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A cryptophane-based "turn-on" ^{129}Xe NMR biosensor for monitoring calmodulin†

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We present the first cryptophane-based "turn-on" ^{129}Xe NMR biosensor, employing a peptide-functionalized cryptophane to monitor the activation of calmodulin (CaM) protein in solution. In the absence of CaM binding, interaction between the peptide and cryptophane completely suppresses the hyperpolarized ^{129}Xe-cryptophane NMR signal. Biosensor binding to Ca^{2+}-activated CaM produces the expected ^{129}Xe-cryptophane NMR signal.

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.[1] CaM binds four Ca^{2+} ions, which activates binding to a variety of helix-forming peptide sequences with μM to nM K_D values.[2] Comparison of crystallographic and solution NMR structures of calcium-free (apo)[3] and calcium-bound (holo)[4] CaM revealed that Ca^{2+} binding induces conformational changes necessary for peptide binding.[5] When Ca^{2+}-CaM binds to a cognate peptide, it undergoes a second dramatic conformational change (Fig. 1), which has been probed using many spectroscopic techniques.[2,6] Here, we investigate the potential for using sensitive hyperpolarized (hp) ^{129}Xe NMR spectroscopy to detect active CaM in solution.

Our sensor design builds on previous work characterizing CaM complexes with olfactory cyclic nucleotide-activated cation channels (OCNCs) that function to mediate olfactory and visual signal transduction.[7] It was determined that CaM binds to a 26-amino acid N-terminal portion of rat, bovine, and fish transmembrane OCNCs and that Ca^{2+} is necessary for substrate binding.[7,8] The CaM binding motif in this and other peptides is characterized by several attributes, namely, a basic amphiphilic structure, the ability to form a helix, and two hydrophobic "anchor residues", typically 10–14 amino acids apart.[7,9] The original 26 amino acid sequence was pared down to a 17-mer truncated peptide that retained the ability to bind CaM, which we here call FRRIAR (Scheme 1).[6a] This truncated peptide retains two aromatic amino acids characteristic of CaM binding motifs: Phe[1] and Trp[14] in a canonical 1–14 binding mode.[7,9b]

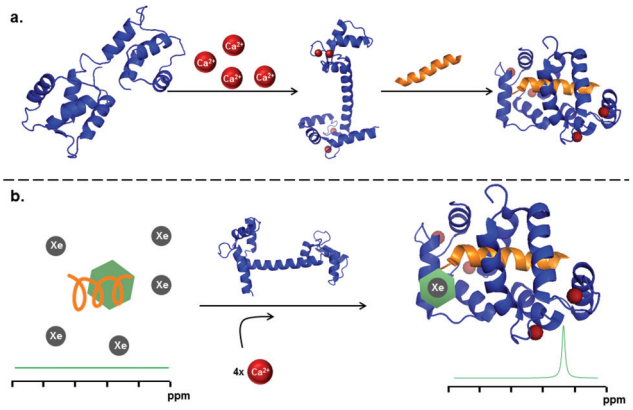

Fig. 1 a. Native conformational change of CaM with the addition of 4 calcium ions and subsequent folding in the presence of FRRIAR peptide. b. "Turn-on" ^{129}Xe NMR signal resulting from FRRIAR-TUC biosensor binding to holo CaM. CaM binding peptide structure taken from PDB 1SY9. Image generated in PyMOL (Delano Scientific, LLC; South San Francisco, CA USA).

