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A Ratiometric pH Reporter For Imaging Protein-dye Conjugates In Living Cells

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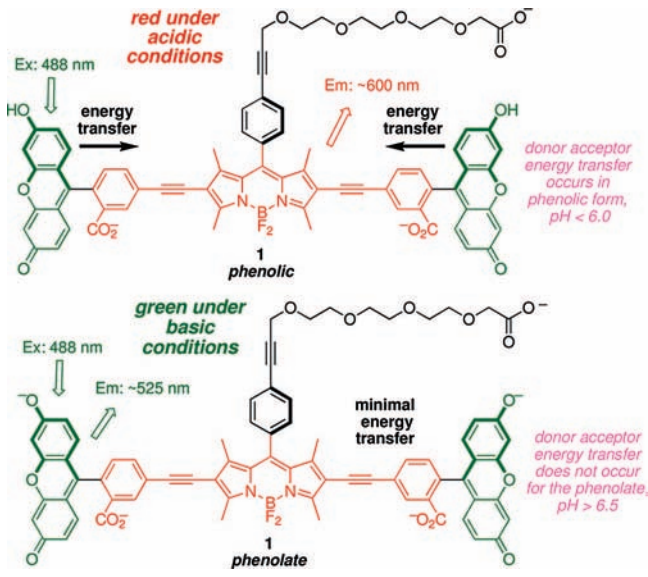
Of the numerous fluorescent small molecule pH probes that have been reported, only a small number are practical for intracellular imaging.[1−7] This is unfortunate because pH changes within cells are indicative of many cellular processes.[8−12] The most useful probes are the ratiometric ones that absorb UV excitation at a fixed wavelength and emit at two different fluorescence wavelengths in a pH dependent manner. These probes do not give dark regions in the cell at extreme pH environments, and simply areas where the dye did not permeate are clear. A widely used, commercially available pH-sensitive probe is C.SNARF-1 (Invitrogen Inc.). This communication focuses on the pH probe **1** based on a through-bond energy transfer cassette.[13−15] Data are presented to demonstrate that probe **1** tends to fluoresce with higher quantum yields than C.SNARF-1 over a physiologically relevant pH range. The ideal pH ranges of operation for **1** and for C.SNARF-1 are complementary (4.0−6.5 and 7.0−8.0, respectively).



Xanthene (the fluorescent core of fluorescein) is highly emissive at pH values > 7 (ϕ = 0.9); under those conditions it exists predominantly in the phenolate form. As the pH is lowered to ~6, it transforms into the phenolic state that is somewhat less emissive (ϕ = 0.4).[16−18] The pH probe **1**, which has two xanthene donors and one BODIPY acceptor, was designed to harness changes in the oxidation potentials associated with these protonation states. We hypothesized that the efficiency of the energy transfer from the xanthene donors to the BODIPY core would be governed by oxidation potentials which in turn depend on the protonation state of the xanthenes, making the whole cassette sensitive to pH changes in the range of cellular physiological processes. Perfect energy transfer, when the probe is excited near the fluorescein absorption maxima (*ca.* 495 nm), would give red fluorescence (600 nm). Conversely, if the energy transfer was completely eliminated at certain pH values, then the cassette would fluoresce from the xanthene core (*i.e.* ~520 nm). In either case, *the cassette would remain fluorescent as the cellular pH changes*.

Cassette **1** was prepared via Sonogashira coupling of two 5′-alkynyl fluorescein diacetate molecules[13] with an appropriate diiodo-BODIPY, followed by deprotection (see Supporting Information, SI). It is slightly water soluble at neutral pH and more so at pH 8.

Absorbance spectra for the conjugate of this probe with bovine serum albumin, *i.e.* **BSA-1**, were measured as a function of pH in aqueous media. The absorption maxima for the acceptor BODIPY part are ~576 nm and are impervious to pH changes between 4.1 and 7.9. However, the extinction coefficient for the fluorescein part at ~495 nm diminishes markedly from pH 7.9 to 4.1 (SI, Figure S2).

Fluorescence spectra for **BSA-1** as a function of pH are shown in SI, Figure S3. Under neutral and basic conditions, pH 7.0 and 7.9, the probe emits almost exclusively at ~520 nm, *i.e.* green fluorescence. Conversely, at the acidic extreme, pH 4.1 and 5.0, the cassette fluoresces almost completely red, *i.e.* from the BODIPY acceptor. The inset of Figure S3 shows that the ratio of red-to-green fluorescence for **BSA-1** is highly sensitive to pH in the 4.0−6.5 range. A crossover occurs around pH 6.0 where significant red and green fluorescence are observed. If the measurement at pH 7.9 is excluded from consideration in Figure S3 (this is justifiable because we do not claim that the probe is sensitive above pH 7), then an isobestic point is apparent. Quantum yield measurements for **BSA-1** at the pH extremes of 4.1 and 8.8 were 0.18 and 0.14. By comparison, literature quantum yields for C.SNARF-1 are 0.03 (pH 5−6) and 0.09 (pH 10−12).[19] The fluorescence response of **1** as the pH is cycled between acidic (3.4) and basic media (8.0) demonstrated that the probe is stable to this treatment (SI, Figure S4).

