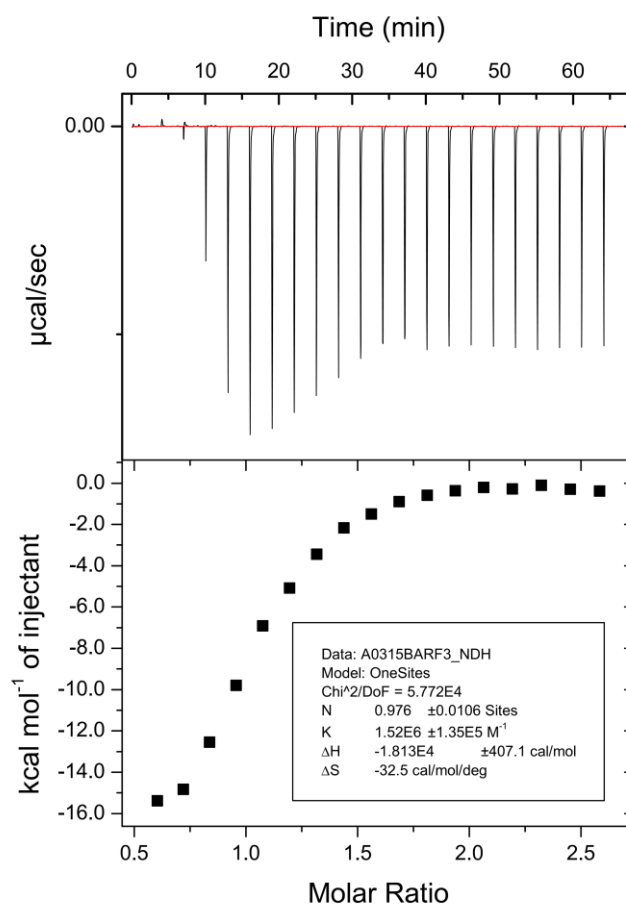


Figure 4. 8. Enthalpogram of FRRIAR-TUC (300 μM) titrated into CaM (30 μM).

Biosensor Binding Studies

The binding interaction between FRRIAR-TUC and CaM was characterized using isothermal titration calorimetry as our laboratory has done with previous biosensors.⁷⁰ Titration experiments were performed in 10 mM HEPES, 1 mM CaCl_2 pH 7.2. We determined $\Delta H = -18.1$ kcal/mol, which gives a sub-micromolar dissociation constant of 0.65 μM at rt, see Figure 4. 8. These data were someone convoluted by the presence of an initial dissociation process which may be the result of alleviating cryptophane-peptide

interactions before calmodulin binding can take place. Reversible cryptophane-FRRIAR association is not surprising as we have previously observed this phenomenon with other peptide-cryptophane constructs.^{67, 77}

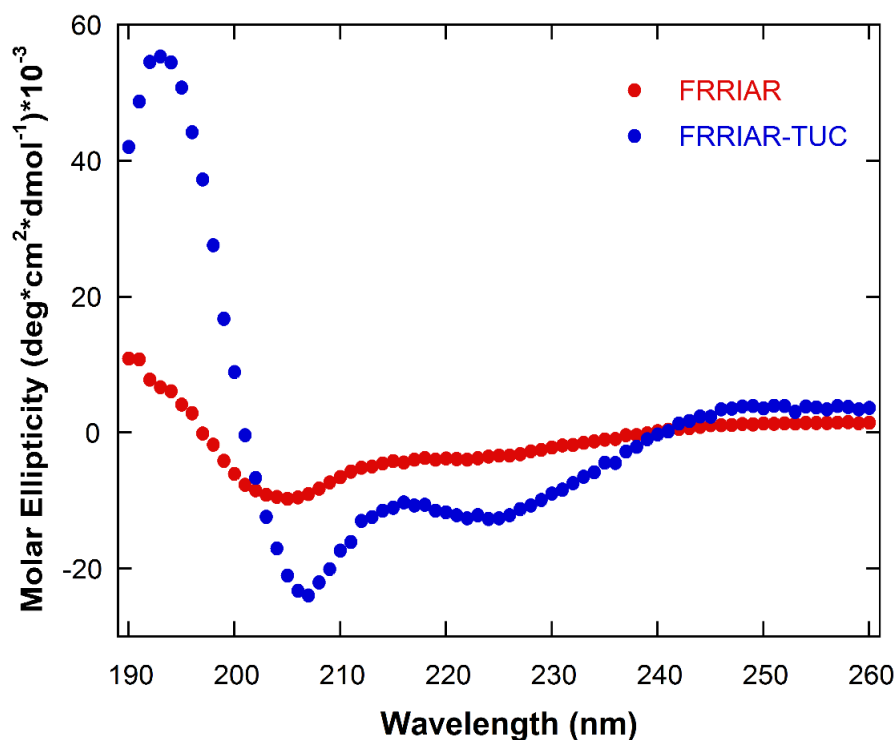


Figure 4. 9. Electronic circular dichroism of FRRIAR (30 μ M) and FRRIAR-TUC (10 μ M) in 1:1 MeCN:H₂O at 298 K. Data are normalized to molar ellipticity (see materials and methods)

Electronic Circular Dichroism

Although NMR^{178, 179} and crystallographic¹⁸⁹ studies indicate FRRIAR peptide is helical upon protein binding (Figure 4. 1), electronic circular dichroism studies demonstrate the peptide is fairly disordered (Figure 4. 9, red trace) when free in solution. Interestingly, after conjugating FRRIAR to cryptophane, the peptide exhibits a large increase in helicity (Figure 4. 9, blue trace) from 11 to 37%, (see Materials and Methods).

Because racemic cryptophane does not produce ECD signal, conjugation to the cage seems to promote helicity in FRRIAR peptide. For ECD studies, samples of FRRIAR peptide (30 μM) and FRRIAR-TUC (10 μM) were prepared in 1:1 MeCN:H₂O, as confirmed by UV-vis spectroscopy (FRRIAR: $\epsilon_{280} = 6,970 \text{ M}^{-1}\text{cm}^{-1}$, FRRIAR-TUC: $\epsilon_{280} = 16,970 \text{ M}^{-1}\text{cm}^{-1}$). HPLC solvents were used to minimize observed light scattering from DMSO and HEPES.¹⁹⁰ These data are in line with previous work where cryptophane was observed to promote helicity in a helix forming peptide, likely through cryptophane-peptide pi-stacking interactions.⁷⁷ These data serve to further explain observations by ITC where there is an initial “dilution” effect before CaM binding.

Table 4.1: α -helical content of FRRIAR vs FRRIAR-TUC in 1:1 MeCN:H₂O

Compound	$-\left[\theta\right]_{222} \text{ (deg}\cdot\text{cm}^2\cdot\text{dmol}^{-1}\text{)}$	Helicity (%)
FRRIAR	3959	11
FRRIAR-TUC	12,596	37

¹²⁸Xe NMR Spectroscopy

Finally, HP ¹²⁹Xe NMR spectroscopy was employed to further investigate the biosensor-CaM interaction. Interestingly, the biosensor alone gave no hp ¹²⁹Xe-cryptophane NMR signal; only the hp ¹²⁹Xe@H₂O resonance was observed, Figure 4. 10. UV-Vis spectroscopy confirmed that significant quantity ($\sim 70 \mu\text{M}$, $\epsilon_{280} = 16,970 \text{ M}^{-1}\cdot\text{cm}^{-1}$) of biosensor was in solution. Upon addition of equimolar calcium-activated CaM, FRRIAR-TUC produced strong ¹²⁹Xe NMR signal corresponding to cryptophane-CaM complex formation Figure 4. 10. The two bound peaks (65.9, 67.6) were assigned to

diastereomers, which are formed when racemic biosensor is conjugated to the chiral peptide. Diastereomeric ^{129}Xe NMR peaks have been observed previously for peptide-cryptophane conjugates as well as for xenon biosensors bound to protein active sites..^{66, 70} Only trace amounts of “free” biosensor was observed in the presence of calcium-bound CaM (at 62.9 ppm), which was assigned from previous literature examples of water-soluble cryptophane. The lack of hp ^{129}Xe NMR signal corresponding to free biosensor is thus consistent in the presence or absence of CaM. Likewise, no hp ^{129}Xe NMR signal was observed for biosensor in the presence of apo CaM, as FRIARR-CaM interaction is much weaker in the absence of calcium (as confirmed by gel electrophoresis, Figure 4. 6). These experiments demonstrate the first example of a “turn-on” xenon NMR biosensor, which results from the binding of FRRIAR-TUC to calcium-activated CaM.

Our ECD and Trp fluorescence data suggest significant cryptophane-peptide interactions as has been previously demonstrated.^{67, 77} Such interactions may contribute to poor solubilisation of the cryptophane and should favour less xenon-accessible conformations resulting in an “OFF” signal from the biosensor. Upon calcium-activated CaM binding to the peptide, cryptophane-FRRIAR interactions are decreased and xenon biosensor signal is turned “ON”.

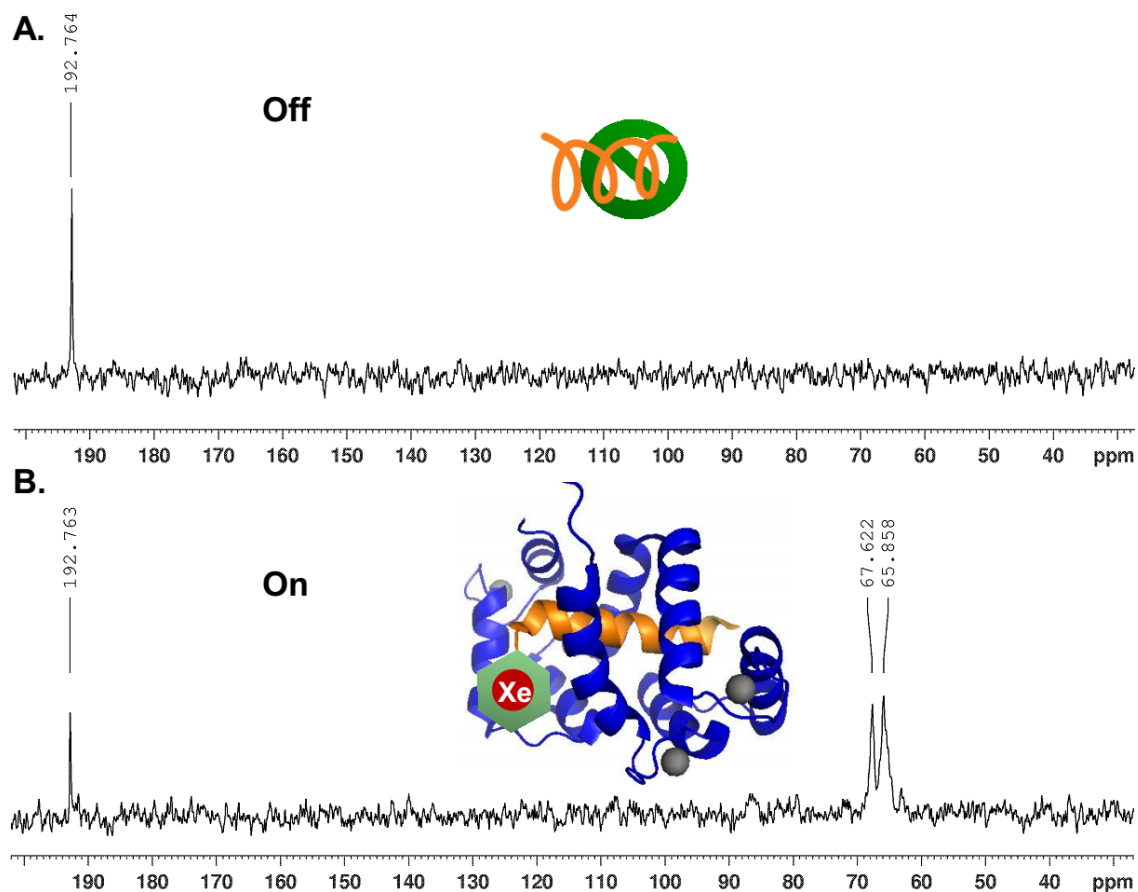


Figure 4. 10. ^{129}Xe NMR spectra of CaM bound FRRIAR-TUC resulting in two bound signals, A. and FRRIAR-TUC alone and in the presence of apo CaM resulted in no bound signal.