The Dmochowski laboratory and others have developed hyperpolarized (hp) ^{129}Xe NMR biosensors for detecting proteins, oligonucleotides, and metal ions in solution.[10] Less investigated is the potential for studying protein–protein interactions using ^{129}Xe NMR spectroscopy.[11] Xenon-129 has a spin $\frac{1}{2}$ nucleus and isotopic abundance of 26.4%.[12] Non-Boltzmann distributions of spins can be achieved in a hyperpolarization process termed spin-exchange optical pumping (SEOP). 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 its

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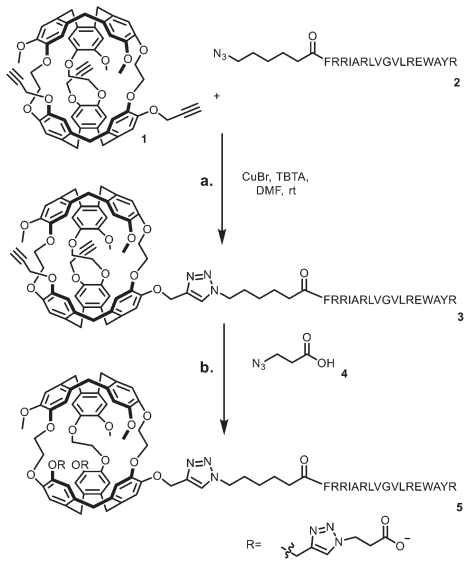

Scheme 1 Synthesis of FRRIAR peptide conjugated to trisubstituted, ultrasensitive cryptophane (FRRIAR-TUC). a. **1** (1 eq.), **2** (0.8 eq.), CuBr (2 eq.), TBTA (3 eq.), 3 h; b. **3** (crude), **4** (10 eq.), 12 h.

polarizable electron cloud, xenon exhibits appreciable affinity for small-molecule hosts such as cryptophane derivatives, and to a lesser extent, cucurbit[6]uril.[13] Cryptophanes can be made water-soluble and provide a hydrophobic cavity with internal volume roughly double that of Xe (D = 4.3 Å, $V \approx 40$ Å^3).[13f–h,14] Our laboratory[11,15] and others[16] have designed cryptophane-xenon biosensors targeted to specific biomarkers indicated in cancer, including integrin[11b] and folate receptors,[15b] EGFR,[16h] mIgM,[16i] MMP-7,[17] and carbonic anhydrase.[15a] Additionally, studies have demonstrated the use of xenon biosensors to target live cells.[15c,18] In these prior examples, Xe binding to cryptophane biosensors produced a specific hp ^{129}Xe NMR chemical shift, which shifted downfield when bound to protein analyte.

Herein, we demonstrate a novel turn-on detection approach where hp ^{129}Xe-cryptophane NMR signal is absent until a protein–peptide interaction occurs. We demonstrate a binary (ON/OFF) ^{129}Xe signal in the presence and absence of Ca^{2+}-CaM (Fig. 1). Building on our earlier synthetic work, we conjugated the FRRIAR CaM-binding peptide and two water-solubilizing moieties to our tripropargyl cryptophane host molecule. Scheme 1 shows the synthesis of the FRRIAR peptide-trifunctionalized, ultrasensitive cryptophane (FRRIAR-TUC) **5**, the details of which are provided in the ESI.† 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%.[15c] Azido-FRRIAR peptide **2** was prepared with standard Fmoc-based synthetic methods. A single peptide was attached to the cryptophane *via* copper(I)-catalyzed [3 + 2] azide–alkyne cycloaddition (CuAAC) to form **3** after a 3 h reaction.[19] Conjugation of a single peptide to the cryptophane was achieved by controlling reaction stoichiometry (1 : 0.8 cryptophane to peptide). Analysis by reversed-phase high performance liquid chromatography (RP-HPLC) indicated complete consumption of azido-peptide and formation of compound **3** and was confirmed by matrix-assisted laser desorption mass spectrometry (MALDI-MS, Fig. S1 and S2†). The triazole-hexyl spacer keeps the peptide in close proximity to the cryptophane-bound ^{129}Xe nucleus while minimizing potential steric clashes with CaM upon peptide binding. A solubilizing linker, 3-azidopropionic acid **4**, was synthesized in one step from the commercially available β-propiolactone (see ESI†).[11b,15a] After consumption of **2**, as confirmed by HPLC (Fig. S1†), **4** was added to the crude reaction containing **3** without further addition of copper catalyst. Starting from tripropargyl cryptophane **1**, FRRIAR-TUC **5** was isolated in ~70% yield after HPLC purification (Fig. S3†) and its identity was confirmed by MALDI-MS (Fig. S4†).

