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A ratiometric two-photon probe for Ca^{2+} in live tissues and its application to spinal cord injury model

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ABSTRACT

Ratiometric imaging with a small-molecule probe is important for the in-situ quantitative analysis of chemical events. We developed a ratiometric two-photon fluorescent probe (**SCa1-I_{REF}**) derived from dual dyes with different Stokes shifts. This probe has two identical windows: a Ca^{2+}-sensing window and an internal reference window, with eliminated FRET interference. **SCa1-I_{REF}** shows a marked change in the ratio upon response with Ca^{2+}, significant two-photon brightness, considerable selectivity for Ca^{2+}, and cell loading ability with low cytotoxicity. The ratiometric two-photon microscopy images revealed that this probe could directly and quantitatively estimate Ca^{2+} in live neurons and various tissues including rat spinal cord tissue. The studies of spinal cord injury model revealed that the Ca^{2+} level was significantly affected by elapsed time after injury. These results will provide useful applications for in-situ $[Ca^{2+}]_i$ imaging and for the development of effective ratiometric probes.

1. Introduction

Precise imaging of chemical events in live specimens is crucial for understanding physiology and pathology [1,2]. A ratiometric probe based on small molecules is a powerful tool to detect analytes in situ quantitatively [3–9]. Two main classes of small-molecule ratiometric probes have been constructed: intramolecular charge transfer (ICT)-based and Förster resonance energy transfer (FRET)-based probes. These systems utilize the ratios of two emission intensities between the free and binding forms of the probe. The ratios can be calibrated for the analyte with minimum interference from experimental artifacts like probe distribution and instrumental variability. However, most of these probes are based on irreversible reactions and are limited to visualizing dynamic concentration changes of the analyte [4–9]. The ICT-based probe often shows excitation ratiometry and not emission ratiometry, which requires two excitation wavelengths that may hamper real-time monitoring [10]. Hence, a straightforward method for designing a ratiometric probe remains a key challenge.

Recently, an internal reference (I_{REF})-based probe was reported for ratio analysis [11,12]. In principle, a turn-on probe can be readily transformed into a ratiometric probe by linking a differently colored emissive dye as an I_{REF}. This system requires that the turn-on signal acts as a sensing window while the I_{REF} signal remains constant with minimum interaction with the other. However, if the FRET efficiency from the I_{REF} to the probe is too high (Scheme 1a), the I_{REF} signal would be negligible, thereby making ratio analysis difficult [12]. To address this issue, we decided to design an I_{REF}-based ratiometric probe using two dyes with different Stokes shift (Scheme 1b). We anticipated that FRET interference could be ruled out if the absorption spectra of the sensing dye and I_{REF} were in a similar region, but their Stokes shifts were different, thereby possessing native fluorescent properties of the two parts and enhancing ratio sensitivity.

Intracellular Ca^{2+} ($[Ca^{2+}]_i$) is a critical signal messenger controlling various cellular functions. Disruption of $[Ca^{2+}]_i$ is directly linked to human disorders such as neurodegeneration, heart disease, and skeletal muscle defects [13,14]. For the ratiometric $[Ca^{2+}]_i$ analysis, ICT-based probes like Fura-2 are widely used [9]. However, these probes are based on excitation ratiometry. Further, most existing small molecule probes for Ca^{2+} also limit their applications in live sample imaging owing to the turn-on sensing process with single detection window, fast photobleaching, and short excitation light, which can cause photodamage, and have limited tissue