§ 4.3 Conclusions

In summary, we report a straightforward synthesis for the first “turn-on” cryptophane biosensor, which was used to detect calcium-bound calmodulin protein. This so called turn-on effect improves signal-to-noise especially in scenarios where there are multiple biosensors with both “free” and “bound” signals. The observation of signal only in the presence of target analyte will also facilitate ultrasensitive detection using hyperpolarized chemical exchange saturation transfer (Hyper-CEST) NMR techniques.⁹⁵ For example, large, high-powered pulse widths that increase detection sensitivity can be

employed without interfering signal from untargeted biosensor(s). This work highlights a new paradigm in xenon biosensing, and future studies will help to elucidate the nature of peptide-cryptophane interactions that can produce the “OFF” state.

§ 4.4 Materials and Methods

General Information

Instrumentation and Methods. ^1H NMR (500 MHz) data were obtained in deuterated chloroform (CDCl_3) or dimethyl sulfoxide ($\text{DMSO}-d_6$) using a Bruker DMX 500 NMR spectrometer. Column chromatography was performed using silica gel (60 Å pore size, 40-75 μm particle size) from Sorbent Technologies. Thin layer chromatography (TLC) was performed using silica gel plates (60 Å pore size, Silicycle) with UV light at 254 nm as the detection method. MALDI-MS data were collected using a Bruker Ultraflex III TOF/TOF mass spectrometer. All HPLC purifications were performed on a Varian Prostar 210 system equipped with a quaternary pump and diode array detector. All air- and moisture-sensitive reactions were performed under inert atmosphere in glassware flamed under vacuum, using anhydrous dry solvents. Standard workup procedures involved multiple (~3) extractions with the indicated organic solvent, followed by washing of the combined organic extracts with water or brine, drying over Na_2SO_4 and removal of solvents *in vacuo*. All yields reported were determined after purification by column chromatography or reverse phase HPLC, unless otherwise noted. All data were collected using instruments in the Chemistry Department at the University of Pennsylvania.

Materials. Organic reagents and solvents were used as purchased from the following commercial sources: Sigma-Aldrich: N,N-diisopropylethylamine (DIPEA); copper(I)

bromide; dimethyl sulfoxide (DMSO, anhydrous, 99.9%); Sigmacote®; Tris-[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA). Fisher: acetone (HPLC grade); chloroform (CH₂Cl₃, HPLC grade); dichloromethane (CH₂Cl₂, HPLC grade); ethyl acetate (EtOAc, HPLC grade); hexanes (HPLC grade); hydrochloric acid; methyl alcohol (MeOH, HPLC grade), perchloric acid (60%); potassium carbonate (anhydrous); sea sand (washed); sodium chloride (NaCl); sodium hydroxide (NaOH); sodium sulfate (anhydrous). Novabiochem (currently EMD Millipore; Billerica, MA, USA): 6-azidohexanoic acid; 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU). MarCor: deionized (DI) water filtered (18 MΩ). Acros Organics: β-propiolactone (90%); cesium carbonate (Cs₂CO₃, 99.5%); chloroform-*d* (CDCl₃); 1,2-dibromoethane; 3,4-dihydroxybenzaldehyde (97%); N,N-dimethylformamide (DMF, 99.8%, anhydrous, across seal); dimethylsulfoxide-*d*₆, 4-hydroxy-3-methoxybenzyl alcohol (99%); propargyl bromide (80% solution in toluene); scandium(III) trifluoromethanesulfonate (Sc(OTf)₃, 95%); sodium borohydride (NaBH₄, powder, 98%); sodium hydride (NaH, 60% dispersion in mineral oil); tetrahydrofuran (THF, extra dry, over molecular sieves); triisopropylsilane (TIS). MG Industries (Linde Group, NJ): xenon gas (scientific grade).

Synthetic Procedures

Cryptophane Synthesis. Tripropargyl cryptophane was achieved in a 6-step synthesis with a 6.4% overall yield from two commercially available compounds, 3,4-dihydroxybenzaldehyde and vanillyl alcohol. Spectroscopic data agreed with literature values.⁷⁷

Peptide Synthesis and Purification. FRRIAR peptide was synthesized using standard solid phase peptide synthesis as described previously,⁷⁷ and then N-terminally capped with

commercially available 6-azidohexanoic acid and purified by reverse-phase HPLC.⁷⁷ Alternatively, azido-FRRIAR was purchased from Anaspec as a purified white powder separated into 10 mg aliquots.

Solubilizing Linker Synthesis. 3-Azidopropionic acid was prepared from β -propiolactone by literature procedure and matched the reported ^1H NMR spectrum.¹⁶⁸

3-azidopropionic acid (4). Briefly, sodium azide (4.5 g, 0.69 mmol, 1 eq) was dissolved in ddH₂O water. β -propiolactone (4.4 mL, 0.069 mmol, 1 eq) was added dropwise and the reaction was allowed to stir at rt for 6 h. The reaction was neutralized with 1 M HCl and then extracted 3 times. The organic layer was then dried over sodium sulfate and filtered through cotton. A clear oil in a 15% yield was recovered, requiring no further purification.

FRRIAR-TUC Biosensor Synthesis. The copper(I)-catalyzed [3+2] azide-alkyne cycloaddition (CuAAC) between N₃-EALA peptide and cryptophane and the subsequent cycloaddition reaction between the cryptophane and 3-azidopropionic acid yielded the FRRIAR-TUC biosensor.

FRRIAR-cryptophane (3). To conjugate the azido-EALA peptide to the tripropargyl cryptophane (**1**) CuAAC was utilized with modified conditions of those previously employed.^{67, 68, 70, 73} Firstly, 5.3 mg (1.0 eq) of **1** and 10 mg of **2** (0.8 eq) were dissolved in 1 mL of dry N,N-dimethylformamide (DMF) in a conical reaction vessel. The reaction mixture was put to stirring and degassed. In a separate vial, 16 mg (6 eq) of tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methylamine (TBTA) copper ligand was dissolved in 100 μL of DMF. A separate solution of 0.35 M copper (I) bromide (CuBr) in DMSO was also prepared. To the TBTA solution, 43 μL of CuBr (3 eq) was added, vortexing after addition.

The reagent mixture was then added to the reaction vessel. The reaction vessel was again degassed and then covered with foil and allowed to stir for 3 h under nitrogen at rt. A small aliquot of reaction was removed (2 μ L) and diluted in HPLC solvent mixture, 50:50 mixture of acetonitrile and water with 0.1% trifluoroacetic acid to 1 mL. To verify product formation, analytical reverse-phase HPLC was performed using a Grace C18 analytical column (250 $\times$ 4.6 mm, 5 μ m beads) and monitored at 215 and 277 nm. The elution gradient was composed of two solvents: 0.1% aqueous TFA (solvent A) and a 0.1% solution of TFA in CH₃CN (solvent B). The purification method went equilibrated at 90% A for 5 min. Then went from 90% A to 60% A over 3 min, then from 60% A to 22% A over 32 min, and finally decreased to 0% A over the next 5 min at a flow rate of 1 mL/min with a 1 mL injection volume. The FRRIAR-cryptophane conjugate eluted at 28.00 min, Figure 4. 11. MALDI-MS m/z calculated for FRRIAR-cryptophane C₁₆₆H₂₂₅N₃₇O₃₃ (M+H⁺) 3265.71; found 3265.76, Figure 4. 12.

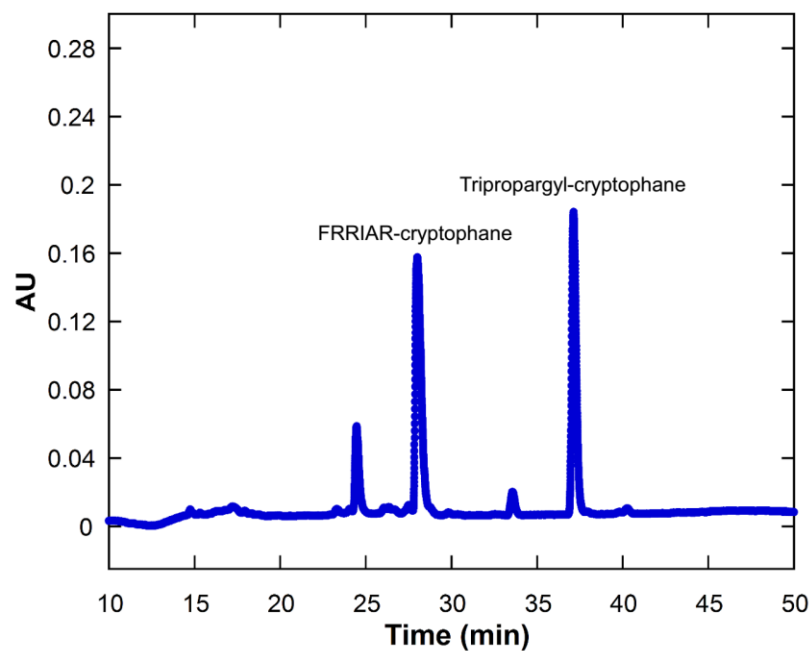


Figure 4. 11. HPLC chromatogram of FRRIAR-cryptophane (**3**). UV absorbance monitored at 277 nm. Product eluted at 28.00 min. Peak assignment based on MALDI-MS.

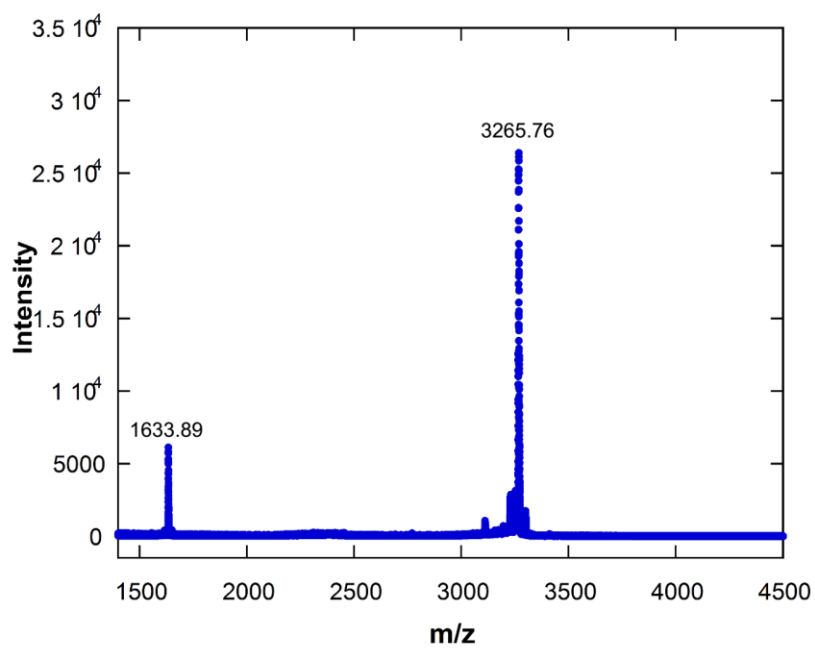


Figure 4. 12. MALDI-MS spectrum of FRRIAR-cryptophane (**3**). Expected mass ($M+H^+$) 3265.71; found 3265.76.

FRRIAR-trisubstituted ultrasensitive cryptophane (FRRIAR-TUC) (5). After 3 h the crude reaction (**3**) was then dosed with 6 μ L 3-azidopropionic acid (**4**) (11 eq) and allowed to stir overnight while covered, to yield the FRRIAR-TUC biosensor. The reaction was diluted 10-fold in 50:50 ACN:H₂O and purified. Purification was achieved through reverse-phase HPLC employing a semi-preparative method and using a Grace C18 semi-preparative column (10 $\times$ 250 mm, 5 μ m beads). The purification method went equilibrated at 90% A for 5 min. Then went from 90% A to 55% A over 3 min, then from 55% A to 51% A over 7 min, and then from 51% A to 47% A over 13 min. Next went from 47% A to 40% A over 6 min and finally decreased to 0% A over the next min at a flow rate of 4 mL/min with a 1 mL injection volume. The biosensor eluted at 20.30 min Figure 4. 13. MALDI-MS m/z calculated for FRRIAR-TUC C₂₀₈H₂₈₉N₄₃O₆₁ (M+H⁺) 3496.02; found 3495.99, Figure 4. 14. The pure fractions were then collected and lyophilized to a white powder and dissolved in a minimal amount of DMSO and then diluted to desired concentrations in 10 mM HEPES at pH 7.5 with and without 1 mM CaCl₂.