To ensure that FRRIAR-TUC binds holo CaM in the expected 1 : 1 stoichiometry, we employed a native gel-shift assay previously demonstrated by Liu *et al.*[7] In this assay, protein migration is retarded after binding peptide, relative to CaM alone. This assay confirmed that the FRRIAR-TUC biosensor binds in a 1 : 1 stoichiometry at 10 μM holo CaM concentration in CaM binding buffer: 10 mM HEPES, 1 mM $CaCl_2$, pH 7.2 with 1% DMSO (Fig. 2 and Fig. S5 and S6†).[7] To investigate whether the biosensor binds to CaM in the same calcium-dependent fashion as peptide alone, apo CaM was prepared (see procedure in ESI†) and characterized by thermal denaturation using electronic circular dichroism (ECD) spectroscopy (Table S2, Fig. S7†). Holo CaM is thermostable with a melting temperature (T_m) greater than 90 °C, and can be easily differentiated from the apo protein for which the reported T_m is approximately 50 °C, depending on buffer conditions.[20] Native gel analysis verified that FRRIAR-TUC, like FRRIAR peptide alone, cannot bind CaM in the absence of Ca^{2+} (Fig. 2, lanes 3 & 5; in 10 mM HEPES, pH 7.2, 1% DMSO). Finally, we investigated if non-specific interactions between CaM and the biosensor could promote protein conformational change. The assay was repeated with CaM and a water-soluble tris(triazole propionic acid) cryptophane-A derivative (TTPC, structure shown in Fig. S11†), which is identical to FRRIAR-TUC, except for a third propionic acid moiety replacing the FRRIAR peptide. Lack of gel shift was observed (Fig. S8†), confirming that cryptophane alone does not generate CaM conformational change.

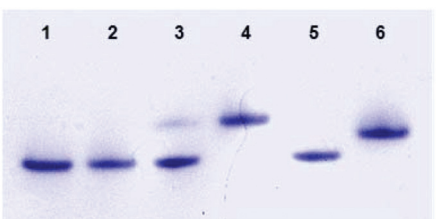

Fig. 2 Native gel-shift assay with samples of 10 μM CaM only or 10 μM CaM plus either 10 μM FRRIAR peptide or 10 μM FRRIAR-TUC biosensor in 10 mM HEPES, ±1 mM $CaCl_2$, pH 7.2. (1) Apo CaM only; (2) Holo CaM only; (3) Apo CaM + FRRIAR peptide; (4) Holo CaM + FRRIAR peptide; (5) Apo CaM + FRRIAR-TUC; and (6) Holo CaM + FRRIAR-TUC.

Although NMR[7,8] and X-ray crystallographic[21] studies indicate that the FRRIAR peptide is helical upon CaM binding (Fig. 1), ECD studies demonstrate that the peptide is less folded (Fig. 3, red trace) when free in solution. Interestingly, after conjugating FRRIAR to cryptophane, the peptide helicity (Fig. 3, blue trace) increased from 11% to 37% (Table S1 and Fig. S9†). Because racemic cryptophane does not contribute to the ECD signal, we conclude that conjugation to the cryptophane promotes FRRIAR peptide folding, through cryptophane–peptide interactions. This provides evidence of interaction between cryptophane and conjugated peptide. These data are consistent with previous data showing that cryptophane promotes helicity in a helix-forming peptide, likely through cryptophane/peptide π-stacking interactions.[15c]

