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Calcium-Induced Morphological Transitions in Peptide Amphiphiles Detected by ^{19}F -Magnetic Resonance Imaging

Adam T. Preslar^{†,‡,§}, Laura M. Lilley^{†,§}, Kohei Sato[‡], Shanrong Zhang^{||}, Zer Keen Chia[§], Samuel I. Stupp^{*,†,⊥}, and Thomas J. Meade^{*,§}

[§]Departments of Chemistry, Molecular Biosciences, Neurobiology and Radiology, Northwestern University, Evanston, Illinois 60208, United States

[⊥]Departments of Chemistry, Materials Science and Engineering, and Biomedical Engineering, Northwestern University, Evanston, Illinois 60208, United States

[‡]Simpson Querrey Institute for BioNanotechnology in Medicine and Department of Medicine, Feinberg School of Medicine, Northwestern University, Chicago, Illinois 60611, United States

^{||}Advanced Imaging Research Center, University of Texas Southwestern Medical Center, Dallas, Texas 75390, United States

Abstract

Misregulation of extracellular Ca^{2+} can indicate bone-related pathologies. New, noninvasive tools are required to image Ca^{2+} fluxes and fluorine magnetic resonance imaging (^{19}F -MRI) is uniquely suited to this challenge. Here, we present three, highly fluorinated peptide amphiphiles that self-assemble into nanoribbons in buffered saline and demonstrate these nanostructures can be programmed to change ^{19}F -NMR signal intensity as a function of Ca^{2+} concentration. We determined these nanostructures show significant reduction in ^{19}F -NMR signal as nanoribbon width increases in response to Ca^{2+} , corresponding to ^{19}F -MR image intensity reduction. Thus, these peptide amphiphiles can be used to quantitatively image biologically relevant Ca^{2+} concentrations.

Graphical abstract

*Corresponding Authors: tmeade@northwestern.edu. Phone: 1-(847) 491-2481 (T.J.M). s-stupp@northwestern.edu. Phone: 1-(847) 491-3002 (S.I.S.).

ORCID: Kohei Sato: 0000-0002-8948-8537

Samuel I. Stupp: 0000-0002-5491-7442

Thomas J. Meade: 0000-0001-6202-1155

[†]Author Contributions: A.T.P. and L.M.L. contributed equally.

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Detailed synthetic procedures, purification, and NMR characterization of PAs, as well as MRI parameters (PDF)

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Keywords

peptide amphiphile; MRI; ^{19}F ; calcium sensing; morphology

Abnormal calcium concentrations have been associated with a variety of pathologies, including Paget's disease and osteoporosis.¹ Osteoporosis is characterized by pathological reduction in bone density and greatly increases risk of severe bone fractures and hospitalizations.² Current methods of measuring bone density use ionizing radiation and can only identify advanced stages of osteoporosis.³ Ca^{2+} fluctuations from 2–10 mM are linked to bone resorption.⁴ Direct observation is difficult and new tools are needed to image Ca^{2+} flux in real time.

Magnetic resonance imaging (MRI) allows noninvasive measurement of biological phenomena without the use of ionizing radiation and produces excellent anatomical contrast with high spatial resolution in soft tissues.⁵ Typically, paramagnetic MR probes produce signal indirectly via ^1H signal enhancement of endogenous water by accelerating magnetic ^1H relaxation.⁶ Despite advances in probe design, relaxation-based MR probes are limited by native tissue background.⁷ We have therefore been interested in investigating alternative methods to develop directly detected MR probes. For example, the absence of fluorine-containing organic molecules in biological systems has encouraged the development of ^{19}F -probes. ^{19}F offers zero-background signal and thus has the potential for quantitative imaging.^{8–10} While ^{19}F -MRI has low sensitivity on a per-atom basis (~ 0.5 mM),^{7,11} the signal can be enhanced by a high local concentration of magnetically equivalent fluorine atoms. This has been accomplished with the use of emulsions or micelles.^{7,12–15}

The tendency of highly fluorinated molecules to aggregate due to their hydrophobicity can be exploited to create biologically responsive agents. Aggregated organofluorine moieties with slow molecular dynamics on the MR time scale (~ 0.1 s) have been reported to be MR silent due to rapid T_2 relaxation.¹⁶ The tunable properties of self-assembling systems can be used to design fluorinated agents that can switch between different aggregation states with distinct MR signals in response to a stimulus, such as enzymatic cleavage or pH.^{17,18} Generally, these responsive probes are designed to switch between assembled and unassembled states.^{16–20} In this work, we have used self-assembling fluorinated peptide amphiphiles (F-PAs) to create nanoscale agents that can switch between “light” and “dark” states without completely disrupting assemblies.

