

Photostable and Proteolysis-Resistant Förster Resonance Energy Transfer-Based Calcium Biosensor

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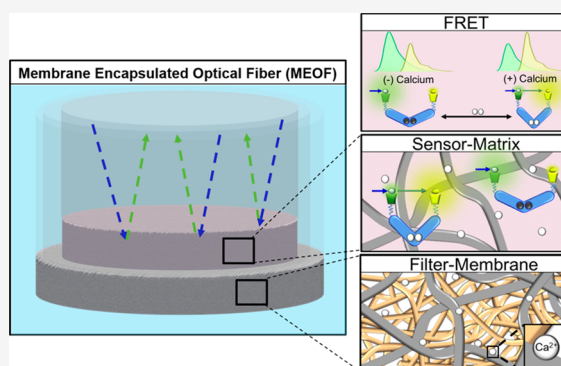
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ABSTRACT: Molecular sensors from protein engineering offer new methods to sensitively bind to and detect target analytes for a wide range of applications. For example, these sensors can be integrated into probes for implantation, and then yield new and valuable physiological information. Here, a new Förster resonance energy transfer (FRET)-based sensor is integrated with an optical fiber to yield a device measuring free Ca^{2+} . This membrane encapsulated optical fiber (MEOF) device is composed of a sensor matrix that fills poly(tetrafluoroethylene) (PTFE) with an engineered troponin C (TnC) protein fused to a pair of FRET fluorophores. The FRET efficiency is modulated upon Ca^{2+} ion binding. The probe further comprises a second, size-excluding filter membrane that is synthesized by filling the pores of a PTFE matrix with a poly(ethylene glycol) dimethacrylate (PEGDMA) hydrogel; this design ensures protection from circulating proteases and the foreign body response. The two membranes are stacked and placed on a thin, silica optical fiber for optical excitation and detection. Results show the biosensor responds to changes in Ca^{2+} concentration within minutes with a sensitivity ranging from 0.01 to 10 mM Ca^{2+} , allowing discrimination of hyper- and hypocalcemia. Furthermore, the system reversibly binds Ca^{2+} to allow continuous monitoring. This work paves the way for the use of engineered structure-switching proteins for continuous optical monitoring in a large number of applications.



The development of continuous biomedical sensors provides clinicians and researchers real-time data on clinically relevant and new physiological signals.¹ Currently, the catalog of continuous sensors is vastly outweighed by the number of clinically relevant analytes, which are largely analyzed with point-of-care (POC) devices or at clinical laboratories. For example, ionized calcium (Ca^{2+}), an essential mineral for muscle contraction, bone development, nerve impulses, blood clotting, and regulating heart beat propagation, is assayed by a calcium blood test; this test requires a healthcare professional to draw blood from a patient's median cubital vein and send it to a clinical laboratory for a complete metabolic panel analysis.^{2,3} The time between depositing a sample and receiving results may be several hours, or approximately 1 h in emergency cases. To eliminate the latency caused by hospital lab delay, POC devices such as Abbott I-STAT can perform on-site assays, including Ca^{2+} , providing results within a few minutes. However, the frequency of assay is still dependent upon typically infrequent, professional blood draws. Though laboratory assays of Ca^{2+} are precise and accurate, the measurements are intermittent as compared to physiological Ca^{2+} dynamics. For example, in clinical cases, such as rapid blood transfusion during liver transplantations, Ca^{2+} concentrations can exhibit rapid

transients at very low concentrations (e.g., drops by 0.1 mM Ca^{2+} in 5 min), underlying the need for a continuous Ca^{2+} sensor.^{2,3}

Advances in protein engineering have yielded new classes of binding macromolecules that display exquisite ligand binding specificity and yield quantifiable signals upon such ligand or target binding.^{4,5} For example, Maeshima et al. developed a Förster resonance energy transfer (FRET)-based molecular Mg^{2+} sensor to monitor Mg^{2+} dynamics during the cell cycle. This sensor comprises the structure-switching *Escherichia coli* (*E. coli*) Mg^{2+} transporter CorA (CorA-CD) positioned between the fluorescent proteins (FPs) enhanced cyan fluorescent protein (eCFP) and Venus (a variant of yellow fluorescent protein, YFP).⁶ Furthermore, a genetically encoded calcium indicator, termed Cameleon, has been reported; this sensor joins structure-switching calmodulin (CaM) and CaM-

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binding peptides of myosin light-chain kinase positioned between eCFP and eYFP to monitor intracellular calcium.⁷ These FRET sensors demonstrate the specificity and sensitivity of genetically encoded protein biosensors.