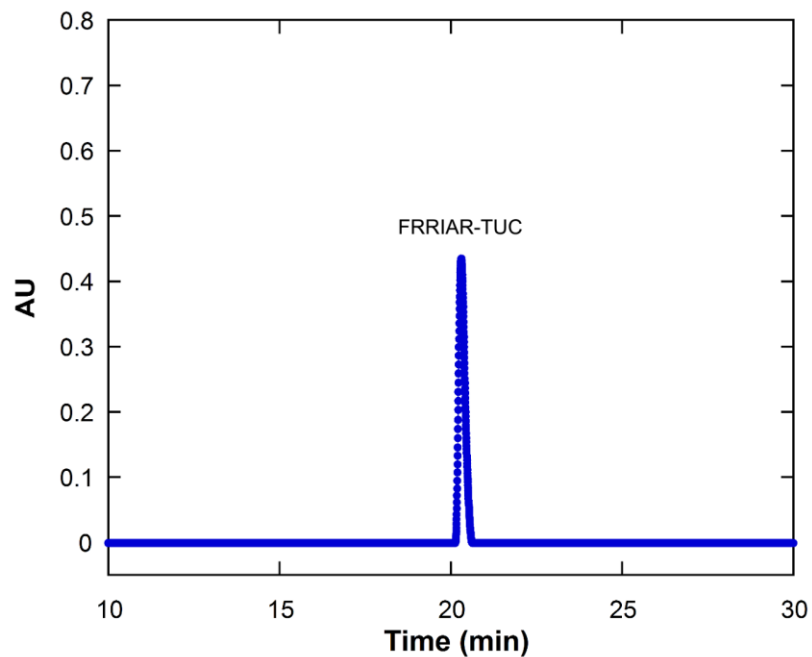


Figure 4. 13. HPLC chromatogram of FRRIAR-TUC (**5**). UV absorbance monitored at 277 nm. Product eluted at 20.30 min. Peak assignment based on MALDI-MS.

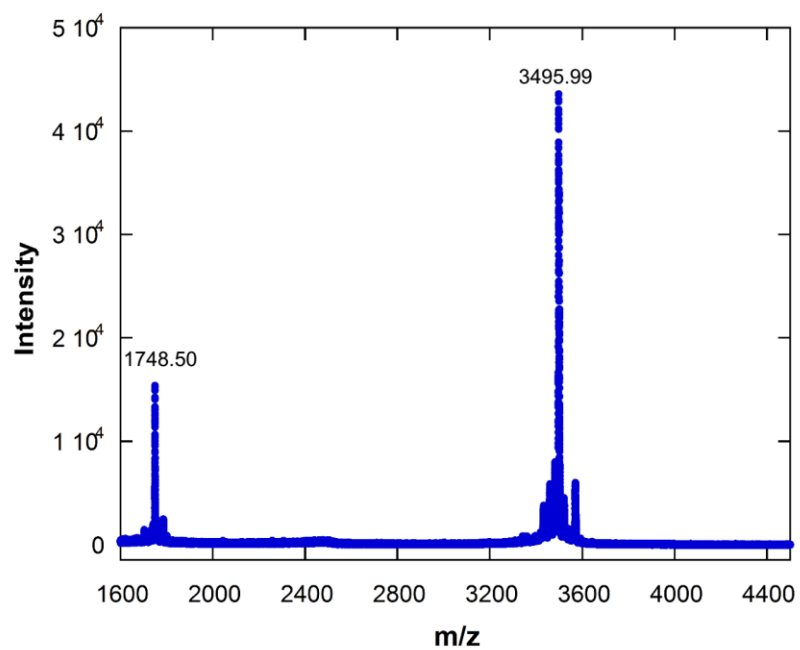


Figure 4. 14. MALDI-MS spectrum of FRRIAR-TUC (**5**). Expected mass ($M+H^+$) 3496.02; found 3495.99.

Calmodulin Protein Expression and Purification. Calmodulin (CaM) protein was expressed and purified following previously established protocols.¹⁷³ Briefly, *Escherichia coli* BL21(DE3) cells were transformed with plasmid containing chicken CaM gene. Transformed cells were selected on the basis of ampicillin resistance. M9 minimal media (50 mL) supplemented with ampicillin (100 µg/mL) was inoculated with single colonies. An M9 salts solution (42.3 mM Na₂HPO₄, 22.0 mM KH₂PO₄, and 8.5 mM NaCl) was prepared and autoclaved. Autoclaved solutions of the following salts were added per liter of M9 salts: 10 mL of 10% NH₄Cl, 1 mL of 2 M MgSO₄, 1 mL of 15 mg/mL FeCl₂ (in 1.0 M HCl), 1 mL of 15 mg/mL ZnCl₂ (in acidified H₂O), and 2 mL of 10% Bacto™ Yeast Extract. The primary 50 mL culture was incubated at 37 °C with shaking at 250 rpm overnight. The cells were harvested at 5000 g for 15 min and the resulting pellet was resuspended in 1 L of M9 minimal media supplemented with ampicillin. The 1 L culture was incubated at 37 °C with shaking at 250 rpm until the absorbance at 600 nm reached an OD of 0.9 AU. The culture was induced with isopropyl D-galactoside (IPTG), and then incubated at 25 °C for an additional 12 h. The cells were again collected at 5000 g for 15 min and the resulting pellet was suspended in 15 mL of MOPS resuspension buffer: 50 mM 3-(N-morpholino)propanesulfonic acid (MOPS), 100 mM KCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol, pH 7.5. Cell were lysed with lysozyme (150 µg/mL) for 1 h at room temperature followed by brief sonication. The cell lysate was cooled on ice for 5 min. CaCl₂ was added to the sonicated lysate to a final concentration of 5 mM prior to centrifugation for 20 min at 30,000 g, 4 °C. CaM was purified from the cleared cell lysate using a phenylsepharose CL-4B column (resin bed

volume = 20 mL) with EDTA as eluent. The column was first equilibrated with 4 column volumes of Buffer A (50 mM Tris base, 1 mM CaCl_2 , pH 7.5). After the clear cell lysate was loaded and allowed to pass through the resin, the column was washed with 4 column volumes of Buffer A, 4 column volumes of high-salt Buffer B (50 mM Tris base, 0.5 M NaCl, 0.1 mM CaCl_2 , pH 7.5), and an additional 2 column volume washes of Buffer A to restore low-salt conditions. CaM was eluted with Buffer C (10 mM Tris base, 10 mM EDTA, pH 7.5) and collected in 4 mL fractions until absorbance at 280 nm was no longer detected. A second column purification was performed on the first batch of eluted fractions (re-saturated with CaCl_2 to a concentration of 20 mM) to obtain CaM with high levels of purity. Column fractions were dialyzed against 10 mM ammonium bicarbonate (pH 8.0) and stored as a lyophilized powder at $-20\text{ }^{\circ}\text{C}$. SDS-PAGE analysis was performed to analyze dialyzed CaM elution fractions.

Characterization Methods

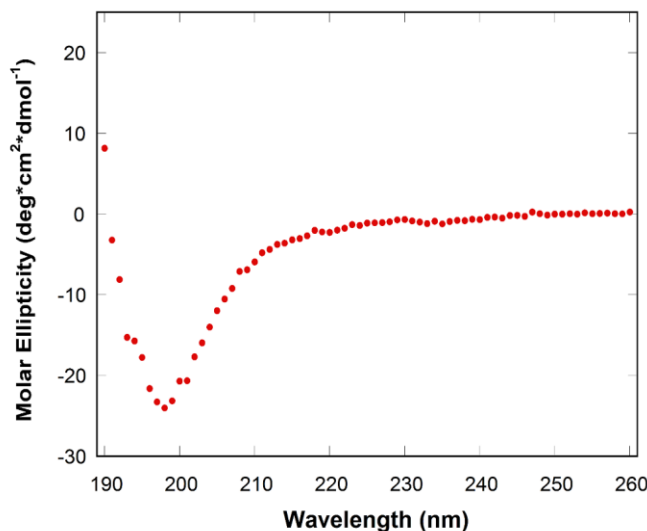


Figure 4. 15. Electronic circular dichroism spectrum of FRRIAR peptide showing a disordered conformation.

Electronic Circular Dichroism (ECD) Spectroscopy. All ECD spectroscopy experiments were performed on the Aviv 410 CD spectrometer. Data were collected at 25 °C from 260-190 nm, with a 30 s averaging time, 1 nm wavelength step, 1 s averaging time, and 1 nm bandwidth. The sample in Figure 4. 15 was prepared by dissolving lyophilized FRRIAR in 10 mM HEPES, 1 mM CaCl₂ pH 7.2 to 30 μM and serves as a control for peptide conformation irrespective of solvent conditions. The concentration for all experiments was confirmed by measuring the absorbance at 280 nm, $\epsilon_{280} = 6,970 M^{-1}cm^{-1}$ [FRRIAR] and $\epsilon_{280} = 16,970 M^{-1} cm^{-1}$ [FRRIAR-TUC] and using an Agilent 89090A UV-visible spectrophotometer.

Data Analysis. The molar ellipticity was calculated from the observed ellipticity (mdeg) and has the units of deg cm² dmol⁻¹. The molar ellipticity is given by equation 4.1 where C

is the concentration of the peptide or biosensor, ℓ is the path length of the cuvette-0.1 cm, and n_r is the number of residues-.¹⁶⁹

$$[\theta] = \frac{\theta_\lambda}{(C * \ell * n_r * 10)} \quad (4.1)$$

The helical content of FRRIAR and FRRIAR-TUC were determined from circular dichroism studies at 30 and 10 μ M concentrations, respectively, and in accordance with literature precedent.¹⁷⁰ The concentration variation is a result of the large difference in extinction coefficients (6970 vs 16970 $M^{-1} \cdot cm^{-1}$), because data are normalized to molar ellipticity they can be directly compared. Studies at various concentrations demonstrated reproducible percent helicity values. Helicity was calculated using the formulas 4.2 and 4.3 where n_r is the number of amino acids in the peptide, in this case 17. Racemic cryptophane was employed for these experiments, and thus did not contribute to the measured CD signal.¹⁴⁵ Data are shown in Tables 4.1 and graphically in Figure 4. 9. These experiments were performed in 1:1 MeOH:H₂O because even samples with 0.25% DMSO contributed to significant scattering below 210 nm as did the buffer to a lesser degree.¹⁹⁰ Comparative studies between FRRIAR in buffer and FRRIAR in 1:1 MeCN:H₂O demonstrated equivalent spectra.

$$\%helicity = 100 * \frac{[\theta]_{222}}{max[\theta]_{222}} \quad (4.2)$$

$$max[\theta]_{222} = -40000 * [1 - \frac{2.5}{n_r}] \quad (4.3)$$

Tryptophan Fluorescence Studies. All fluorescence studies with the peptide and CaM were carried out on Photon Technology International (PTI) QuantaMaster™ 40

fluorescence spectrometer (Birmingham, NJ, USA). Samples containing 0, 7.5, 15, 22.5, or 30 μM CaM and 30 μM FRRIAR peptide were prepared in 10 mM HEPES, 1 mM CaCl_2 pH 7.2 and placed in the fluorimeter. Fluorescence spectra were collected at 25 °C in quartz cuvettes with a 1-cm path length. The samples were excited at 295 nm and emission data were collected from 300-400 nm. For all spectra the slit widths were 5 nm, scan rate was 60 nm/min, averaging time was 1 s, and the data interval was 1 nm. All fluorescence studies with the biosensor and CaM were prepared in the same manner and carried out on Cary Eclipse Fluorescence Spectrophotometer from Agilent (formally Varian).