PAs that self-assemble into high aspect ratio nanofibers were first reported by Stupp et al.²¹ These molecules contain short peptide sequences that include a domain with the propensity

to form β -sheets, charged residues, and a single hydrophobic moiety covalently grafted to one terminus. These sequences can be programmed to exhibit a diverse variety of one-dimensional morphologies and dimensions.^{20–22} Additionally, these bio-compatible PAs have been shown to be highly functional in imaging applications.^{23–25} Designing PA molecular imaging probes with variable morphology shows promise for sensing biochemical events. Previously, we reported on a series of F-PAs that self-assembled into various morphologies.²⁶ Some of these agents exhibit changes in morphology, but not necessarily secondary structure, and consequently ^{19}F -MR signal as a function of pH.²³ In this work, we examine the self-assembly behavior and the ^{19}F -MR response of three F-PAs to titration with Ca^{2+} with the long-term objective of sensing Ca^{2+} in vivo. A series of three F-PAs bearing the β -sheet-forming sequence V_2A_2 were synthesized incorporating K_2 , E_2 , or E_3 charged groups for solubility (Figure 1).

The V_2A_2 sequence is known to promote β -sheet formation and the assembly of 1D nanostructures.²⁷ Glutamic acid was chosen for its ability to chelate Ca^{2+} and cross-link negatively charged peptide nanofibers.²⁸ A positively charged, lysine bearing sequence was selected as a control without affinity for Ca^{2+} . The peptides were prepared by solid-phase peptide synthesis and tridecafluoroheptanoyl chloride was condensed to the terminal amine of each peptide.²⁶ The resulting fluorinated peptide amphiphiles (F-PAs) were cleaved from the resin and purified by reverse-phase high-pressure liquid chromatography (HPLC). Purity was assessed by analytical HPLC-MS (Figures S1 and S2). Unless otherwise indicated, solutions and gels were prepared for analysis by dissolving each F-PA at 2 mM concentration in 100 mM NaCl and 30 mM Tris buffer adjusted to pH 7.4. The 2 mM peptide concentration had previously been optimized for similar F-PAs, such systems are employed as implants and persist in vivo over long periods without diminishing signal.^{26,29} Solutions were allowed to equilibrate for at least 12 h.²⁹ CaCl_2 was added from concentrated stock solutions to minimize changes in the total solution volume during titrations. In the case of NMR experiments, 10% D_2O was included as the lock solvent and trifluoroethanol (TFE) at 3 mM was used as an internal standard. Samples were allowed to equilibrate for at least 5 min between CaCl_2 additions.

Characterization of F-PA supramolecular structures by cryogenic transmission electron microscopy (Cryo-TEM) revealed that the morphology was dependent on Ca^{2+} (Figure 2) in negatively charged C_7E_2 and C_7E_3 , but not in positively charged C_7K_2 . Persistence length is a comparative metric of fiber rigidity in soft matter assemblies such as F-PAs.³⁰ Notably, C_7E_2 formed fibers with partial ribbon-like character before the addition of CaCl_2 . C_7E_3 formed wider, more rigid nanoribbons than C_7E_2 , likely arising from intermolecular interactions from the additional glutamic acid. At 6 mM CaCl_2 , it was observed that flat, ribbon nanostructures were the dominant morphology while at 30 mM Ca^{2+} the solution became a turbid gel. Cryo-TEM indicated that the gelled nanoribbons did not have a regular morphology. When compared across the same concentration range, C_7E_3 did not exhibit the same low- Ca^{2+} transition as C_7E_2 , but maintained ribbon morphology at all CaCl_2 concentrations tested. In general, higher concentrations of Ca^{2+} produced shorter, more bundled ribbons (Figure 2). C_7K_2 morphology did not change with CaCl_2 addition (Figure S4).

