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Design and Investigation of a Series of Rhodamine-Based Fluorescent Probes for Optical Measurements of pH

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Abstract

A series of structurally similar fluorescent probes (1-4), synthesized from rhodamine B, were designed to optically measure pH. Each probe had a unique “off-on” response as the solution went from basic to acidic. Probes 1-3 exhibited a spiro-cyclic quenching of the pyronin B fluorophore, whereas probe 4 is quenched by PET from the amine moiety.

A chemical sensor capable of measuring pH by optical methods has important implications in analytical and biological chemistry. Fluorescence based probes have demonstrated a superior ability in the detection of various analytes with high sensitivity and low detection limits. In cellular biology, the pH of specific and localized areas of the cell can be an important indication of cellular events.¹ Such information could potentially be useful in the diagnosis of specific diseases. For example, colorectal cancer,² breast cancer^{2b,3}, cystic fibrosis,⁴ and neurodegenerative disorders⁵ have all been linked to abnormal pH regulation, resulting in localized pH values of 4.5-6.0 that are referred to as the acidic window. Currently, a large number of fluorescence based pH sensors have been reported in the literature and are commercially available.⁶ However, many of these probes lack sensitivity or are simply non-responsive in the so-called “acidic window”. Therefore, it would be of great benefit to aid in the diagnosis of these diseases by developing new and more sensitive probes for the measurement of pH.

Many fluorescent probes operate in an “off-on” fashion, where in the “off” state the fluorescence is quenched, while the “on” state the fluorophore is fully fluorescent with a high quantum yield. Among the many fluorophores that potentially could be used as pH sensors, pyronin B has particular advantages in biological imaging because of its high quantum yield ($\Phi = 0.35$), longer excitation and emission wavelength (550-600 nm), spectral insensitivity to solvent polarity, pH, and a high tolerance to photobleaching.⁷ With these criteria in mind, we set out to design a mode of regulating the fluorescence of this fluorophore by synthesizing various benzylamine derivatives at the 9-position, which have

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Supporting Information **Available:** Detailed synthetic procedures and spectra. This material is available free of charge via the Internet at <http://pubs.acs.org>.

different interactions with the fluorophore. Herein, we describe a new series of rhodamine-based analogs designed for the optical detection of pH.

Starting with commercially available rhodamine B, we synthesized various benzylamine structures; (see supporting information for synthesis details), where the amine moiety is primary, secondary, and tertiary (Figure 1). The rhodamine-based literature is rife with fluorescent probes that are regulated by a spirocyclic structure. These spirocyclic compounds lack absorbance and fluorescence, in visible spectrum, when engaged in the spirocyclic form but will open upon binding to specific analytes to restore the fluorophore and fluorescence intensity of that compound. Typically, these compounds are in the form of a rhodamine lactone or lactam type derivative,⁸ which have been mostly applied in the detection of heavy metal cations. However, Nagano et. al. recently published work on a probe for hypochlorous acid involving a spiro-thiophene derivative.^{7c} We envisioned that our series of structurally related analogs should interact with the pyronin B fluorophore in a similar fashion but differ in their response to pH based on the nucleophilicity and steric hinderance of the amine moiety. Specifically, probes **1-3** should take on a spiro-cyclic structure in the absence of protons at high pH, with **3** maintaining the most spiro-like character. Compound **4**, however, contains a tertiary amine, which will avoid intramolecular cyclization and should thus maintain its fluorescence. This is analogous to rhodamine derivatives bearing a tertiary amide or an ester functionality at the 2' position, e.g. rhodamine 6g.