Here, the calcium FRET sensor Twitch-2B is considered for use in a continuous Ca^{2+} monitor. Twitch-2B was developed by Thestrup et al. for intracellular monitoring of Ca^{2+} transients during T-lymphocyte activation and action potential propagation in mouse cortical neurons.⁸ Twitch-2B is derived from toadfish *Opsanus tau* troponin C (TnC), a muscular actin-associated protein that undergoes structure switching upon Ca^{2+} binding. Twitch-2B comprises a modified TnC (equilibrium dissociation constant for Ca^{2+} , $K_D = 200$ nM) spaced by linkers, each fused at their free ends to the FPs mCerulean3 (cyan FP variant) and cpVenus^{cd} (yellow FP variant), at the N- and C-termini, respectively.⁸ Twitch-2B was determined to be a candidate sensing molecule for a continuous physiological Ca^{2+} probe because of its reversible binding kinetics, stability in vivo, and sensitivity to varying Ca^{2+} concentrations.

A number of calcium sensing modalities have been developed to monitor calcium.⁹ Asif et al. developed an electrochemical sensor to Ca^{2+} comprising functionalized biocompatible ZnO nanorods. In vitro testing shows a log-linear relationship between sensor voltage and Ca^{2+} ranging from 100 nM to 10 mM.¹⁰ Shortreed et al. functionalized the distal end of an optical fiber with the calcium-sensitive dye Calcium Green and reported a unique emission spectrum for Ca^{2+} concentrations ranging from 37.6 nM to 39.8 μM .¹¹

These reported strategies lack a method to prevent interactions with physiological macromolecules, including antibodies, proteases, and other soluble proteins, upon device implantation. Proteins from the foreign body response (FBR) can foul the surface and adversely affect sensing for in vivo applications.¹² In consideration of an implantable Ca^{2+} sensor, a new type of Ca^{2+} sensor is presented that combines a FRET-based sensing molecule with a new membrane to provide the requisite protection for in vivo applications.

An optical fiber device was developed and referred to here as the membrane encapsulated optical fiber (MEOF, Figure 1). MEOF first comprises a hydrophilic poly(tetrafluoroethylene) (PTFE) sheet impregnated with a solution of a protein FRET sensor and placed in contact with the end of an optical fiber. MEOF also includes an outer membrane composed of a size-

selective poly(ethylene glycol) dimethacrylate (PEGDMA) 2000 hydrogel interpenetrating a second PTFE sheet. Results are presented for a Ca^{2+} -sensing MEOF wherein the FRET sensor is a new photoresilient variant of Twitch-2B, SNAP-Twitch-2B-CLIP (STC), which replaces the readily photo-bleached FPs with photostable Alexa Fluor 488 and Alex Fluor 546 as the donor and acceptor fluorophores, respectively.¹³ This Ca^{2+} probe shows a reversible response curve for concentrations ranging between 0.01 and 10 mM and is photostable over prolonged excitation scans, a necessary capability for future implantation. The probe also operates in real time, which is critical for a continuous Ca^{2+} biosensor.

EXPERIMENTAL SECTION

Filter Membrane Fabrication. Monomeric PEGDMA 2000 precursor (Sigma-Aldrich) and Milli-Q water were mixed with a flea-sized micro stirring bar (Grainger) in a 30%/70% (w/w) ratio in a dark room until a homogeneous solution was obtained. Photoinitiator 2-hydroxy-4'-(2-hydroxyethoxy)-2-methylpropiophenone (Irgacure 2959, Sigma-Aldrich) was then mixed at 1% (w/w), and the mixture was illuminated by a UV (365 nm), 8 W VWR dual UV transilluminator for 10 min to form a hydrogel. Subsequently, filter membranes were fabricated by polymerizing the hydrogel precursor solution while in PTFE (FHLC01300, 80% porosity, 0.45 μm pore size, 145 μm thick, Sigma-Aldrich).