Hyperpolarized ^{129}Xe NMR Spectroscopy. HP ^{129}Xe was generated using spin-exchange optical pumping (SEOP) method with a home-built version of the previously commercially available Nycomed-Amersham (now GE) model IGI.Xe.2000 ^{129}Xe hyperpolarizer. A gas mixture of 89% helium, 10% nitrogen, and 1% natural abundance xenon (Linde Group, NJ) was used as the hyperpolarizer input. 795 nm circularly polarized diode laser was used for optical pumping of Rb vapor. ^{129}Xe was hyperpolarized to 10–15% after being cryogenically separated, accumulated, thawed, and collected in controlled atmosphere valve NMR tubes (New Era). After hp Xe collection, NMR tubes were shaken vigorously to mix cryptophane solutions with hp Xe. All ^{129}Xe NMR measurements were carried out on a Bruker BioDRX 500 MHz NMR spectrometer (138.12 MHz frequency for ^{129}Xe), using a 5-mm BBO NMR probe. Sample temperature was controlled by VT unit on NMR spectrometer to 300 ± 1 K. Eburp1 shaped pulse was used to selectively excite Xe@FRRIAR-TUC biosensor peak. Spectra were averaged over 16 scans. A delay of 0.15s was given between scans to allow for xenon exchange. All acquired NMR spectra were

processed with 25 Hz Lorentz broadening. Chemical shifts were referenced to free xenon gas of 0 atm at 0 ppm.

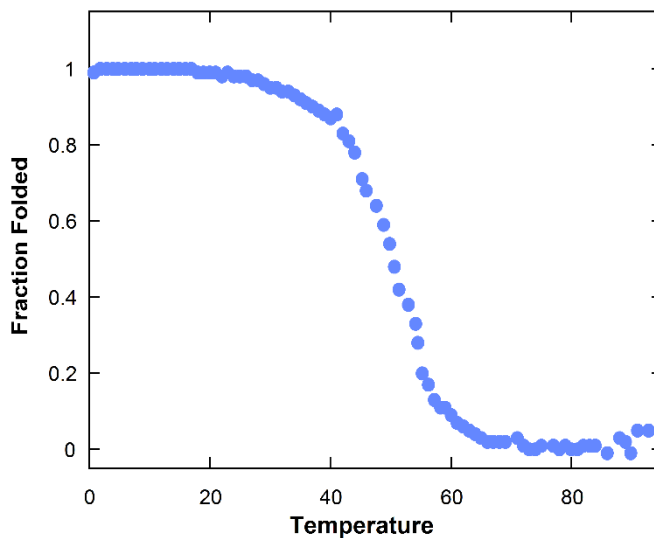


Figure 4. 16. Temperature-dependent circular dichroism spectroscopy of 30 μ M apo calmodulin

Thermal Melt of Calmodulin. To confirm that Apo CaM had been achieved temperature-dependent circular dichroism (CD) spectroscopy was performed following literature protocol.¹⁷³ Briefly, calcium containing CaM (holo-CaM) is thermostable ($T_m > 90$ °C) thus, we measured the thermal unfolding of the apo protein which has a reported T_m of 55 °C.¹⁹¹ CD data were obtained from approximately 30 μ M protein sample monitored at 222 nm between 0 and 95 °C using the variable temperature module with the Aviv 410 CD spectrometer. Data were collected every 1 °C, using a 30 s averaging time, 2 min temperature equilibrium, and 1 nm band width. The resulting ellipticity (θ_D) measurements were converted to molar residue ellipticity values (θ) using equation 4.1, described above.

The fraction folded (f_f) for apo protein was determined using linear baselines to fit the low (θ_F) and high (θ_U) temperature data, equations **4.4** and **4.5**, respectively.

$$\theta_F = m_F T + b_F \quad (4.4)$$

$$\theta_U = m_U T + b_U \quad (4.5)$$

The entirety of the data range was then fit to equation **S6** where $K = e^{-(\Delta H - T\Delta S)/RT}$, where ΔH and ΔS are adjustable parameters and $R = 8.3145 \text{ J} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$. The resulting plot is shown in Figure 4. 16.

$$\theta = \theta_F(T)f_f(T) + \theta_U(T)(1 - f_f(T)) \quad (4.6)$$

Where: $f_f = K/(1 + K)$

Our values agree quite favorably with the measured values associated with experiments performed in Goldberg *et al.* (not published) and are shown in **Table S2**.¹⁷³

Table 4.2: Calculated values for ΔH and ΔS from the thermal melt of apo calmodulin

	Our Calculated Value	Literature Value ^a
ΔH	$1.43 \times 10^5 \text{ kJ} \cdot \text{mol}^{-1}$	$1.43 \times 10^5 \text{ kJ} \cdot \text{mol}^{-1}$
ΔS	$444 \text{ J} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$	$438 \text{ J} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$

^aThese values are associated with data analysis performed in Goldberg *et al.* while these values were not published in that manuscript they were provided by the authors for comparison.

Gel Shift Assay. All gels were run on a Bio-Rad PowerPac™ Basic gel setup in 1x Tris-glycine solution (prepared from a dilution of commercially available Tris-Glycine 10x Solution for Electrophoresis in ddH₂O). Mini-protean®TGX™ precast gels, 4-15%, 30 μL /well from Bio-Rad. Initial characterization of CaM gel shift upon binding was achieved by preparing samples with 0, 10, or 20 μM FRRIAR (final concentration) with 10 μM CaM

(final concentration) and is shown in Figure 4. 17. For the FRRIAR-TUC titration gel, samples containing 0, 7.5, 15, 22.5, or 10 μ M FRRIAR-TUC and 10 μ M CaM were prepared in 10 mM HEPES, pH 7.2 with 1% DMSO, Figure 4. 18. For the Apo/Holo binding gel, 10 μ M (final concentration) of either apo or holo CaM was combined with 10 μ M FRRIAR (final concentration), 10 μ M FRRIAR-TUC, or buffer, Figure 4. 6. Native Tris-Glycine Sample Buffer 2x from Novex (25 μ L) was combined with 25 μ L of each sample. Samples were incubated for 1 min after prep before being loaded onto a 4-15% gradient gel. The gel box was put on ice and run at 120 V for 1.5 h before being stained with Coomassie brilliant blue stain mixture (20 min) and then destained. The gel were imaged on the Typhoon FLA 7000 gel imager.

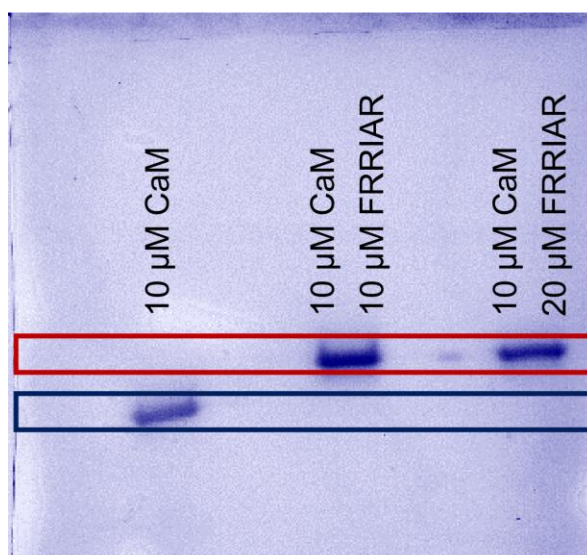


Figure 4. 17. Native gel shift assay demonstrating retarded gel migration of CaM after binding FRRIAR and that it does so in 1:1 stoichiometry.

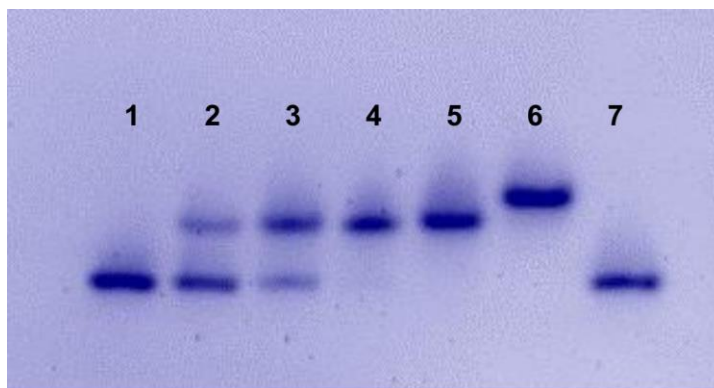


Figure 4. 18. Native gel shift assay, where sample are all 10 μM CaM (final concentration) plus 1. buffer; 2. 0.25 μM FRRIAR-TUC; 3. 0.50 μM FRRIAR-TUC; 4. 0.75 μM FRRIAR-TUC; 5. 10 μM FRRIAR-TUC; 6. 10 μM FRRIAR peptide; and 7. buffer.

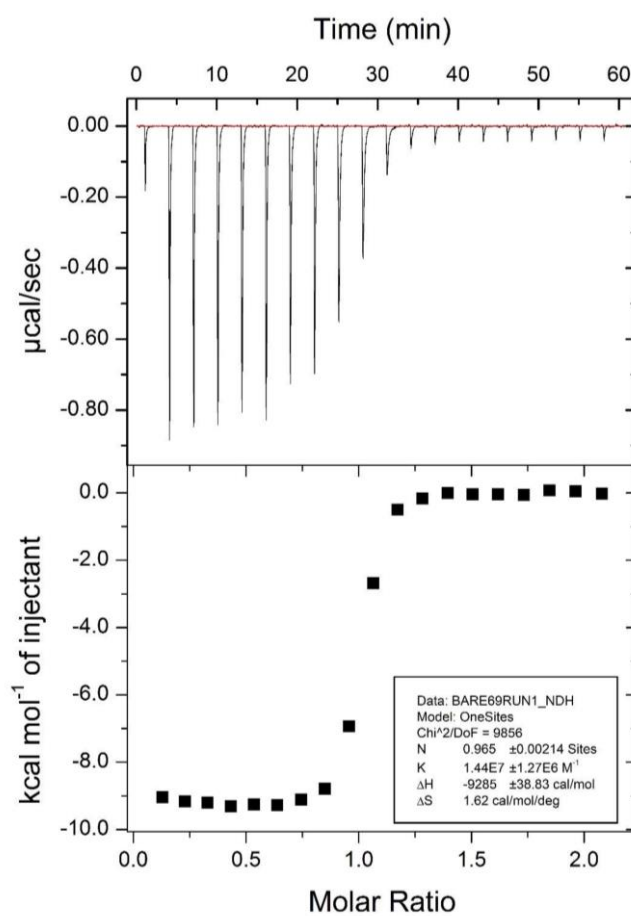


Figure 4. 19. Enthalpogram of FRRIAR peptide injected into calmodulin protein.

Isothermal Titration Calorimetry. All experiments were performed on a GE MicroCal™ ITC₂₀₀ titration microcalorimeter at 298 K. Experiments were performed in high feedback mode with 1000 rpm stir speed, an initial injection delay of 60 s, 180 s spacing between injections, a differential power of 60, and a filter period of 5 s. An initial injection of 0.6 μ L was measured and removed from subsequent data analysis due to initial drop dilution this was followed by 19- 2 μ L injections. Buffers were prepared in 1 L stock using ddH₂O water and were purified by vacuum filtration at 0.22 μ M. CaM protein was dialyzed against the desired buffer 2-3x. The buffer from the last dialysis was saved for subsequent use. Protein concentration was determined using UVvis A₂₈₀, $\epsilon = 3000 \text{ M}^{-1} \text{ cm}^{-1}$ and was diluted in the buffer used for dialysis to a final concentration of 30 μ M, 300 μ L. Purified FRRIAR peptide as a lyophilized powder was dissolved in the buffer from dialysis as well as to ensure perfect buffer match to a final concentration of 300 μ M, 100 μ L as determined by UV-vis: A₂₈₀ with $\epsilon = 6970 \text{ M}^{-1} \text{ cm}^{-1}$. The heat of binding was measured for each injection by measuring the power applied to the reference cell to maintain a temperature equal to that of the sample cell and was measured in microcalories per second. Values for binding enthalpy (ΔH), binding entropy (ΔS), binding stoichiometry (n), and association constant (K_A) were determined by peak integration and data fitting of the heat evolved per mole of peptide injected using Origin software (version 7). Enthalpogram is shown in Figure 4. 19. A control was performed of buffer injected into buffer and of peptide injected into buffer (Figure 4. 20).

Repeated attempts were next made to calculate binding affinity between FRRIAR-TUC and CaM at various concentrations using ITC, however, no reliable data were

achieved. Resulting enthalpograms were somewhat suggestive of a “two-site” binding model which is likely reflective of an initial unfolding event to alleviate peptide-cryptophane interactions or micelle disassembly to free up peptide for binding. Examples are shown in Figure 4. 8.