Variation in fiber width was quantified for each condition based on Cryo-TEM measurements (Figure 3). The width of C₇E₂ ribbons increased significantly ($p < 0.001$) upon the addition of CaCl₂, from 16 ± 4 nm to 22 ± 5 nm with at least 100 independent measurements. This increase in width corresponds to the same calcium window where NMR signal decreases substantially (Figure 4B). A small effect was observed for C₇E₃, where ribbon width increased slightly from 39 ± 10 nm to 45 ± 9 nm ($p < 0.001$). At 30 mM, further changes in the width did not occur in C₇E₃ nanostructures, and the helicity of C₇E₂ became irregular, making an accurate measurement by TEM challenging. Structural evidence for β -sheet character in all F-PAs is provided by solid-state FT-IR spectra featuring a carbonyl band at 1630 cm^{-1} (Figure S5). Furthermore, we observed changes in the circular dichroism spectra upon the addition of Ca²⁺ in the C₇E₂ and C₇E₃ sequences (Figure S6). These changes upon calcium binding are consistent with our recent observation by Overhauser dynamic nuclear polarization relaxometry that the water dynamics around amino acids of peptide amphiphiles change significantly upon the addition of calcium.³¹

Nuclear magnetic resonance spectroscopy (NMR) and magnetic resonance imaging (MRI) measure the same physical phenomenon. Because NMR is typically orders of magnitude more sensitive, we first quantified the response of our probes to Ca²⁺ using NMR spectroscopy. 1D ¹⁹F-NMR spectra for each amphiphile were obtained using an Agilent DD2 500 MHz spectrometer with HFX probe tuned to 470 MHz, and collected over a range of concentrations. Figure 4A follows the trifluoromethyl peak of the perfluorinated heptanoyl tail and integration of the perfluoromethyl group (at about -81 ppm) was quantified as a measurement of T_2 quenching (Figure 4B).

It was observed that C₇E₃ showed an NMR response to Ca²⁺ only at high concentrations (>10 mM). Additionally, the slope of the C₇E₃ signal as a function of Ca²⁺ concentration was much less negative than the corresponding slope for C₇E₂ (Figure 4). C₇E₂ displayed a sharp, order of magnitude signal change, as the nanostructures transition from narrow to wider nanoribbons in response to Ca²⁺ over the physiologically relevant range (2 to 6 mM). This range corresponds to where the C₇E₂ nanostructure increases in ribbon width and rigidity. No change in C₇K₂ signal integration was observed as a function of Ca²⁺ (Figure S7), indicating that Ca²⁺ chelation by glutamic acid residues is responsible for the observed effects in C₇E₂ and C₇E₃. The NMR signal of each F-PA appears to be stable over time when incubated with Ca²⁺ and analyzed over 24 h (Figure S10).

When CaCl₂ is titrated into a solution of F-PA and centrifuged, the Ca²⁺ concentrations in the supernatant do not vary for C₇K₂, but are significantly reduced in C₇E₂ and C₇E₃ as quantified by inductively coupled plasma-optical emission spectroscopy (ICP-OES). This supports Ca²⁺ chelation by C₇E₂ and C₇E₃ but not C₇K₂ (Figure S11). Interestingly, when F-PA is titrated with MgCl₂, no change in NMR signal is observed by any of the F-PAs, suggesting selectivity for Ca²⁺ versus Mg²⁺ (Figures S11 and S12). This supports previous studies that found Ca²⁺ binds cooperatively, rather than relying on purely electrostatic interactions.³²

¹⁹F-MR imaging of the F-PAs was performed using a ¹⁹F surface coil at 9.4 T. A steady-state free precession (SSFP) sequence was utilized, including a sinc pulse (1.5 ms in length)

as an excitation pulse to excite the desired ^{19}F signal (ca. -76 to -80 ppm).^{19,20} An internal standard of TFE (3 mM) was added to enable signal comparison between different scans. Two mM C_7E_2 and C_7E_3 were each imaged after the addition of 0, 1.7, 3.4, 5.1, and 25.5 mM CaCl_2 (Figure 5).