Permeability Testing. Permeability of hydrogels was tested using 3 mm diameter disks of PEGDMA 2000. The hydrogels were first allowed to swell overnight in phosphate-buffered saline solution (PBS, pH 7.4, Thermo Fisher Scientific). Swelling reaches a steady state in 5 h (Supporting Information, Figure S1). The swollen hydrogels were then placed in a custom-made rubber chamber (frame size, 20 mm $\times$ 20 mm; mat opening, 15 mm $\times$ 15 mm; height, 1 mm; McMaster-Carr) and incubated in PBS solutions containing Alexa Fluor 488-labeled insulin (5.8 kDa, Supporting Information) or Precision Plus Protein Kaleidoscope pre-stained protein standards (Bio-Rad; 10–250 kDa) for 30 min.¹³ Confocal microscopy was performed using either a Leica Sp8 or Zeiss LSM700 inverted microscope using 488 and 633 nm laser lines and 10 $\times$ or 20 $\times$ objective lens. Emission was collected from 520 to 700 nm and 650–700 nm under 488 and 633 nm laser line excitation, respectively. Images were acquired every 30 s.

The permeability of the filter membranes was tested by first creating 1 mm PTFE disks with a 1 mm biopsy punch (World Precision Instruments). The disks were then glued (Loctite 4981, Henkel) to the face of MRE-025 Microrenathane tubing (i.d., 0.30 mm; o.d., 0.63 mm, Braintree Scientific), and the hydrogel precursor solution was polymerized within the pores of the PTFE. A bare optical fiber (FG200LEA, 0.22 NA, 200 μm core, 220 μm cladding, Thorlabs) was guided down the tubing and placed in contact with the membrane. Loctite 4981 glue was used to seal the back end of the silica optical fiber to the Microrenathane tubing. The distal end of each optical fiber terminated in an SMA thread and was coupled to a custom benchtop spectrometer apparatus (Figure S2). A set of membrane-fiber units were fabricated for each test solution described below. After incubation in PBS, the tip of each optical fiber construct was immersed in a solution containing fluorescein (FITC, 389.4 Da, Sigma-Aldrich), green fluorescent protein (GFP, 26.9 kDa), Alexa Fluor 488-labeled insulin

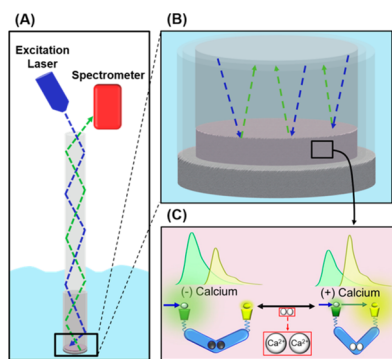


Figure 1. Schematic of the FRET calcium ion probe. (A) Excitation light results in a FRET signal analyzed by a spectrometer. (B) A size-excluding filter membrane and sensor matrix encapsulate the optical fiber. (C) The FRET signal is modulated by calcium ion concentration.

(5.8 kDa), or fluorescein isothiocyanate–dextran (3–5 kDa or 150 kDa, Sigma-Aldrich).^{14,15}

Fabrication of the Membrane Encapsulated Optical Fiber. MEOF probes were fabricated as illustrated in Figure 2.

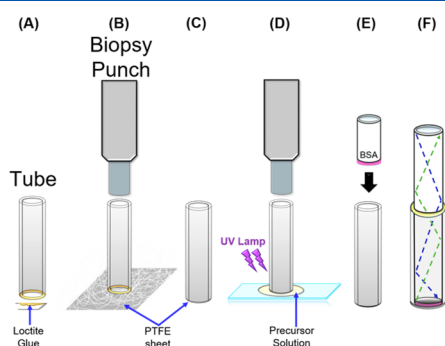


Figure 2. Fabrication of the membrane encapsulated optical fiber (MEOF). (A) Microrenathane tubing is cut, and Loctite 4981 glue is applied by a small needle to its tip. The tubing is placed onto a sheet of PTFE and cut by a biopsy punch (B) to produce a capped tube (C). PEGDMA 2000 hydrogel precursor solution is allowed to impregnate the PTFE sheet (D) such that, after polymerization, a hybrid PEGDMA2000/PTFE membrane is produced. An optical fiber guides a PTFE sheet, wetted with FRET protein solution (shown in pink), to the end of the tubing (E), and it is then sealed on the back end with Loctite 2981 glue (F).