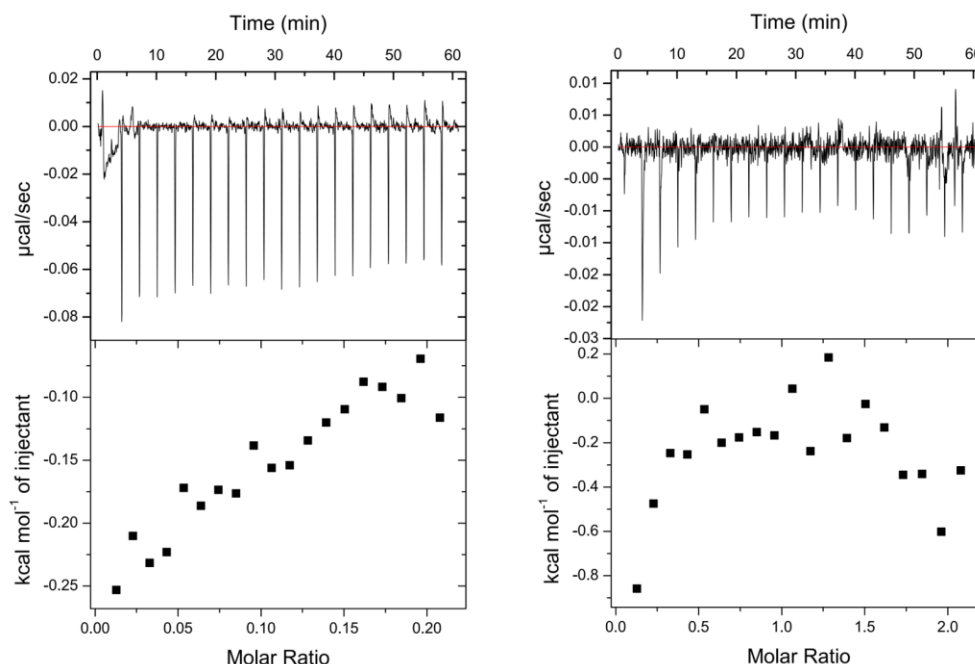


Figure 4. 20. Enthalpograms of ITC controls. a. buffer titrated into buffer and b. FRRIAR titrated into buffer. Buffer is 10 mM HEPES 1 mM CaCl_2 pH 7.2.

§ 4.5 Acknowledgments

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Chapter 5: Epilogue

This thesis has explored the nature of cryptophane-based xenon biosensors and has endeavored to elucidate design and synthesis strategies to generate biosensors with a high degree of target specificity coupled with large changes in chemical shift. The long-term goal is for this work to culminate in an array of targeted biosensors that can be employed in animal models, and ultimately humans, as a means of accurately diagnosing cancer in an ultrasensitive fashion.

§ 5.1 Carbonic Anhydrase as Cancer Biomarkers

Chapter 2 discussed our work with carbonic anhydrase targeting biosensors and our studies attempting to explicate the chemical shift variation achieved from differing linker lengths and the presence of multiple bound peaks. Our initial work was performed with ubiquitously studied carbonic anhydrase isozymes I and II but as with all work in the selective targeting and inhibition of specific carbonic anhydrase isozymes, we have also considered isozymes indicated in cancer, CA IX and XII. These membrane-bound isozymes are upregulated in a multitude of cancers where they acidify the extracellular milieu¹⁹²⁻¹⁹⁵ and have been challenging to express and work with in an “*ex cellula*” context. However, there are a variety of cell lines that up-regulate these proteins. Thus, moving forward we hope to generate fluorescent versions of our CXB (Figure 5.1) biosensors to allow us to employ confocal microscopy and flow cytometry to visualize and quantify the specificity of our biosensors. Because these proteins are membrane-bound we anticipate an increase in the $\Delta\delta$ ppm as a result of bringing the cryptophane in close proximity to the cellular membrane, as discussed in Chapter 3. Furthermore, there are a wide array of targeting ligands for CA, some of which exhibit a degree of isozyme specificity that we

plan to couple with our fluorescence strategies to develop biosensors highly specific for CA IX and XII.^{80, 196} With these derivatives we plan to achieve large isozyme-dependent chemical shifts that can be used in cellular NMR/MRI for ultrasensitive detection.

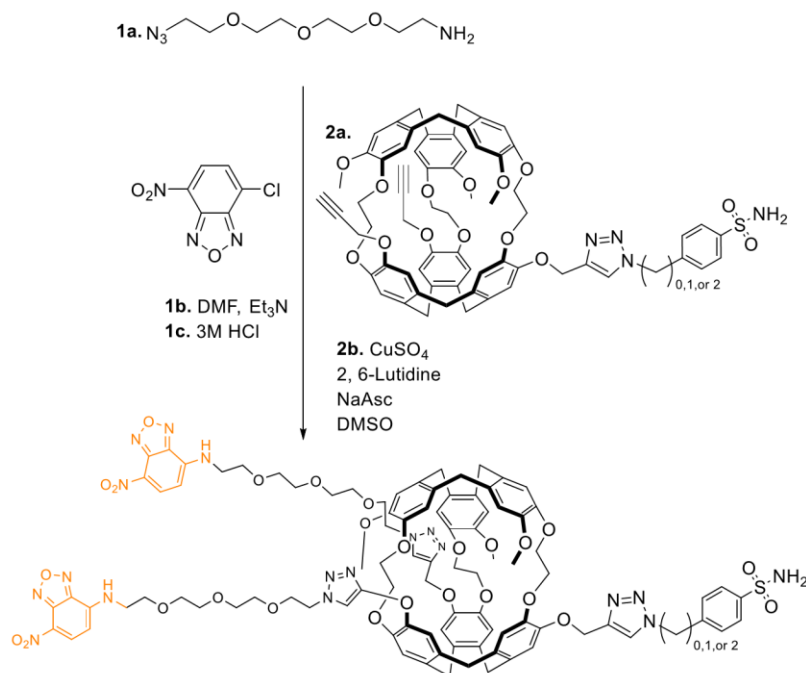


Figure 5. 1. Proposed synthesis of fluorescently labeled CXB derivatives where X=0, 1, or 2 carbon spacers.

§ 5.2 Stimuli-Responsive Cell Labeling as a Mechanism for Cancer Detection

Building on our work with carbonic anhydrase and its role in extracellular acidification, Chapter 3 described a peptido-cryptophane that was used to label cells with acidic local environments. This system was unusual in the field of targeted cryptophane biosensors in that it targets a specific “stimulus” rather than an over-expressed receptor or up-regulated enzyme. While the WEC biosensor does not provide a read-out of specific pH values like some other studies have demonstrated,^{127, 134, 136} we were able to label cells only

when placed in an acidic environment and detect cell labeling at 10^3 -fold improved concentration limits over other CEST reporters. Importantly, using the WEC biosensor we achieved the largest chemical shift change published to date.

Moving forward this work could be improved by a peptide that undergoes a conformational change over a narrower pH range and by studying if relative peak ratios of the “bound” and “free” conformations can be utilized to provide a pH measurement. More importantly, this work introduced a new paradigm to the field of cryptophane biosensors. These methods can be applied to detect a wide range of stimuli in a wide variety of fashions. One important aspect to bear in mind when designing stimuli responsive biosensors is to generate a large change in chemical shift. Secondly, the biosensor can be targeted both directly to the stimulus, such as enzymatic cleavage of a peptide-substrate or indirectly as in the case of WEC where the xenon senses a change in conformation resulting in membrane insertion rather than directly sensing pH change. In a living system, biosensors that bind to receptors or proteins will either label the cells or be internalized via an endocytotic mechanism. Stimuli-responsive biosensors may physically bind to the diseased cell further necessitating a need to generate strong chemical shifts post targeting to deconvolute the resultant signal. Our work targeting MMP-7, described in Section 1.9, produced very small chemical shift variation pre- and post- enzymatic cleavage.⁶⁷ Because the chemical shift change is a result of shielding/deshielding effects, we propose to improve the $\Delta\delta$ ppm for an enzymatic substrate tethered to cryptophane by using a highly fluorinated version of the peptide. Finally, for many studies, an “on-off” response will be sufficient, whereby cryptophane-labeled cells will produce observable hp ^{129}Xe NMR/MRI

signal. Accumulation of cryptophane at diseased cells should provide a useful diagnostic agent, similarly to how FDG has enabled early cancer detection via PET imaging.

§ 5.3 Employing Xe as an Ultrasensitive Probe for Applications in Chemical Biology

Building on our work with WEC, Chapter 4 considers new uses of cryptophane- ^{129}Xe NMR spectroscopy to probe protein dynamics. In the field of chemical biology there is a wide range of tools used to label proteins to study conformational changes and protein-protein interactions. The development and use of these tools tends to be a tradeoff between sensitivity and level of perturbation. X-ray crystallography and neutron diffraction can be employed with macromolecular samples and can provide high-resolution structures but this information is obtained from solid crystals and thus may not represent dynamic information.¹⁹⁷ Producing stable crystals can also require large screens to obtain ideal conditions which can consume large quantities of protein. Time-resolved crystallography helps to mitigate some of these concerns by generating molecular movies at atomic resolution.¹⁹⁸ This technique is limited, however, to conformational rearrangements that do not disrupt crystal contacts and cannot be performed *in vivo*. Nuclear magnetic resonance (NMR) can be used to study protein samples in solution to obtain both dynamic and structural information through distance constraints determined by homo- and heteronuclear through-bond and through-space correlations.^{199, 200} The limitation of this technique is the difficulty to perform these experiments and that inferences, as opposed to direct measurements, must be made about line-broadening patterns. Importantly, the NMR time scale is typically too slow to capture the full range of dynamic motions in large conformational changes. Fluorescence spectroscopy methods require relatively small

amounts of protein and can be performed in live tissue and in complex solutions such as cell lysates. Fluorescence spectroscopy can also be performed relying on intrinsic characteristics of proteins such as tryptophan (W) signal. Conversely, proteins can be labeled with a wide variety of fluorophores from Green Fluorescent Protein (GFP) to much smaller molecules like fluorescein, acridone, coumarin, and Alexa Fluors.²⁰¹ With smaller molecules you potentially have much decreased perturbation of the native structure but the fluorescence intensity is also reduced, thus precluding ultrasensitive detection. Thus the use of cryptophane labeling for xenon monitoring of protein dynamics may provide a complementary method to monitor protein dynamics in dilute solutions and in turbid media.

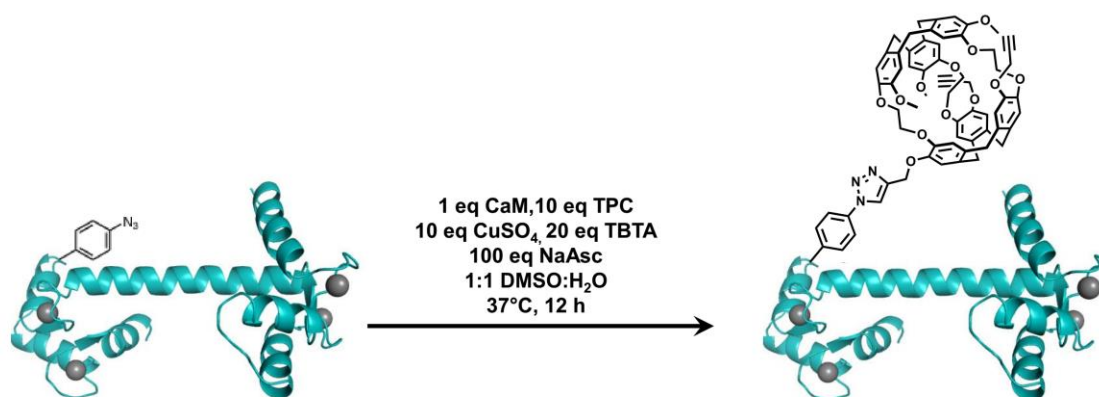


Figure 5. 2. Synthesis of cryptophane labeled calmodulin protein.

Although our initial foray into this field involved labeling a peptide-substrate involved in bringing about a conformational change, this method is not limited to labeling peptides. Indeed through the incorporation of unnatural amino acids, bioorthogonal azides can be introduced into proteins, allowing protein labeling as demonstrated by Wissner *et*

*al.*¹⁷⁴ We have initiated studies directly labeling calmodulin protein with cryptophane, following the reaction scheme in Figure 5.2 where the labeled protein was achieved as confirmed by MALDI-MS, Figure 5.3. The reaction was complicated by the presence of unlabeled protein as a result of aryl azide reduction, thus requiring further purification for accurate concentrations of labeled protein to be determined. Moving forward, however, it is reasonable to work with partially labeled batches of proteins as long as sufficient concentrations are used to obtain xenon signal. More importantly, this system should be optimized to increase xenon binding by first generating a cryptophane molecule that has been solubilized to promote an open, xenon binding conformation.⁶⁵ One possible method to both improve solubility and improve labeling is to use a cryptophane such as the one depicted in Figure 5.4.

