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COMMUNICATION

A rational approach to tuning the pK_a values of rhodamines for living cell fluorescence imaging†

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A novel strategy to systematically tune the pK_a values of rhodamines is described. This strategy was applied to rationally develop compound 1e with a pK_a of 6.5, the highest among rhodamine amide derivatives, and it could be employed to detect acidic pH variations in living cells with a turn-on signal.

Fluoresceins have been extensively employed as fluorescent pH probes, as their fluorescence properties are sensitive to pH variations in the environment.[1] Under basic or neutral conditions, fluoresceins are fluorescent, as they exist largely in the ring-opened form (Fig. 1A). By contrast, under acidic conditions, fluoresceins are nonfluorescent, as the spirocyclic form dominates. Thus, when the pH values of the environment decrease (for instance, from neutral to acidic conditions), intensity-based fluorescein probes may display a fluorescence quenching signal. Since fluorescent quenching probes could provide false positive data by other fluorescent quenchers in real samples and fluorescence quenching response often leads to a low signal-to-noise ratio,[2] it is much more desirable to detect the diminution of pH values by an enhanced fluorescence signal.

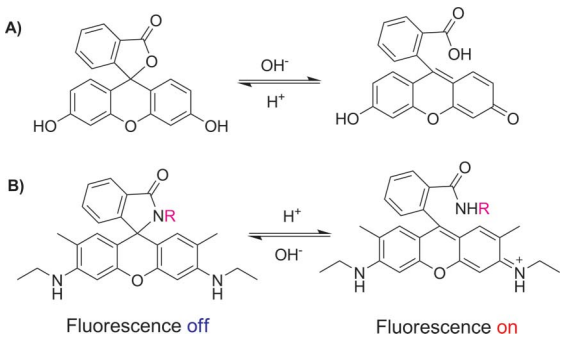

Fig. 1 (A) pH-dependent equilibrium between the spirolactone form and the ring-opened form of fluoresceins; (B) pH-dependent equilibrium between the spirolactam form and the ring-opened form of rhodamine amide derivatives.

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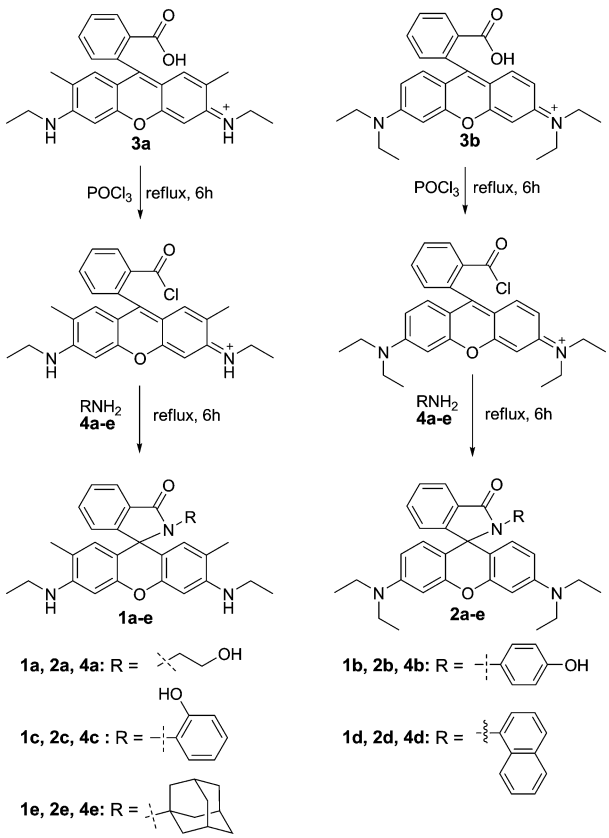
Since the seminal work by Czarnik's group about the first rhodamine-based fluorescent chemodosimeter for Cu^{2+},[3] rhodamines have been widely exploited as the scaffold of fluorescence turn-on probes for various metal ions (*e.g.* Cu^{2+}, Hg^{2+}, Fe^{3+}, Cr^{3+}, Pb^{2+}, Zn^{2+}, *etc.*) and biologically relevant species (*e.g.* NO, ClO^-, *etc.*).[4–5] When compared to fluoresceins, rhodamines appear to have better photostability. Another major distinction between rhodamines and fluoresceins is that they have the opposite trend of fluorescence response to pH variations. Unlike fluoresceins, at basic or neutral conditions, rhodamine amide derivatives are non-fluorescent, as they exist largely in the spirocyclic form (Fig. 1B), whereas rhodamine amide derivatives are fluorescent under acidic conditions, as the ring-opened form dominates. Thereby, when the pH values of the environment decrease, intensity-based rhodamine probes may exhibit a fluorescence enhanced signal. From the fluorescence response point of view, rhodamines are superior to fluoresceins in monitoring attenuation of pH values.

It is known that the pK_a values of fluoresceins can be tailored by introducing electron-withdrawing groups nearby the phenolic alcohol on the xanthene ring.[6] However, to our best knowledge, no strategy has been previously developed to tune the pK_a values of rhodamines. Although scarce examples of rhodamine-based fluorescent pH probes have been constructed by empirical experience,[7] the rational design of rhodamine-based fluorescent pH probes is still very challenging due to the lack of a suitable approach to systematically tune the pK_a values of rhodamines. Thus, it is of interest to develop new molecular strategies for modulating the pK_a values of rhodamines.

It is known that rhodamine 6G derivatives are much more sensitive than rhodamine B derivatives as fluorescent turn-on probes.[8] We reasoned that this behavior may be ascribed to the steric effect of the methyl group on the xanthene ring of rhodamine 6G derivatives. In other words, the steric methyl group may facilitate the conversion of the nonfluorescent spirocyclic form to the fluorescent ring-opened form. Since the pK_a of rhodamine defines the equilibrium between the spirocyclic form and the ring-opened form, we reasoned that the pK_a values of rhodamines could be systematically modulated by introducing a steric group on the nitrogen atom of the amide moiety.