Microrenathane tubing was cut into 5 mm segments (Figure 2A), and the glue was then applied to the bottom annulus of the tubing (Figure 2A) to adhere the tubing to a PTFE sheet (Figure 2B). A 1 mm biopsy punch was slipped over the tubing to cut the PTFE sheet into a circle of 1 mm diameter (Figure 2C). Next, the hydrogel precursor solution was pipetted on a glass slide and the PTFE-capped tubing was pressed into the solution. The hydrogel precursor solution permeates throughout the PTFE due to their compatible hydrophilicity and was polymerized with a 365 nm 8 W VWR dual UV trans-illuminator for 10 min, producing a membrane-capped tube (Figure 2D). A second hydrophilic PTFE sheet was wetted with 2 μ L of FRET sensor solution (4 mg/mL) by first pipetting the FRET protein solution onto the PTFE material, and then compressing this sheet between two microscope slides to form the sensor matrix. In parallel, a silica optical fiber was incubated overnight in a 2% bovine serum albumin (BSA, Chromatopur)/water solution to block the optical fiber's surface and prevent surface-induced aggregation of the FRET sensor. Next, the sensor matrix was placed onto the tip of the BSA-coated optical fiber (Figure 2E) and the assembly was pushed through the tubing until it contacted the filter membrane (Figure 2F). Glue was used to seal the back end of the silica optical fiber to the Microrenathane tubing, and the resulting MEOF was incubated in protein buffer media at 4 °C for at least 5 h. The assembly quality was assessed by MEOFs encapsulating GFP to monitor for leakage. These MEOFs were incubated in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer for 5 h (Figure S3), and the solution was tested for leaking GFP using a fluorescence plate reader (Spark, TECAN). No signal intensity was observed, confirming encapsulation of the protein within the sensor matrix.

FRET Calcium Sensing. Each spectral measurement was acquired in triplicate with exposure times of 100 ms for the Twitch-2B sensor or 2 s for the STC sensor. Background (dark

signal)-subtracted spectra were processed in MATLAB. Custom MATLAB code was used to process the digitized signals and calculate the FRET ratiometric signal, which here is defined as the peak acceptor intensity signal divided by the peak donor intensity signal ($I_{\text{acceptor}}/I_{\text{donor}}$), a metric commonly used to quantify FRET efficiency.¹⁶

Calcium solutions ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, Sigma-Aldrich) at the indicated concentrations were placed in microcentrifuge tubes capped with Parafilm to prevent evaporation. MEOF probes were tested by dipping the devices into these calcium solutions. GraphPad Prism was used for to fit data points onto nonlinear regression models and for statistical analysis.

RESULTS AND DISCUSSION

Characterization of the Filter Membrane. A well-known event, the FBR activates upon device implantation.¹² Of particular importance to the MEOF Ca^{2+} probe, the FBR recruits immune cells that secrete proteases, which could cleave the Twitch-2B and render the device inoperable. Thus, the device must exclude protease access to the molecular sensor.

Here, PEG hydrogels are formulated to size-exclude proteases secreted by immune cells. Such proteases are considerably smaller than immunoglobulins (~150 kDa) and much larger than calcium ions.¹⁷ The smallest of these known proteases is Chymase, which has a molecular weight (MW) of approximately 30 kDa.¹⁸ PEG-derived hydrogels were selected for protein exclusion due to the tunability of their mesh size, swelling, and tensile strength.^{19,20} Additionally, PEG is considered to be generally nonfouling and biocompatible.²¹ Screening of various PEG formulations and derivatives led to the selection of PEGDMA 2000 hydrogels, formulated as described in the Experimental Section, and these were shown to exclude proteins as small as 10 kDa but permit transport of insulin (5.8 kDa). Fluorescence signals from the fluorescently labeled insulin at the hydrogel/fluid interface show very rapid kinetics (Figure 3, parts A and C); in comparison, the 10 kDa and larger proteins show no penetration of the hydrogel as measured by fluorescence (Figure 3, parts B and C).

The PEGDMA 2000 hydrogels are very soft when hydrated, and too fragile to place on the tip of a probe for implantation.

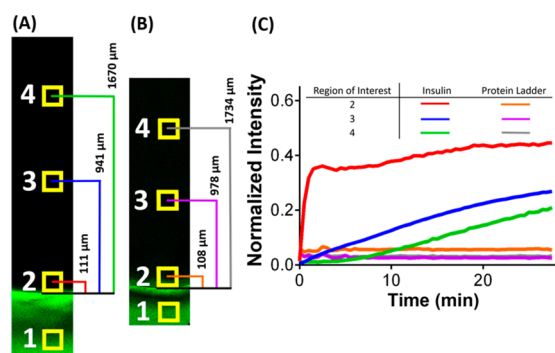


Figure 3. Examining the permeability of the PEGDMA 2000 hydrogel. (A) Confocal micrographs of the PEGDMA 2000 hydrogel surrounded by Alexa Fluor 488-labeled insulin (20X image) or (B) a protein ladder (Bio-Rad Protein Kaleidoscope; 10X image) were captured at $t = 0$. (C) Intensity profiles highlight permeabilization by proteins into the indicated regions of interests (ROIs) over time with normalization for fluorescence in ROI 1. ImageJ software was used to analyze ROI intensity.