Endosomes within cells (pH 5.0−5.5) are markedly more acidic than the cytosol.[20] We have observed that when the noncovalently bound carrier peptide "Pep-1"[21] imports dye labeled proteins into COS-7 cells, the protein−dye conjugates tend to be encapsulated in endosomes.[22] Consequently, we anticipated that when **BSA-1** was imported into cells using Pep-1, it would localize in endosomes, and in that acidic environment the probe would emit at ~600 nm. When **BSA-1** was imported into COS-7 cells using Pep-1 (1 μM protein; 1:20 mol ratio protein/carrier, 37 °C, 1 h), irradiation at 488 nm resulted predominantly in red fluorescence localized in punctate vesicular structures (Figure 1a).

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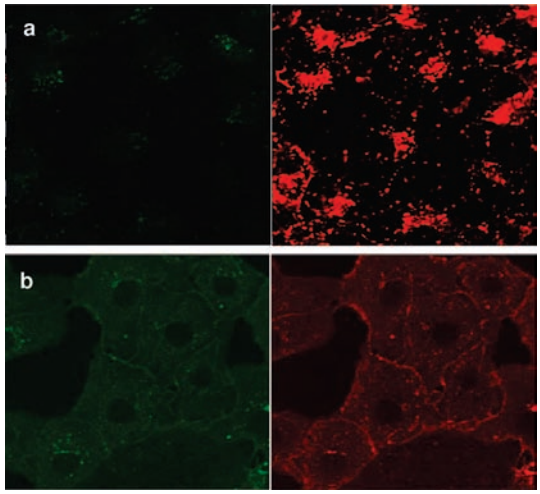

Figure 1. Pep-1 mediated cellular uptake of **BSA-1** (1 μM) into COS-7 cells after 1 h incubation at (a) 37 and (b) 4 °C. The cells were irradiated at 488 nm, and fluorescence from donor (503−553 nm) and acceptor (575−625 nm) was detected, respectively.

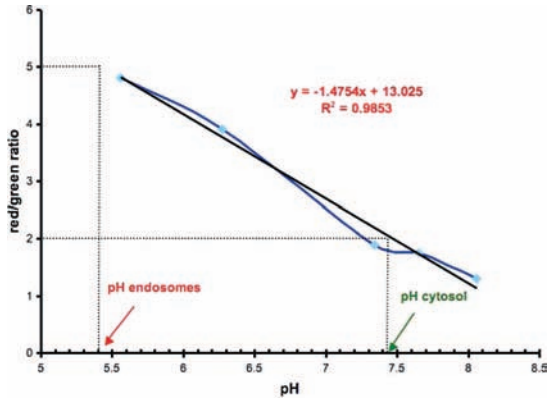

Figure 2. *Ex vivo* calibration curve with pH values corresponding to those observed within endosomes (red/green = 5.03; import at 37 °C) and the cytosol (red/green = 2.03; import at 4 °C).

We recently discovered that Pep-1 mediated import into COS-7 cells tends to deposit the dye-labeled protein cargoes *into the cytosol when the experiment is performed at 4 °C*.[22] Thus, **BSA-1** under these conditions would be expected to fluoresce with diminished red-to-green ratios. The fluorescence intensity for **BSA-1** is distributed within the cytosol; hence the images in Figure 1b appear to be deceptively weak relative to situations (*e.g.* Figure 1a) where the probe is concentrated in punctates. A better impression of the relative intensities in the red and green channels for both experiments (37 and 4 °C) after correction for autofluorescence and donor bleed through is shown in the SI (p. S8).

Quantitative data for pH measurements in cells were obtained via a calibration experiment. Details are provided in the SI, but the salient feature is that the ionophore nigericin was used to produce "leaky cells" that were then bathed in buffers. This is a standard approach that has been used for the same purpose to calibrate other pH measurements *ex vivo*.[7,23,24] A curve generated with the calibration experiment (Figure 2) was used to determine pH values for the endosomes and the cytosol for the experiments shown in Figure 1. The pH values, obtained from the red/green ratio (R/G = 5.03 and 2.03 at 37 and 4 °C, respectively), were 5.4 and 7.4 and in good agreement with those expected for such intracellular regions. When C.SNARF-1 AM ester (not protein conjugated) was imported into COS-7 cells (at 37 and 4 °C) as a lipophilic, hydrolyzable form, a pH of 7.1 was determined (see SI); this is very close to the value cited above for probe **1** (*i.e.* 7.4).

Our interpretation of the SNARF literature indicates that this probe is usually used as a nonconjugated form (*i.e.* not attached to proteins); presumably this is because it has a low quantum yield and cannot easily be visualized when present at low concentrations.[19] By contrast, the data presented in this study show that visualization of proteins conjugated to **1** is possible. This is highly significant because the new probe should facilitate observation of *processes* within the cell. Further, the energy transfer approach to pH probes for intracellular imaging has considerable potential for modifications with other pH sensitive donors to give a spectrum of probes with systematically varied pH response transitions. Design of probes of this type requires an understanding of the parameters underlying the fluorescence energy transfer processes. These parameters, although not simple, have been elucidated in a parallel study that focuses on the redox behavior of the cassette components; this is to be reported elsewhere in detail.

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Supporting Information Available: Experimental procedures for the preparation of **1** and **BSA-1** conjugate. This material is available free of charge via the Internet at http://pubs.acs.org.

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