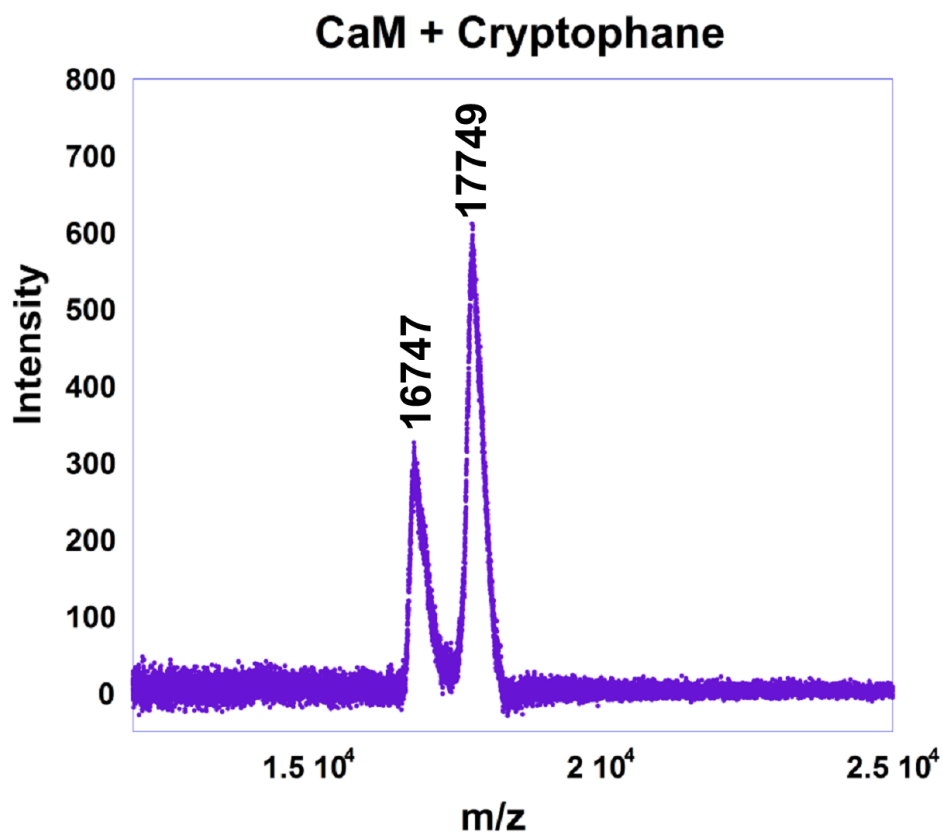


Figure 5. 3. MALDI-MS of cryptophane labeled calmodulin. Expected mass, $M+H^+=17702.5$; Found, $M+H^+=17749$. The mass 16747 corresponds with para-amino-phenylalanine 16709.4 CaM. MALDI taken from crude reaction in DMSO and resulted in broad peaks.

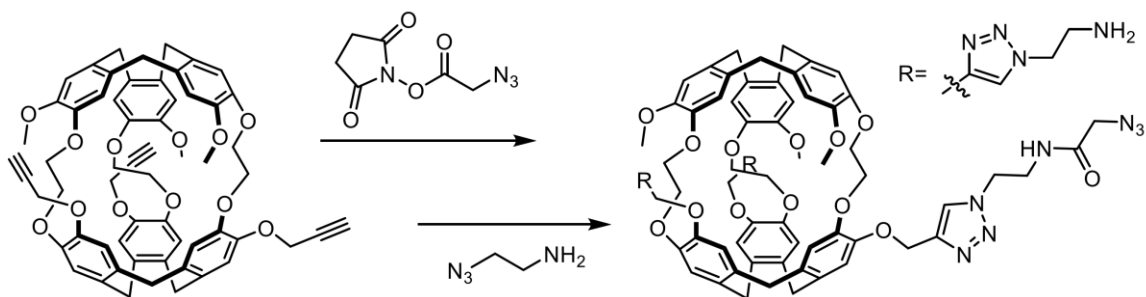


Figure 5. 4. Proposed synthesis of water-soluble, azido cryptophane for labeling alkyne-incorporated protein.

§ 5.4 Final Remarks

In this thesis we have demonstrated the utility of xenon-129 as an ultrasensitive contrast agent for NMR/MRI. Xenon hyperpolarization can be used to generate strong signal enhancement ($>10^5$) over thermally equilibrated samples. The chemical environment sensitivity of xenon can be exploited through host-guest chemistry using cryptophanes for which xenon has a very high affinity. The cryptophane host molecules can be functionalized with a wide variety of chemical handles to target any number of medically relevant disease biomarkers. Further increases in sensitivity can be achieved through indirect detection via hyperpolarized chemical exchange saturation transfer (HyperCEST). Indeed, through this technique we have demonstrated picomolar detection of targeted biosensors. This host-guest system can also be applied as a biophysical probe in chemical biology to facilitate the study of protein dynamics.

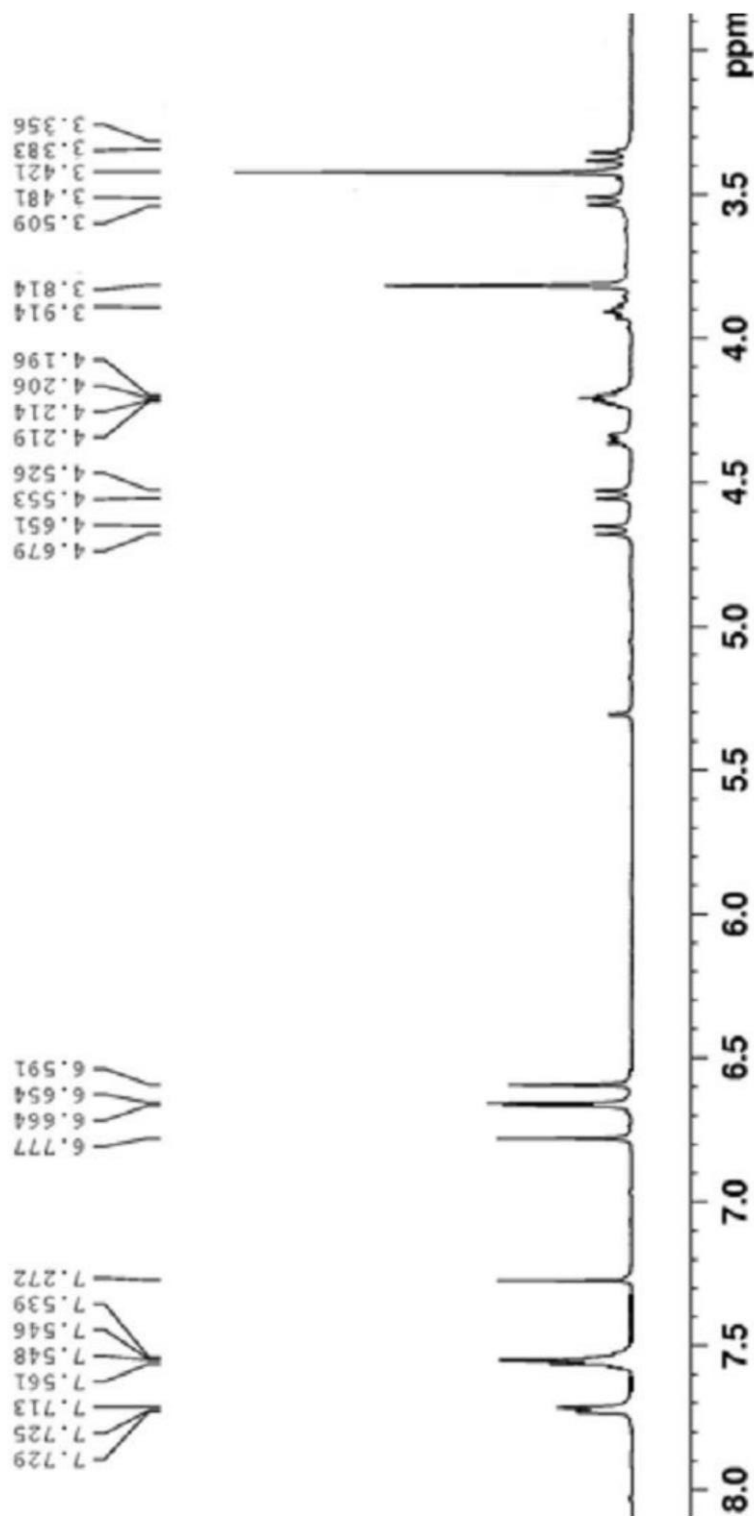
Moving forward, the field must demonstrate the utility of xenon biosensors in living systems. Thus far, synthetic and equipment limitations have limited our ability to study the efficacy of cryptophane *in vivo*. However, recent acquisition of a xenon polarizer and small animal MRI at the medical school of the University of Pennsylvania may prove to facilitate *in vivo* experiments in the near future. This will be a crucial test to ascertain the ultimate applicability of this approach to disease imaging. In parallel with cryptophane biosensors, the Dmochowski laboratory will continue to develop other small molecule (i.e., cucurbituril) as well as genetically encoded (i.e., protein) xenon biosensors, which will expand the range of applications.

Appendix A: List of Abbreviations

Abbreviation	Meaning
(HLA) DR1	human leukocyte antigen
AsPC-1	Human pancreas adenocarcinoma ascites metastasis cells
c[RGDyK]	cyclic-[D-arginine-D-glycine-D-aspartic acid-L-tyrosine-D-lysine peptide
C6B	cryptophane 6-bonds to benzene sulfonamide, soluble
C7B	cryptophane 7-bonds to benzene sulfonamide, soluble
C8B	cryptophane 8-bonds to benzene sulfonamide, soluble
CaM	calmodulin protein
CAX	carbonic anhydrase, X=isozyme number
CD	circular dichroism
CEST	chemical exchange saturation transfer
CT	computed tomography
CTG	cyclotriguiacylene
CTV	cyclotrimeratrylene
CuAAC	copper(I) catalyzed [3+2] azide-alkyne cycloaddition
FID	free induction decay
FRRIAR	N-terminal section of bovine olfactory nucleotide gated channel known to bind CaM
HA	hemagglutinin
HeLa	human epithelial cell line
HFL-1	Human foetal lung fibroblast cell line
HP ¹²⁹ Xe	hyperpolarized xenon-129
HT-1080	human fibrosarcoma cell line
hyper-CEST	hyperpolarized chemical exchange saturation transfer
ITC	isothermal titration calorimetry
KB	oral carcinoma cell line
MALDI-MS	matrix assisted laser desorption ionization-mass spectrometry
MHC	major histocompatibility complex
MON	monocrystalline iron-oxide particles
MMP-7	matrix metalloproteinase-7
MPIO	micron-sized iron-oxide particles
MRI	Magnetic Resonance Imaging
MRS	magnetic resonance spectroscopy
NMR	Nuclear Magnetic Resonance
PET	positron emission tomography
PFOB	perfluorooctyl bromide nanodroplets
rf	radio frequency
SEOP	spin-exchange optical pumping
SPECT	single photon emission computed tomography
SPIO	super paramagnetic iron oxide particles
TAAC	triacetic acid cryptophane-A
TAC	triallyl cryptophane
THC	trihydroxy cryptophane
TPC	tripropargyl cryptophane
TTEC	tris-(triazole ethylamine) cryptophane
TTPC	tris-(triazole propionic acid) cryptophane
USPIO	ultrasmall dextran coated iron-oxide particles
WEC	water-soluble EALA-cryptophane