Thus, a protective PTFE layer was devised. The hydrogel precursor solution was polymerized within the nonsolid phase of commercially available hydrophilic PTFE sheets. Scanning electron microscopy (FEI Magellan 400 XHR, Electron Microscopy Solutions) after iridium sputter coating (10 nm, EMS 150T, Electron Microscopy Solutions) reveals the natural porosity of PTFE sheets (Figure 4A) and their impregnation

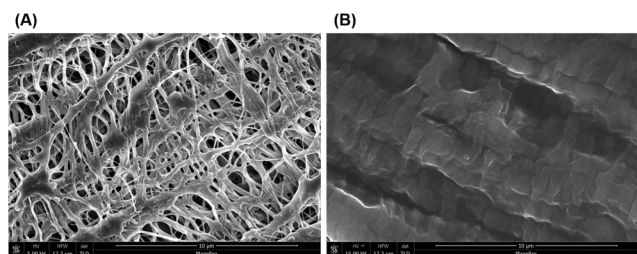


Figure 4. SEM images of PTFE and the filter membrane. Scale bar = 10 μm . (A) The ePTFE sheets contain 0.45 μm pores that are infiltrated with PEGDMA 2000 hydrogel (B) after UV polymerization.

by the PEGDMA 2000 hydrogel (Figure 4B). This composite, termed the filter membrane, combines the size-exclusion properties of the PEGDMA 2000 hydrogel with the durability of PTFE sheets and offers a robust composite material for the tip of the Ca^{2+} sensing device.

Filter membranes were tested to ensure they retain the size selectivity of the hydrogel alone. For each test, a filter membrane was coupled to the end of an optical fiber as described in the Experimental Section. Figure 5A reports that

(A)	Fluorescein	Dextran	Insulin	GFP	Dextran
Molecular Weight (kDa)	0.38	3-5	5.8	26.9	150
Membrane Permeable?	Yes	Yes	Yes	No	No

Figure 5. Molecular weight cutoff properties of the filter membrane. (A) Tabulated permeability results indicate the observed penetration by fluorescently labeled analytes.

FITC, 3–5 kDa fluorescein isothiocyanate–dextran, and Alexa Fluor 488-labeled insulin can diffuse through the filter membrane, while large macromolecules GFP and 150 kDa fluorescein isothiocyanate–dextran cannot. Figure S4A and Figure S4B display the time-dependent emission spectra of Alexa Fluor 488-labeled insulin and GFP. These results show the filter membrane retains the size-exclusion properties of the hydrogel.

Twitch-2B Ca^{2+} Probe. Calcium sensing MEOF biosensors were fabricated as detailed in the Experimental Section, where the FRET molecular sensor was Twitch-2B. Important to the FRET sensor, the sensor matrix allows Twitch-2B to remain in solution, preserving Twitch-2B structure switching upon Ca^{2+} binding and unbinding. The resulting MEOF probe has the following key properties of (1) enabling the passage of small target analytes (e.g., ions and small peptides) yet exclusion of larger proteins (such as serine proteases), (2) allowing FRET sensors to undergo conformational changes upon binding and releasing target analytes, and (3) guiding excitation light to the sensor matrix and fluorescently emitted light to the spectrometer.

In Vitro Testing of MEOF Ca^{2+} Probes. To determine the efficacy of the Twitch-2B MEOF Ca^{2+} probe, probes were incubated in test buffer [25 mM 3-(*N*-morpholino)-propanesulfonic acid (MOPS), 100 mM KCl, pH 7.6] supplemented with increasing concentrations of Ca^{2+} . For each measurement, 0.7 μW of 405 nm laser light was used to excite the protein sensor for 100 ms. Results show the FRET ratio ($I_{\text{acceptor}}/I_{\text{donor}}$) being sensitive to increasing Ca^{2+} concentration from 0 to 14 mM, above which the sensor response saturates (Figure 6A). Importantly, the MEOF sensor