The FRRIAR peptide contains a single tryptophan residue (Trp^{14}) towards the C-terminus that we hypothesized should provide a useful probe of peptide–protein binding, as well as changes in peptide–cryptophane interaction. Fluorescence binding studies (λ_{ex} = 295 nm) with FRRIAR demonstrated a hypsochromic shift and increased Trp emission intensity upon binding holo CaM (Fig. 4a). The emission maximum for the FRRIAR peptide decreased from 352 nm to 332 nm with increasing concentrations of CaM protein. This is a blue shift of 20 nm. In contrast, the emission maximum for FRRIAR-TUC is 328 nm in the absence of CaM, with a slight additional blue-shift of 3 nm and similar trend of increasing intensity upon CaM titration (Fig. 4b). The observation of Trp fluorescence quenching in FRRIAR-TUC is consistent with cryptophane–peptide interaction, and, more specifically, the Trp residue interacting with the hydrophobic cryptophane moiety. Upon CaM binding, the cryptophane quenching of FRRIAR is reduced. Notably, the cryptophane also fluoresces (λ_{max} = 317 nm) upon 295 nm excitation, which contributes to the observed blue-shifted emission spectrum for the cryptophane–FRRIAR peptide conjugate. For comparison, the emission spectrum of TTPC is shown in Fig. 4b (black trace) and agrees with our previous studies.[13g,17]

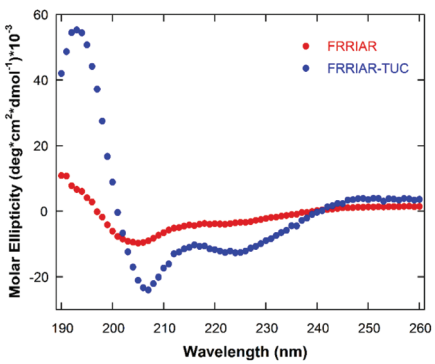

Fig. 3 Circular dichroism comparison of FRRIAR peptide (red trace, 30 μM) *vs.* FRRIAR-TUC biosensor (blue trace, 10 μM) in 1 : 1 acetonitrile to water.

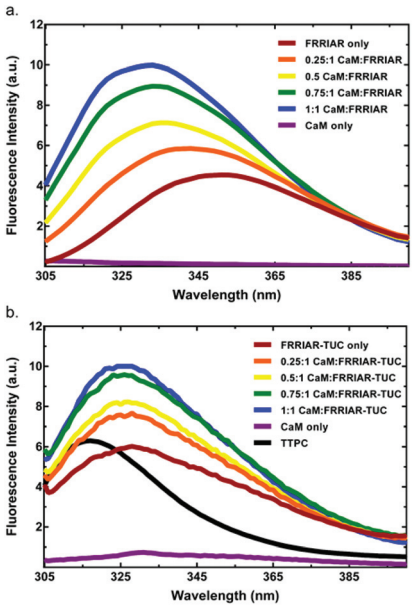

Fig. 4 CaM binding monitored by Trp fluorescence for a. FRRIAR peptide (30 μM) with varying concentrations of CaM protein (0, 7.5, 15, 22.5, or 30 μM in 10 mM HEPES, 1 mM $CaCl_2$, pH 7.2) and; b. FRRIAR-TUC (30 μM) with varying concentrations of CaM protein (0, 7.5, 15, 22.5, or 30 μM in 10 mM HEPES, 1 mM $CaCl_2$, pH 7.2). Water-soluble cryptophane TTPC (15 μM in 1 mM PBS).