The results of these imaging experiments paralleled the NMR results in Figure 4. It should be noted that voxel size was increased in the case of ^{19}F for detection. Imaging parameters and phantom preparation are described in the Supporting Information. Specifically, the ^{19}F -MR signal of C_7E_2 was observed to fall off rapidly to background signal levels by 5.1 mM Ca^{2+} . The C_7E_3 signal did not visibly change until Ca^{2+} concentrations increased to a much higher value (25.5 mM). These results verify that the response in C_7E_2 signal over the physiological Ca^{2+} range (2–6 mM) can be detected a MR imaging experiment. The MR signals of each sample were normalized using the external TFE sample and are compared in Figure S13. Signal decay upon Ca^{2+} addition is analogous to the responses observed (Figure 4), where a precipitous change in MR signal occurs with ~ 5 mM Ca^{2+} for the C_7E_2 sequence and signal is retained to high concentrations of Ca^{2+} for the C_7E_3 sequence.

New supramolecular nanostructures have been reported that are capable of measuring calcium ion concentrations using ^{19}F -MRI. These nanostructures are formed by fluorinated peptide amphiphiles that undergo width changes in response to Ca^{2+} and thereby modulate the intensity of their fluorine signals. As the width of the F-PA nanostructure increases, the ^{19}F -MR signal decreases. In particular, C_7E_2 nanostructures were shown to respond strongly to Ca^{2+} between 2 and 6 mM, a range encompassing physiological extracellular calcium concentration fluctuations. We hypothesize this difference in Ca^{2+} response arises from the nanostructural differences between the F-PAs, namely small nanostructure width results in higher Ca^{2+} response. In the case of C_7E_2 the transition from a thin ribbon to a wide ribbon is highly correlated with a decrease in the ^{19}F -MR signal. The C_7E_2 nanostructures were also found to exhibit excellent Ca^{2+} sensitivity, which disappears by the addition of an additional glutamic acid residue to the peptide sequence in C_7E_3 . We believe these results demonstrate how subtle changes in PA chemical structure have a significant impact on the intensity of ^{19}F -NMR signals, thus making the nanostructures useful as ^{19}F -MR imaging agents.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

F-PAs bearing a perfluorinated 7-C hydrophobic tail, a β -sheet forming V₂A₂ sequence, and charged residues C₇K₂ (top), C₇E₂ (middle), and C₇E₃ (bottom). Charged residues were varied to tune Ca²⁺ affinity and modulate nanostructure assembly. The cationic, C₇K₂ sequence was not expected to interact with Ca²⁺.

Figure 2.

Cryo-TEM of the C₇E₂ and C₇E₃ nanostructures in Tris-buffered saline as a function of CaCl₂ concentration. C₇E₂ transitions from a mixture of nanofibers with some ribbon-like character at 0 mM Ca²⁺ to a mixture of less rigid nanoribbons at 6 mM. A turbid gel of rigid ribbons is formed at 30 mM. For C₇E₃, twisted ribbon structures appearing at 0 mM CaCl₂ become more rigid and show greater bundling at higher concentrations of Ca²⁺.

Figure 3. Measurement of nanoribbon width as a function of calcium added for C₇E₂ and C₇E₃ using cryogenic TEM. The increase in ribbon width between 0 mM and 6 mM is significant ($p < 0.002$) for both compounds. Error bars represent standard deviation. C₇E₂ at 30 mM CaCl₂ is omitted because gelation of C₇E₂ prohibited accurate width determination.

Figure 4.

(A) NMR spectra of perfluoromethyl resonance for (i) C₇E₂ and (ii) C₇E₃ as a function of CaCl₂ concentration. The full spectra can be found in Figures S5–S7. (B) Integrated signal of the perfluoromethyl peak for each amphiphile as a function of CaCl₂ concentration. All signals are normalized to a trifluoroethanol (TFE) internal standard. Error bars represent the standard deviation.

Figure 5. ^{19}F -MRI signals of C_7E_2 and C_7E_3 (2 mM) upon titration of CaCl_2 . Signal is observed to drop for C_7E_2 at much lower Ca^{2+} concentrations than for C_7E_3 , demonstrating that the probe is calcium-responsive in the physiological calcium concentration range. Each image is $1.8 \times 1.8 \text{ cm}^2$. Normalized average intensities are plotted in Figure S10.