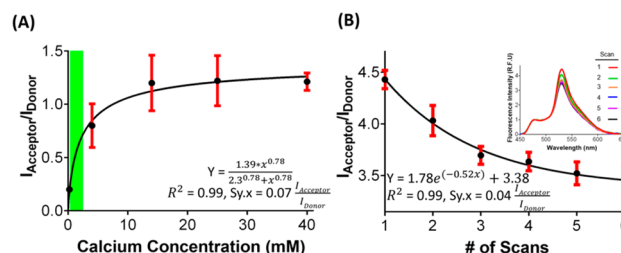


Figure 6. Efficacy of Twitch-2B to sense calcium ions. (A) The dose-dependent response of the MEOF and its data fit to a Hill equation demonstrate the MEOF's usefulness for sensing physiological concentrations of Ca^{2+} (green). $I_{\text{acceptor}}/I_{\text{donor}}$ is the emission ratio after subtracting the spectra for calcium ion-free MOPS buffer. (B) Photobleaching of mCerulean3 and cpVenus^{cd} in calcium-free MOPS buffer is significant and renders Twitch-2B inappropriate for a MEOF calcium ion monitor; the inset depicts the emission spectra normalized by mCerulean3 peak intensity.

responds in the physiological concentration range of Ca^{2+} ; normal non-protein-bound Ca^{2+} concentration in human blood plasma ranges from 1.1 to 1.3 mM with pathological values ranging from 0.33 mM (hypocalcemia) to 2.5 mM (hypercalcemia).^{11,22,23} The probe response curve was fit to a Hill equation, which determined the K_D and Hill constant to be 2.3 mM and 0.78, respectively [R^2 (coefficient of determination) = 0.99, Sy.x (standard error of the estimate) = 0.07] consistent with binding affinity in a useful range. These results demonstrate that the intracellular FRET sensor Twitch-2B can be adapted to function in a hand-held optical device.

However, the Twitch-2B acceptor fluorophore, cpVenus^{cd} (a YFP derivative), is susceptible to photobleaching, particularly as compared to the donor fluorophore mCerulean3 (a CFP derivative).²⁴ The photostability of the Twitch-2B MEOF device was therefore investigated by repeated exposure to 405 nm light while in calcium-free test buffer. The FRET ratio was measured after each exposure and showed significant photobleaching over time (Figure 6B), following an exponential decay in FRET ratio ($R^2 = 0.99$, Sy.x = 0.04), as expected for exponential photobleaching.^{25,26} Further, it has been shown that the CFP–YFP FRET pair may suffer from phototoxicity when excited with violet wavelengths, resulting in inactivation of the FRET pair.²⁴ To account for possible effects specific to the MEOF, photobleaching experiments were conducted using solutions of Twitch-2B. Twitch-2B solutions were exposed to 45 mW, 405 nm laser light for 3 min; a nonilluminated solution served as a negative control. Solutions were analyzed with the spectroscopic fluorescence plate reader under three conditions: donor excitation in test buffer, acceptor excitation in test buffer, and donor excitation in test buffer supplemented with CaCl_2 . Spectroscopic data (Figure S5) confirm photobleaching and consequential loss of sensitivity to Ca^{2+} .

Development of a Photostable Twitch-2B FRET Construct. To overcome cpVenus^{cd} photobleaching during MEOF measurements, photostable fluorescent molecules were sought to replace the mCerulean3-cpVenus^{cd} FRET pair. Small-molecule Alexa Fluor dyes are highly suitable candidates for the MEOF FRET sensor as they provide greater photostability and brightness compared to genetically encoded fluorescent proteins and other fluorescent dyes like Cy3 and fluorescein.^{27–29} To conjugate Alexa Fluor dyes, genetically encoded SNAP and CLIP tags were integrated into Twitch-2B. SNAP and CLIP tags, both derived from the human O⁶-alkylguanine transferase, catalyze the covalent attachment of an O⁶-benzylguanine (O⁶-BG) and O⁶-benzylcytosine (O⁶-BC), respectively.^{30–34}

The Twitch-2B sensor was re-engineered with SNAP and CLIP tags flanking its N- and C-termini, resulting in SNAP–Twitch-2B–CLIP, termed STC. The plasmid encoding STC and plasmid map are shown in Figures S6 and S7. The new construct was expressed at high yields and purified using affinity and size exclusion chromatography (Figure S8A). Commercially available O⁶-BG-Alexa Fluor 488 and O⁶-BC-Alexa Fluor 546-BC (New England Biolabs) were covalently attached onto the SNAP and CLIP tags of STC (Figure S8, parts B and C), and the FRET construct retained its dose-dependent response to Ca²⁺ in 96-well fluorescence plate assays (Figure 7). The STC construct was, henceforth, used in further investigations of the MEOF probes.