Finally, hp ^{129}Xe NMR spectroscopy was employed to further investigate the FRRIAR-TUC/CaM interaction. Notably, FRRIAR-TUC alone gave no hp ^{129}Xe-cryptophane NMR signal at a concentration of 70 μM in CaM binding buffer; only hp $^{129}Xe@H_2O$ resonance was observed (Fig. 5a). At this concentration (determined by UV-vis spectroscopy Fig. S10†), the

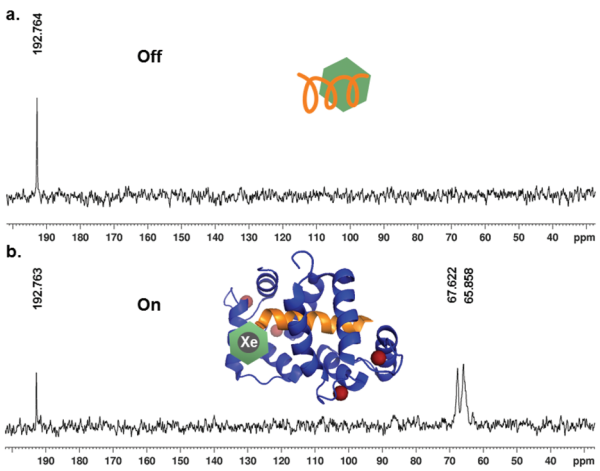

Fig. 5 FRRIAR-TUC alone and in the presence of apo CaM resulted in no hp ^{129}Xe NMR signal (a); CaM bound to FRRIAR-TUC resulted in two distinct ^{129}Xe NMR resonances (b). NMR performed in 10 mM HEPES, 1 mM $CaCl_2$, pH 7.2 with 1% DMSO. $^{129}Xe_{aq}$ peak shown at 192.8 ppm. CaM binding peptide structure taken from PDB 1SY9. Image generated in PyMOL (Delano Scientific, LLC; South San Francisco, CA USA).

^{129}Xe-cryptophane signal has been easily observable in prior constructs studied in our laboratory.[11b,15] Additionally, TTPC at the same concentration and in the same buffer conditions generates a strong ^{129}Xe NMR signal (Fig. S12†). Upon addition of equimolar holo CaM, FRRIAR-TUC in CaM binding buffer, produced a strong ^{129}Xe NMR signal corresponding to cryptophane-CaM complex formation (Fig. 5b). The two bound peaks (65.9, 67.6 ppm) were assigned to diastereomers, which are formed when racemic cryptophane 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.[15a,16a] No hp ^{129}Xe NMR signal was observed for FRRIAR-TUC in the presence of apo CaM (Fig. S13†), as the FRRIAR/CaM interaction is much weaker in the absence of calcium (as confirmed by gel electrophoresis, Fig. 2). Additionally, there was no observed signal when hp ^{129}Xe NMR spectroscopy was performed on holo CaM in the presence of FRRIAR alone (Fig. S14†). These experiments demonstrate the first example of a "turn-on" cryptophane-based xenon NMR biosensor, which results from the binding of FRRIAR-TUC to Ca^{2+}-loaded CaM.

Our ECD and Trp fluorescence data indicate significant cryptophane–peptide interaction, as has been previously demonstrated for related xenon biosensors with a pendant peptide incorporating aromatic residues.[15c,17] Such interactions, particularly when aromatic residues are positioned near the cryptophane, are likely to favor less xenon-accessible cryptophane conformations resulting in an "OFF" signal from the biosensor. We hypothesize that upon FRRIAR binding to holo CaM—which has high affinity for the 17-mer peptide anchored by conserved Phe^{1} and Trp^{14} residues—cryptophane–FRRIAR interactions are decreased, making the cryptophane more accessible to xenon binding.[7] The result is that xenon biosensor signal is turned "ON".

In conclusion, we report a straightforward synthesis for the first "turn-on" cryptophane-xenon biosensor, which was used to detect Ca^{2+}-bound CaM 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.[16e] For example, large-spectral-width, high-power pulses that increase detection sensitivity can be employed without interfering signal from untargeted biosensor(s). Thus, this work highlights a potential new paradigm for cryptophane-based xenon biosensors. Future studies will help to elucidate the nature of peptide–cryptophane interactions that can produce the "OFF" state.

Conflicts of interest

There are no conflicts of interest to declare.

Acknowledgements

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