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Design of a SNARF-based Ratiometric Fluorescent Probe for Esterase

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A novel ratiometric fluorescent probe for esterase has been developed based on the SNARF scaffold. The new SNARF derivative was used to monitor the activity of porcine liver esterase by ratiometric emission spectrum change.

Fluorescent probes are molecules whose fluorescence characteristics, such as fluorescence intensity, wavelength, and life time, change as a result of specific reactions with target molecules. Many procedures for analyzing important biological phenomena have been developed based on the use of fluorescent probes as innovative analytical tools.[1] Among such approaches, the use of ratiometric measurements, monitoring emission or excitation wavelength change, has several advantages, including enhanced dynamic range, precise correction without interference by environmental effects, and convenient visual monitoring.[2,3] However, there are only limited reports on rational approaches to the design of functional ratiometric fluorescent probes,[3–6] and an increase in the available varieties of these probes is urgently needed. Here we propose a design strategy for ratiometric fluorescent probes based on fluorescence wavelength changes resulting from modification of the phenolic moiety present in seminaphthorhodafluor (SNARF). SNARF is well known as a ratiometric fluorescent pH indicator because it has dual-emission properties that are dependent on the concentration of H^+.[7] The phenolic substituent of SNARF is critical for such properties in that an equilibrium exists between the phenol with a λ_{em} of 583 nm (λ_{max} of 544 nm) (written as acidic form) and the phenolate anion with a λ_{em} of 627 nm (λ_{max} of 573 nm) (written as basic form). It follows from this that SNARF derivatives in which the phenolic substituent is protected should have spectral characteristics of the acidic form that are independent of pH. Following removal of the protecting group, either chemically or biologically, the resulting SNARF will exist in equilibrium as the free phenol or phenolate anion, the concentration ratio of which will be dependent on the environmental pH.

As a proof-of-principle study, we used SNARF-OAM[8] as a ratiometric fluorescent probe for esterase (Figure 1). The phenolic moiety of this derivative is masked by an acetoxymethyl group, a functionality that is readily cleaved by esterases.[7a] We first compared the absorption and the fluorescence spectrum of SNARF-OAM with that of the reference compound SNARF at pH 9.0 Figure 2a). SNARF had maximal absorption and fluorescence at 573 and 627 nm ($\lambda_{ex} = 488$ nm), respectively. In contrast, SNARF-OAM had maximal absorption and fluorescence at 544 and 583 nm ($\lambda_{ex} = 488$ nm), respectively. This indicated that under these conditions, SNARF-OAM exists as the acidic form, the spectral characteristics of which are significantly different from SNARF. In order to clarify the applicable scope of this sensing mechanism, the relationships between the pH of the environment and the spectral ratios ($R = I_{583\,nm}/I_{627\,nm}$) of SNARF-OAM and SNARF were determined (Figure 2b), R values of SNARF-OAM were constant and stable (1.41) from pH 5.0 to pH 9.0.

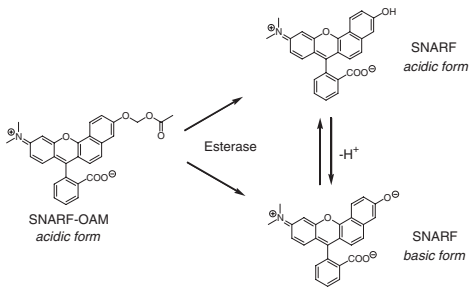

Figure 1. Reaction scheme of SNARF-OAM with esterase to yield SNARF as the hydrolyzed products.

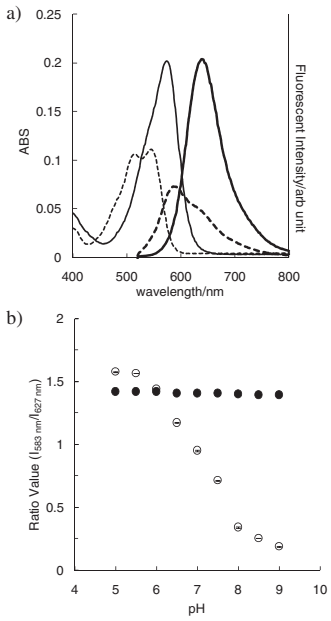

Figure 2. a) Absorbance (fine line) and fluorescence (bold line) spectra ($\lambda_{ex} = 488$ nm) of SNARF (10 μM: solid line) and SNARF-OAM (10 μM: dashed line) at pH 9.0 (10 mM Tris, HEPES and acetate). b) The ratio of fluorescence intensities at 583 and 627 nm plotted versus pH (10 mM Tris, HEPES and acetate buffer from pH 5.0 to 9.0). SNARF: open circle, SNARF-OAM: filled circle. These spectra were measured by Infinite M200 from Tecan.