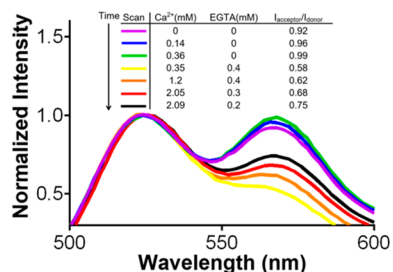


Figure 7. STC Ca²⁺ response in a fluorescence plate reader normalized by Alexa Fluor 488 peak intensity. The FRET ratio response is sensitive to increasing concentrations of Ca²⁺.

Characterization of MEOF Probes with the STC Construct. Here, probe photostability was assessed using a 450 nm laser light to excite the donor fluorophore Alexa Fluor 488. Each MEOF was incubated in reference buffer [RB, 50 mM HEPES, 50 mM NaCl, 10% glycerol, 10 mM β-mercaptoethanol (βME), pH 7.6] and exposed to a 0.71 mW laser for 2 s per reading. Laser light energy per measurement is 20 × 10³ times greater for STC as compared to Twitch-2B. No significant FRET ratio change was observed during the experiment (Figure 8A, relative standard deviation, RSD = 0.57%). Thus, integration of the new donor and acceptor fluorophores provides sufficient photostability for continuous Ca²⁺ monitoring.

The sensor rise time was assessed by first incubating MEOF probes in RB, and then in RB supplemented with 1.25 mM CaCl₂. The FRET ratio was recorded every 3 min for 30 min after media exchange. The data follow an exponential plateau function ($R^2 = 0.98$, $Sy.x = 0.004$) with a rise time of approximately 6 min, without detectable photobleaching within the plateau (RSD = 0.47%) (Figure 8B). The 6 min rise time is similar to that of commercial analytical devices, for

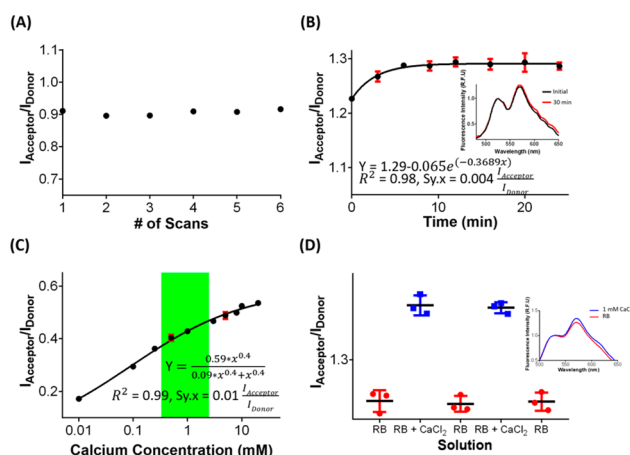


Figure 8. Ca²⁺ sensing with the MEOF probe. (A) The STC exhibits excellent stability in reference buffer. (B) Steady-state measurements are observed 6 min after the addition of calcium ions; the inset depicts the emission spectra normalized by donor peak intensity. (C) The STC exhibits a dose-dependent response, and the data fits to a Hill equation. $I_{\text{acceptor}}/I_{\text{donor}}$ is emission ratio after subtracting spectra in calcium ion-free RB. (D) The MEOF device has excellent reproducibility after serial exposures to either buffer supplemented with Ca²⁺ (1 mM) or RB.

example, continuous glucose monitors, known to be effective in guiding treatment of conditions having rapid and slow transients such as seen in type 1 diabetes.³⁵

Next, MEOF probes were incubated in RB solutions having Ca²⁺ concentrations ranging from 0 to 20 mM. The data shows sensitivity to Ca²⁺ across the measured range (0.01–10 mM) and a functional form consistent with the Hill equation (Figure 8C; $R^2 = 0.99$, $Sy.x = 0.01$) having a Hill constant and K_D values of 0.4 and 0.1 mM, respectively. As indicated by the green region in Figure 8C, the MEOF is sensitive across the physiological range of ionized Ca²⁺ concentration in the blood and extends the dynamic range of the Ca²⁺ sensing modalities previously mentioned.^{11–13} This extended sensitivity range correlates with the Hill constant being less than one, affording a stretched “linear region” of sensitivity.³⁶