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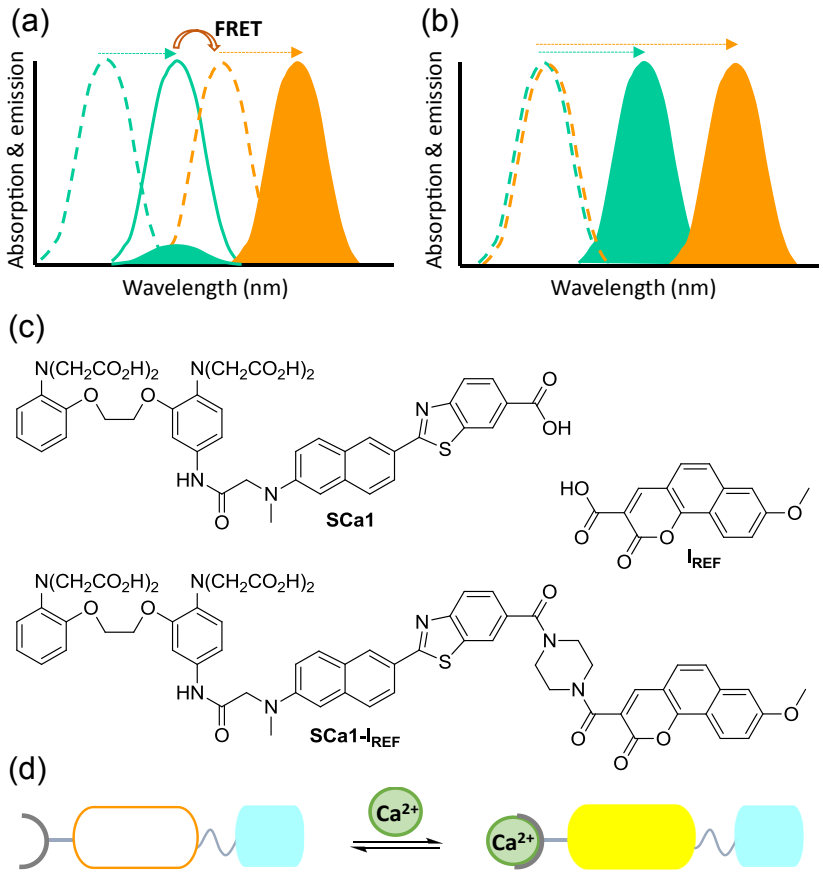

Scheme 1. (a,b) Conceptual absorption (dotted line) and emission spectra (solid line) of a conventional FRET system (a) and a system with two dyes with different Stokes shifts (b). (c) Structure of **SCa1**, $\mathbf{I_{REF}}$, and **SCa1-$\mathbf{I_{REF}}$**. (d) Sensing motif of **SCa1-$\mathbf{I_{REF}}$**.

imaging depth (Scheme S1 and Table S1).

An alternative approach is the use of an emission ratiometric probe with two-photon microscopy (TPM), which employs two near-infrared photons as the excitation source [15]. The TPM has become one of the most powerful technique for imaging studies in living systems, owing to its advantages including greater tissue penetration depth, localization of excitation, low photo-damage, and longer observation times. Recently, small-molecule two-photon probe for Ca^{2+} was reported but these probes are based on turn-on process that limit to quantitative detection (Table S1). Also, two-photon probes derived from FRET and TBET based dual-dye system for ratiometric imaging H_2S and Cu^{2+} were reported [16,17]. The sensing mechanism of these probes are chemoselective reaction with analyte that showed high selectivity and sensitivity. However, the reaction is irreversible, so they limit to visualize the dynamic concentration changes of the analyte. Therefore, there is a critical need for the ratiometric two-photon probes for precisely assessing the dynamics of target analyte in living systems.

Herein, we report a ratiometric two-photon probe (**SCa1-$\mathbf{I_{REF}}$**) for $[Ca^{2+}]_i$ derived from dual dyes that have different Stokes shifts and significant two-photon brightness. Using ratiometric two-photon microscopy (TPM) imaging, we investigated the quantitative analysis of $[Ca^{2+}]_i$ in live neurons and various tissues.

2. Experimental section

2.1. Materials and methods

All reagents were purchased from supplying companies and used without additional purification unless otherwise described. Solvents used in synthesis were previously purified for synthesis by standard methods. Normal phase column chromatography was used for the purification of compounds using MERCK silica gel 60. The progress of reaction was monitored using 60 silica gel by thin layer chromatography (TLC). A full synthesis description of probe and intermediates is presented in the Supplementary Methods section. The synthesis results were characterized by ^{1}H NMR, ^{13}C NMR and ESI mass spectrometry. ^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker 400 MHz spectrometer. Absorption spectra and fluorescence spectra were obtained by using S-3100 UV–Vis spectrophotometer and FluoroMate FS-2 fluorescence spectrophotometer with a 1 cm standard quartz cell, respectively. The fluorescence quantum yield was determined by using coumarin 307 ($\Phi = 0.95$ in MeOH) as the reference by the literature method [18].

2.2. Measurement of two-photon cross section

The two-photon cross section (δ) was determined by using femtosecond (fs) fluorescence measurement technique as described [19]. $\mathbf{I_{REF}}$, **SCa1**, and **SCa1-$\mathbf{I_{REF}}$** (2.0×10^{-6} M) was dissolved in MOPS buffer (30 mM MOPS, 100 mM KCl, pH 7.2) and the two-photon induced fluorescence intensity was measured at 720–900 nm by using rhodamine 6G as the reference, whose two-photon property has been well characterized in the literature [20]. The intensities of the two-photon induced fluorescence spectra of the reference and sample emitted at the same excitation wavelength were determined. The TPA cross section was calculated by