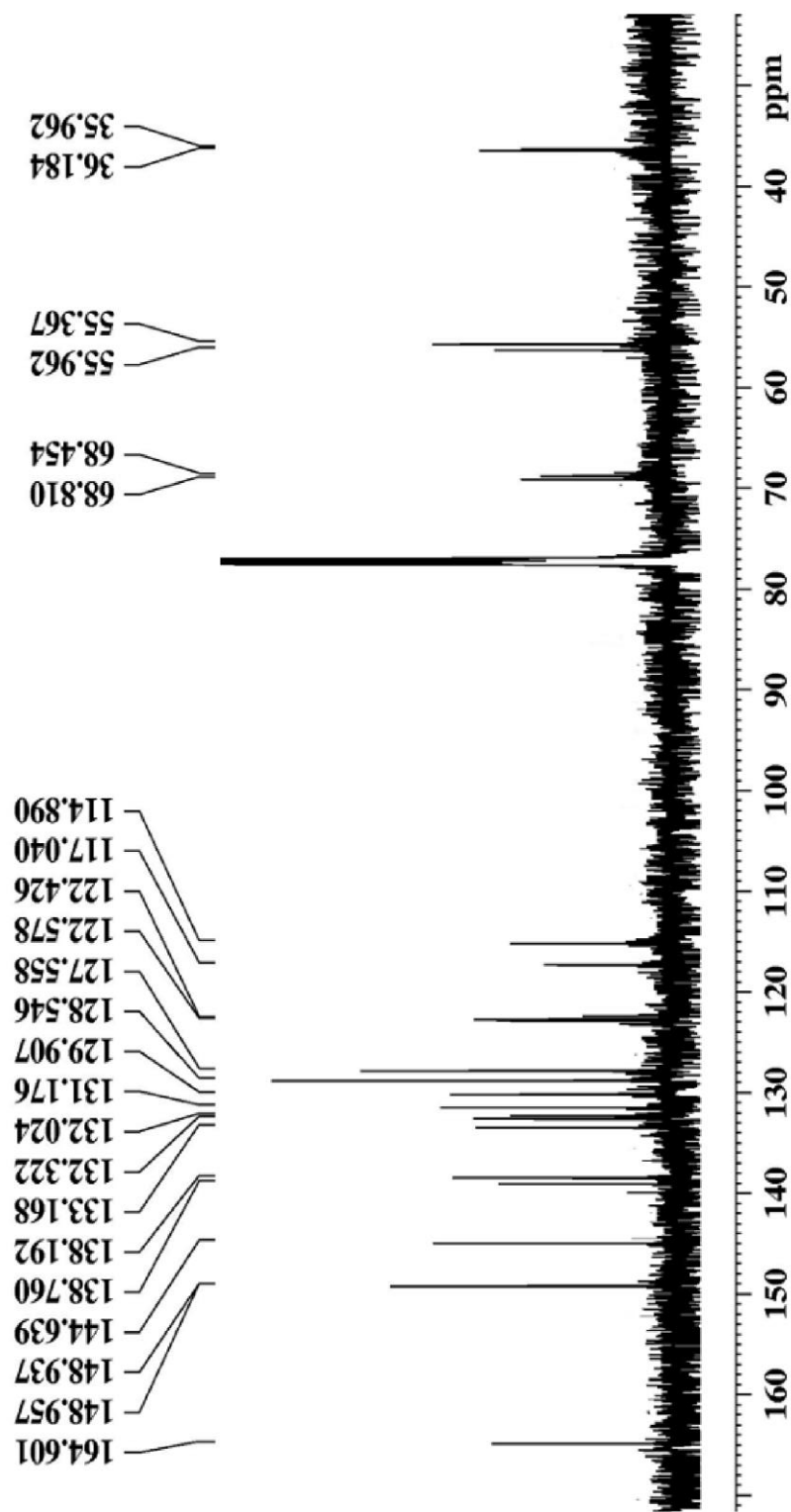
Appendix B: NMR Spectra

^1H NMR of 2a-(S)-(-)



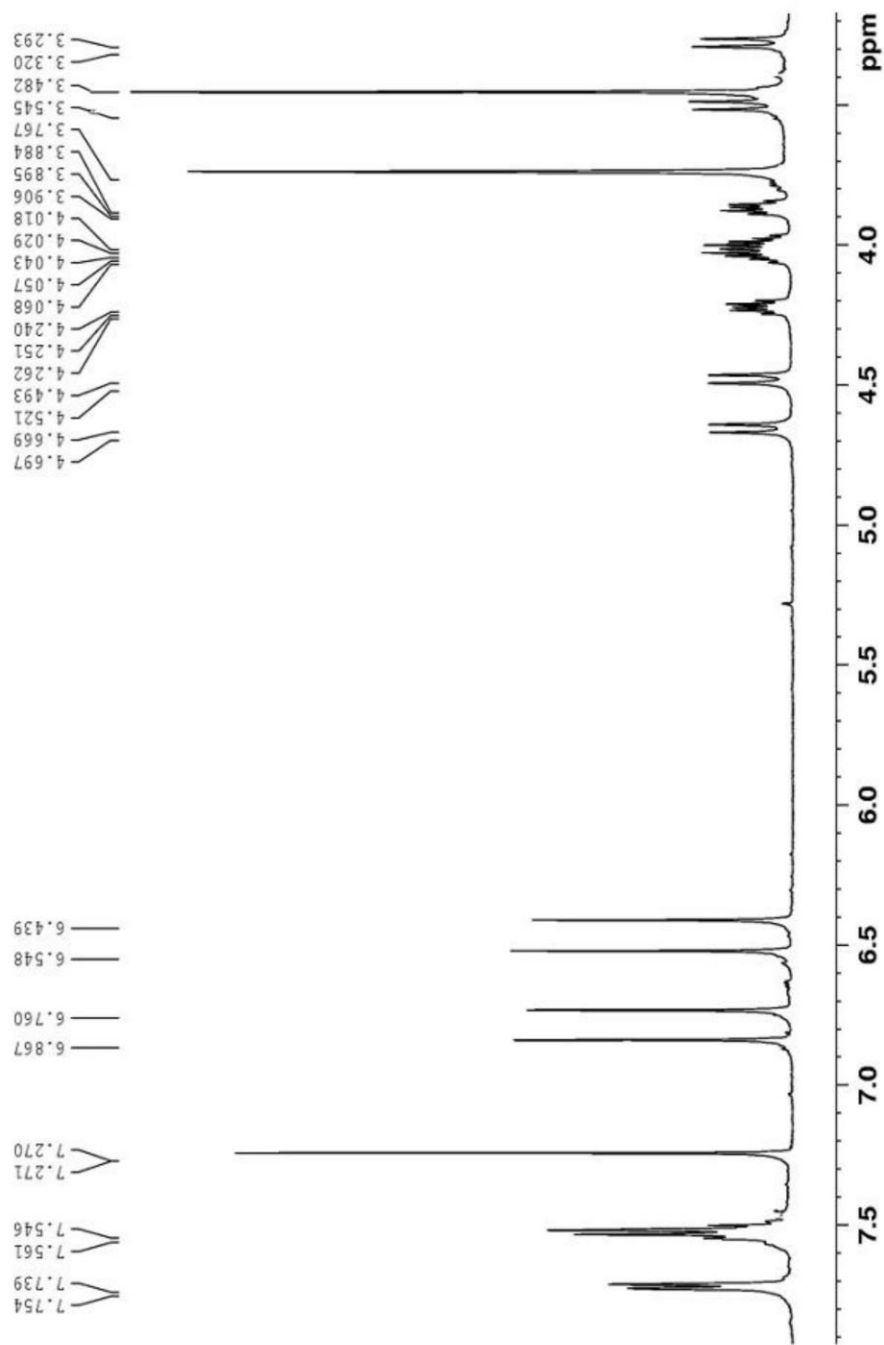
^1H NMR spectra for 2a-(S)-(-). Reprinted with permission from O. Taratula; M.P. Kim; Y. Bai; J.P. Philbin; B.A. Riggle; D.N. Haase; and I.J. Dmochowski. Synthesis of Enantiopure, Trisubstituted Cryptophane-A Derivatives. *Org Lett.* **2012**, *14*, (14) 3580-3583. Copyright 2012 American Chemical Society.

^{13}C NMR of 2a-(S)-(-)



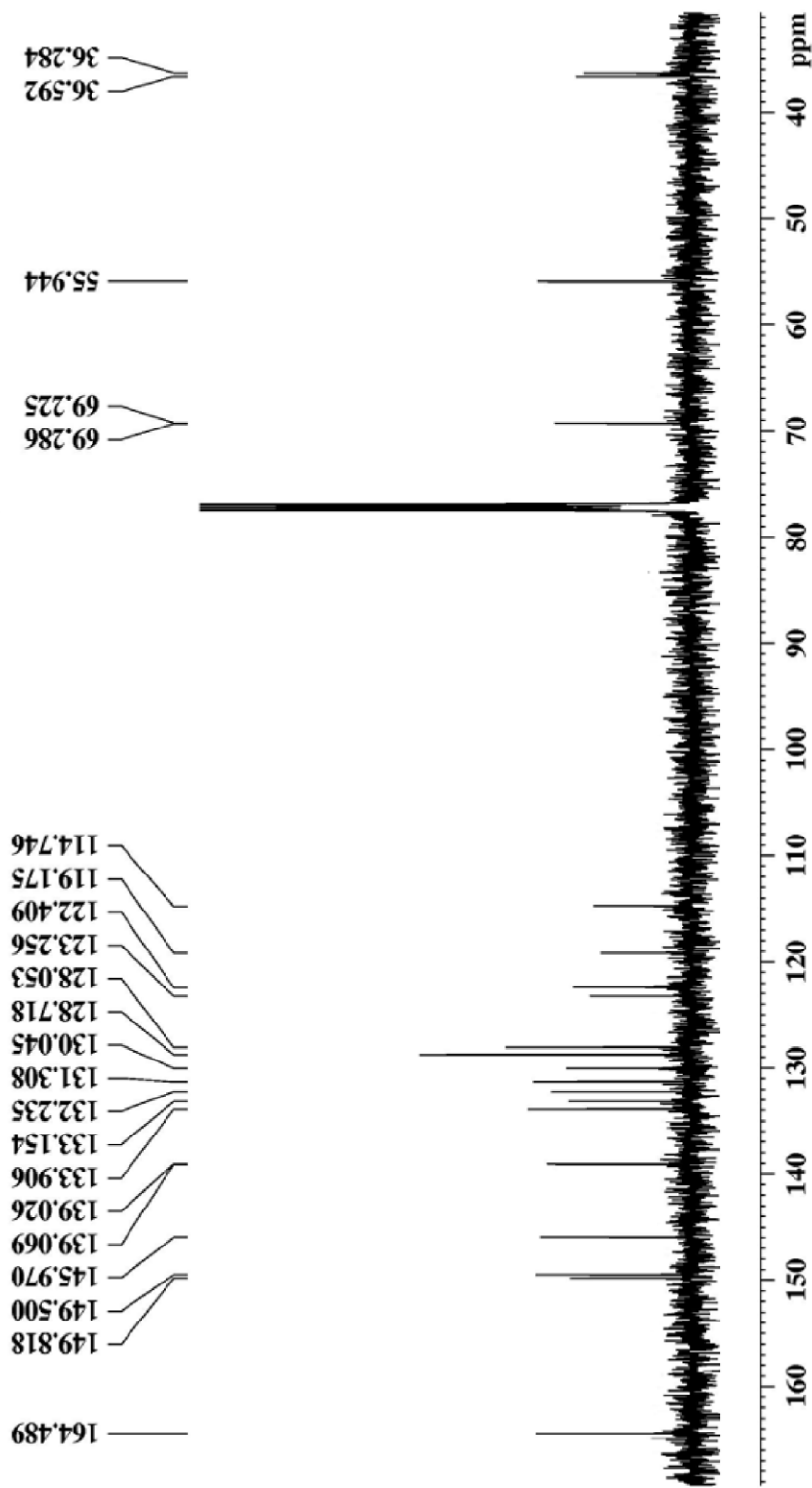
^{13}C NMR spectra for 2a-(S)-(-). Reprinted with permission from O. Taratula; M.P. Kim; Y. Bai; J.P. Philbin; B.A. Riggle; D.N. Haase; and I.J. Dmochowski. Synthesis of Enantiopure, Trisubstituted Cryptophane-A Derivatives. *Org Lett.* **2012**, 14, (14) 3580-3583. Copyright 2012 American Chemical Society.