Upon titrating the fluorescence response to pH for probes **1-4**, (see supporting information for fluorescence spectra) we obtained the following curves shown in Figure 2. The results for probes **1-3** were as expected, with each compound showing a completely non-fluorescent form under basic conditions, which gradually increases in fluorescence intensity by 4-fold when exposed to acidic conditions. Probe **1** is the most sensitive to proton concentration; likely due to better solvation and a higher pK_a than the other three. Probe **4**, however, had a very interesting response. The tertiary amine moiety is incapable of undergoing intramolecular cyclization; therefore, it was surprising that this compound had a response to pH. The relative fluorescence intensity remains above 0.1 at all times, and triples as the pH changes from ~7 to 4. Of the four analogs used to detect pH, probe **3** in Figure 2 has the most responsive curve in the “acidic window”, which is regarded to be of great interest in diagnosis and understanding for some of the aforementioned diseases.

Given that spiro-cyclic structures have been thoroughly investigated in other rhodamine systems, and their existence is proven by x-ray crystallography, ^{7c,8a,b} we thought an NMR study should be sufficient in distinguishing between a spiro-cyclic and an open structure for our series. Specifically, we examined the ¹³C and DEPT spectra for insight on our various derivatives.

As shown in Figure 3, probes **1-3** all have a quaternary carbon atom in the alkyl region of their ¹³C spectra. This signal corresponds to the carbon atom at position 9, indicating that probes **1-3** exist in a spiro-cyclic structure. Probe **4**, however, has no such quaternary carbon in the alkyl region; therefore carbon atom 9 must lie in the aromatic region. Further NMR experiments are needed to confirm the exact chemical shift of carbon atom 9 in the aromatic region. This is consistent with ¹³C spectra of commercial rhodamine ester derivatives.⁹

Further evidence obtained by UV-vis titrations verifies a spiro-cyclic structure for probes **1-3** and proposes a mode of quenching for probe **4**. Probes **1-3** undergo dramatic changes in the absorbance spectra (Figure 4) as the pH is adjusted from acidic to basic conditions, **1-3** loses almost all of their absorbance, while probe **4** remains nearly invariant to changes in pH. This type of behavior where the fluorescence is modulated upon binding to some

analyte, while the absorbance remains constant, is indicative of a photo-induced electron transfer mode of quenching.¹⁰ In this proposed PET mechanism, one of the lone pair electrons on the nitrogen moiety is transferred to the excited fluorophore, which allows for a nonradiative relaxation pathway. This mechanism is disrupted when the amine becomes protonated; thus the fluorescence is fully restored. The efficiency of this quenching process depends on the thermodynamics, allowed by Rehm-Weller formalism,¹¹ and could therefore be “tuned” for future analogs of **4** by the direct attachment of electron-donating or withdrawing substituents on the amine. Further characterization of the fluorescence properties are ongoing, e.g. fluorescence lifetime experiments.

In summary, a series of rhodamine-base analogs were synthesized from rhodamine B for the fluorescence based detection of pH. Probes **1-3** exhibited “off-on” quenching as solutions went from basic to acidic, which was characterized as a spirocyclic structure by UV-vis and NMR. Scheme 1 gives a visual summary of the quenching mechanism for probes **1-3**. Probe **3** has been proposed to be the most biologically relevant probe, because of its dynamic response in the “acidic window”. Probe **4** has interesting photophysical behavior and is related to a PET type mechanism; further spectroscopic interrogation is underway.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Structures of pyronin B, rhodamine B, and benzylamine-moiety-substituted pyronin B derivatives (**1-4**).

Figure 2.

Fluorescence response to pH for compounds **1-4** measured using 0.37 μ M solutions in 15% EtOH/H₂O (v/v). Excitation wavelength was 550-560 nm. Fluorescence intensities are relative to a 0.37 μ M solution of rhodamine B in H₂O.

Figure 3.

The alkyl region of ^{13}C NMR spectra for compounds **1-4**, with the quaternary carbon atom, C9, labeled in **1-3**. The chemical shift of C9 was determined by the corresponding DEPT spectra (see Supporting Information).

Figure 4.

Absorbance spectra for probes **3** (a) and **4** (b) at various values of pH. Measured using 0.37 μM solutions in 15% EtOH/H₂O (v/v).

Scheme 1.
The quenching mechanism for probes 1-3