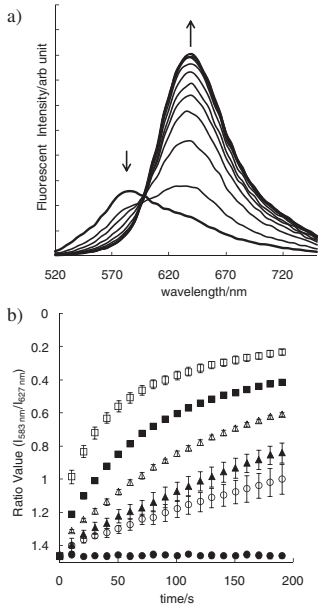

Figure 3. a) Fluorescence spectral change of SNARF-OAM (10 μM) after addition of porcine liver esterase (1 units mL^{-1}) to the buffer (10 mM Tris, HEPES and acetate at pH 9.0) ($\lambda_{ex} =$ 488 nm). b) Fluorescence ratio ($I_{583\,nm}/I_{627\,nm}$) increase of SNARF-OAM (10 μM) after addition of different concentration of porcine liver esterase (PLE) (0, 0.125, 0.25, 0.5, 1.0, and 2.0 units mL^{-1} of PLE from the lowest to the top trace). The error bar in the graph represents the standard deviation of three independent measurements.

On the other hand, R values of SNARF drastically changed from 1.58 (pH 5.0) to 0.19 (pH 9.0), a shift that can be ascribed to the ionization of the SNARF ($pK_a = 7.62$).[9] The changing ratio due to the conversion of SNARF-OAM to SNARF thus would be readily observable above neutral pH values (at least above pH 6.5). On the basis of these results, the schematic illustration of the ratiometric detectable methodology of esterase activity was proposed as shown in Figure 1.

We then examined in detail the suitability of SNARF-OAM as a ratiometric fluorescent probe for esterase. We first confirmed that SNARF-OAM was stable at pH 9.0 in the absence of enzyme.[10] When porcine liver esterase (PLE) is added to a 10 μM solution of SNARF-OAM, the emission spectrum ($\lambda_{ex} = 488$ nm) shows a decrease at 583 nm (acidic form) and an increase at 627 nm (basic form), respectively (Figure 3a). The enzymatic conversion of SNARF-OAM to SNARF was confirmed by HPLC (data not shown). As shown in Figure 3b, this reaction was accelerated by an increase in the PLE concentration, thus demonstrating that PLE activity could be determined by monitoring the increase of fluorescence ratio of the probe. To examine the properties of SNARF-OAM more quantitatively, we estimated the parameters K_m and k_{cat} of PLE for SNARF-OAM by fitting the plot of the initial velocity of the enzyme reaction versus substrate concentration with the Lineweaver–Burk plot.[11] The determined values of K_m, k_{cat}, and k_{cat}/K_m were 4.65×10^{-6} M, 1.08×10^{-2} min^{-1}, and 2.32×10^3 M^{-1} min^{-1}, respectively.[12]

In conclusion, we have successfully demonstrated the utility of SNARF-OAM as a ratiometric fluorescent probe for esterase. Our design strategy is based on locking the equilibrium chromophore of SNARF in the acidic form by introducing a protecting group on the phenolic substituent. This group is then removed under the conditions of the chemical or biological process that is being investigated. It follows that our strategy should be widely applicable to the design of ratiometric fluorescent probes for various processes simply by choosing an appropriate protecting group, for example, a substrate for a targeted enzyme. It should be noted that the SNARF scaffold can also be modified to alter the effective pH range and wavelength because SNARF derivatives which have a range of fluorescent properties have been synthesized.[13] In addition, our new ratiometric fluorescent probes should be particularly useful for monitoring enzymatic activities in living cells, because ratiometric detection is a preferred method for quantitative bioimaging in living cells. Based on our strategy, we plan to design and synthesize additional ratiometric fluorescent probes that can be used to monitor the activities of important druggable enzymes in living cells.

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