¹H NMR of 2a-(S)-(+)



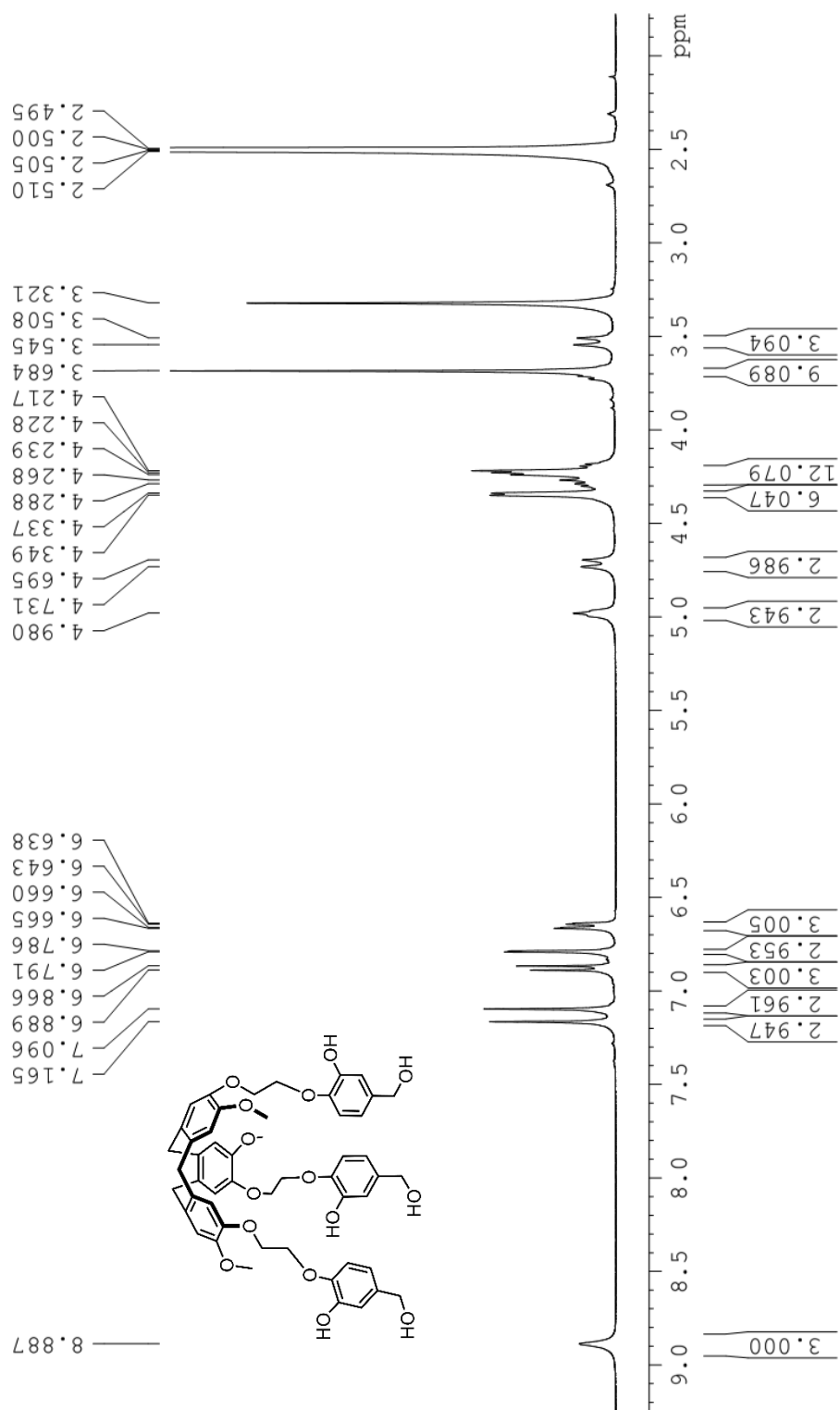
¹H NMR spectra for 2a-(S)-(+). Reprinted with permission from O. Taratula; M.P. Kim; Y. Bai; J.P. Philbin; B.A. Riggle; D.N. Haase; and I.J. Dmochowski. Synthesis of Enantiopure, Trisubstituted Cryptophane-A Derivatives. *Org Lett.* **2012**, 14, (14) 3580-3583. Copyright 2012 American Chemical

¹³C NMR of 2a-(S)-(+)

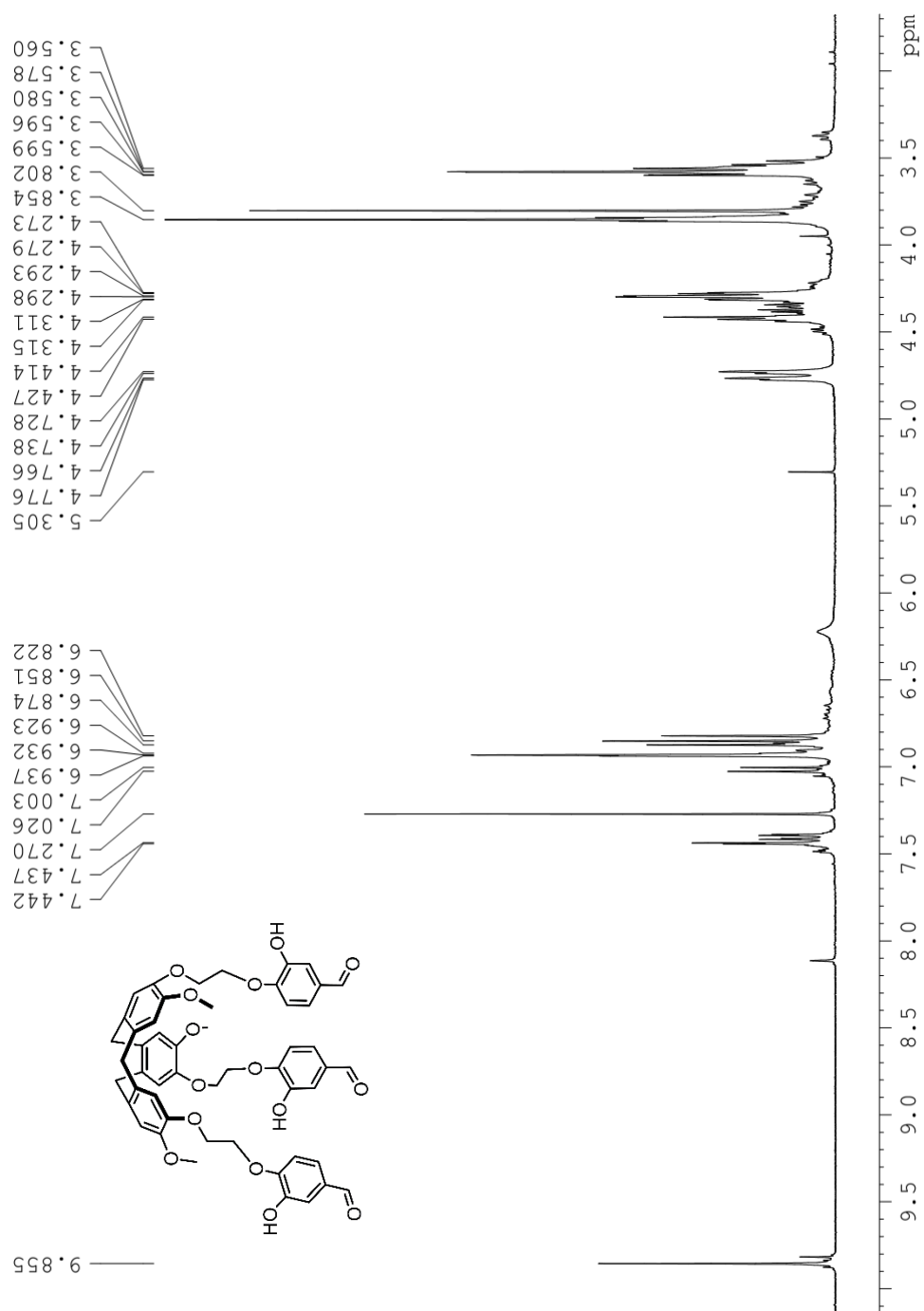


¹³C NMR spectra for **2a-(S)-(+)**. Reprinted with permission from O. Taratula; M.P. Kim; Y. Bai; J.P. Philbin; B.A. Riggle; D.N. Haase; and I.J. Dmochowski. Synthesis of Enantiopure, Trisubstituted Cryptophane-A Derivatives. *Org Lett.* **2012**, 14, (14) 3580-3583. Copyright 2012 American Chemical Society.

NMR of trihydroxy cryptophane precursor from Route 1 in Chapter 2.

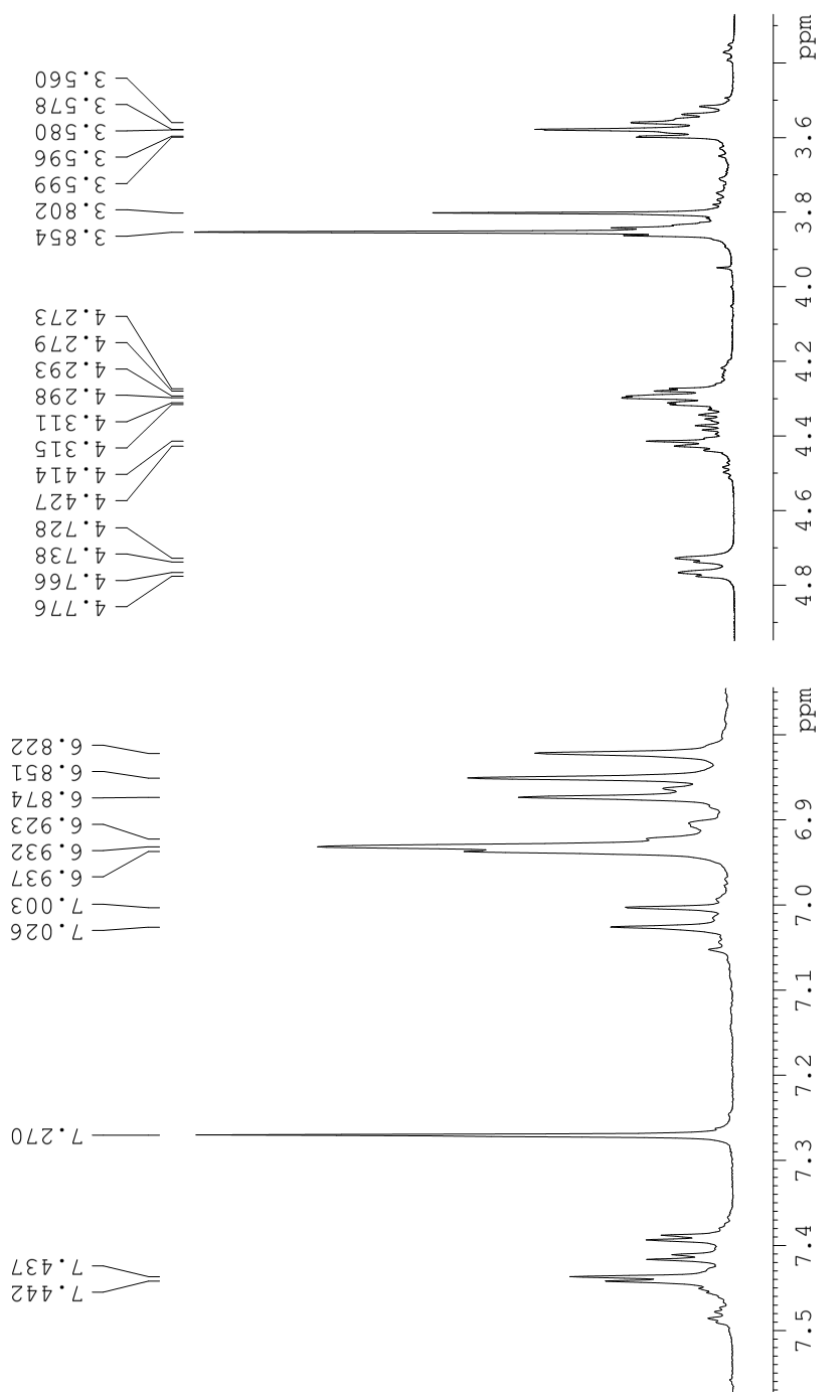


Crude NMR of trihydroxy- aldehyde precursor to trihydroxy cryptophane from Route 3 in Chapter 2.



Crude NMR of trihydroxy- aldehyde precursor to trihydroxy cryptophane from Route 3 in

Chapter 2, zoom.



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