To test for sensor reversibility, MEOF probes were serially placed in RB solutions with or without 1 mM CaCl₂ (Figure 8D). A one-way analysis of variance (ANOVA) was conducted to compare effects of Ca²⁺ on FRET ratio. There was a significant effect of Ca²⁺ across all groups ($P < 0.0001$). The Tukey posthoc comparison with adjusted P values shows no significant differences between the three RB solution groups or the two RB and 1 mM CaCl₂ groups ($p \geq 0.99$ for each comparison) but significant differences between each pairing of the RB or RB and 1 mM CaCl₂ groups ($p < 0.0001$ for each comparison). Notably, sensor dynamics are considerably slower when comparing Ca²⁺ unbinding (Figure S9) to binding (Figure 8B). Although the cytoplasmic Twitch-2B has previously demonstrated rapid unbinding kinetics in cellulo, cells contain orders of magnitude lower calcium concentration as compared to the blood and contain unique pumps and transporters that rapidly reduce cytoplasmic Ca²⁺ concentration.³⁷ And so, it is unsurprising that MEOF fall times are relatively long given the passive diffusion of Ca²⁺ ions at concentrations mimicking the blood.

Protection from Proteolytic Enzymes. To model immune system proteolytic enzymes secretion during the FBR, the STC MEOF was incubated with the endopeptidase

serine protease Proteinase K (28.9 kDa, New England Biolabs).³⁸ The solution comprises 692 μ M proteinase K in a solution of 20 mM Tris-HCl, 1 mM CaCl₂, and 50% glycerol by volume. Emission spectra confirm that proteinase K in the Ca²⁺ solution rapidly cleaves the STC and results in a 26% loss in acceptor fluorescence (Figure 9A). Conversely,

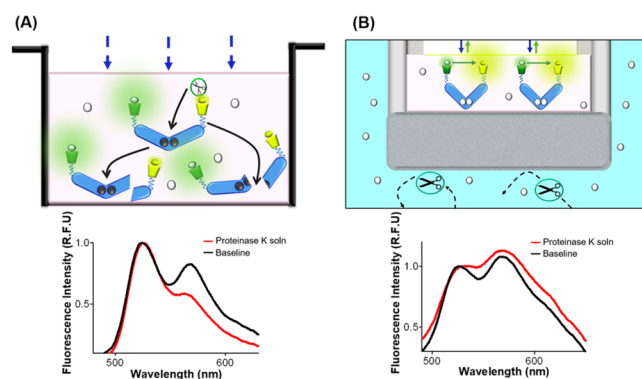


Figure 9. Sensor isolation from proteinase K. (A) Proteinase K cleaves STC resulting in a diminished acceptor intensity signal. (B) However, the MEOF successfully protects the protein sensor as demonstrated by the observed increase in FRET efficiency.

uncleaved STC should show an increase in acceptor fluorescence over time due to FRET. This increase is observed for the MEOF incubated in the Proteinase K solution for 30 min, demonstrating that the filter membrane successfully excludes the Proteinase K (Figure 9B). These findings highlight the need for and the key role of the size-excluding filter membrane to circumvent the FBR.

CONCLUSION

This effort has produced an optical fiber-based probe, MEOF, designed for prolonged measurements of Ca²⁺ concentrations in vivo. Such capabilities result from (1) encapsulating protein FRET sensors within the pores of a mechanically robust PTFE sheet, such that FRET sensors are free to undergo conformation changes, (2) excluding host proteases by a hybrid PTFE-PEGDMA membrane, and (3) elimination of deleterious photobleaching of FPs in the Twitch-2B FRET sensor by installing small molecules for the donor and acceptor FPs, respectively. This approach is generalizable to the larger class of protein FRET sensors (e.g. ATP and Zn²⁺).^{39,40} Further, the molecular weight cutoff properties of the hybrid filter membrane can be tuned for other applications or FRET sensors by new hydrogel formulation comprising PEG derivatives, or other commonly used hydrogels such as alginate and hyaluronic acid. In summary, the MEOF probe can be easily adopted to include new FRET sensors to generate a new class of analytical probes.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.analchem.0c00573>.

Nucleotide sequence and plasmid map of STC, hydrogel swelling dynamics, construction of the spectroscopic benchtop system, GFP encapsulation by the MEOF probe, selective permeability of the filter membrane, Twitch-2B photobleaching, protein and fluorescent gels

of STC after expression, illustration of Alexa Fluor dye conjugation onto STC, and STC unbinding of Ca²⁺ (PDF)

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Notes

The authors declare no competing financial interest.

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