In this work, to validate our working hypothesis, we constructed a series of new rhodamine 6G derivatives **1a–e** and rhodamine B derivatives **2a–e** containing different steric bulky substituents (Scheme 1) to examine their pK_a values. Indeed as anticipated, there is a good correlation between pK_a values and the steric bulky



Scheme 1 Synthesis of rhodamine 6G derivatives **1a–e** and rhodamine B derivatives **2a–e**.

substituents. Based on this key finding, we were able to rationally design novel rhodamine 6G derivative **1e** with a pK_a of 6.5, which makes it suitable for monitoring acidic pH variations in living cells with a fluorescence turn-on signal by fluorescence imaging.

The synthesis of rhodamine 6G derivatives **1a–e** and rhodamine B derivatives **2a–e** is outlined in Scheme 1. rhodamine 6G acid **3a** was treated with $POCl_3$ to afford the acid chloride intermediate, which was further reacted with various substituted amines **4a–e** to give the desired products **1a–e**. A similar synthetic strategy was used to prepare rhodamine B derivatives **2a–e** from rhodamine B **3b**. The structures of the products were confirmed by ^{1}H NMR, ^{13}C NMR, and MS spectroscopy.

With these compounds in hand, we set out to investigate their spectral properties at different pH values. As expected, the rhodamine B derivatives **2a–d** only showed slight fluorescence with attenuation of pH values from 7.4 to 2.0 (Figures S1–4A). Furthermore, when compared to rhodamine B **3b**, rhodamine B derivatives **2a–d** have a much weaker fluorescence at pH 2.0 indicating that the non-fluorescent spirocyclic form is still dominating even at a pH as low as 2.0 (Figures S1–4B). The same conclusion can be drawn from the absorption profiles (Figure S5). By sharp contrast, the rhodamine 6G derivatives **1a–d** displayed intense fluorescence with decrease of pH values from 7.4 to 2.0 (Figures S6–9A), and they have the emission close to rhodamine 6G acid **3a** at pH 2.0 (Figures S6–9B). These findings are further supported by the studies of absorption (Figure S10). Thus, the comparison of rhodamine 6G derivatives **1a–d** and rhodamine B derivatives **2a–d** collaborates the premise that

Table 1 The pK_a values of compounds **1a–e** and **2e**

	pK_a[a]		pK_a[a]
1a	2.8	**1d**	3.3
1b	3.2	**1e**	6.5
1c	3.6	**2e**	5.6

[a] The pK_a was calculated according to the Henderson–Hasselbach-type mass action equation.[7a,9]

the steric methyl group in rhodamine 6G promotes the formation of the fluorescent ring-opened form. The analysis of fluorescence intensity changes at 556 nm as a function of pH by using the Henderson–Hasselbach-type mass action equation[7a,9] gave pK_a values of rhodamine 6G derivatives **1a–d** as shown in Table 1. Indeed, there is a correlation between pK_a values and the steric bulky substituents on the nitrogen atom of the amide moiety, in good agreement with our working hypothesis.

Although compounds **1a–d** have marked fluorescence under acid conditions, the pK_a values of these compounds are too low (< 4.0) to be useful for biological applications. In order to further increase pK_a, based on our above finding, we decided to introduce a highly sterically bulky group, 1-adamantanamine, to the nitrogen atom of the amide moiety in rhodamines 6G and B scaffolds to give compound **1e** and **2e** (Scheme 1), respectively.

As shown in Fig. 2 and Table S1, compound **1e** is essentially nonfluorescent at pH 9.0. However, compound **1e** showed a

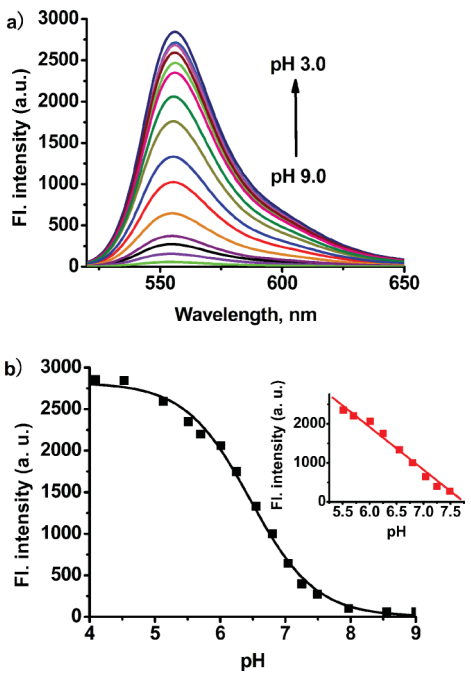


Fig. 2 (a) pH-dependence of the fluorescence intensity of probe **1e** (2 μM) with the arrow indicating the change of the fluorescence intensities with pH decrease from 9.0 to 3.0. Spectra were obtained with excitation at 500 nm in 25 mM PBS aqueous solution (containing 20% DMF as a co-solvent). (b) Fluorescence responses (fluorescence intensity at 556 nm) of probe **1e** (2 μM) to different pH values (from 9.0 to 3.0). The inset shows the linear relationship of fluorescence intensity at 556 nm and varying pH values from 5.5 to 7.5.

very large fluorescence enhanced signal (70-fold enhancement of fluorescence intensity at 556 nm) with attenuation of pH values from 9.0 to 3.0. The pH-dependent absorption spectra are in good agreement with the pH-dependent emission spectra (Fig. S11). The fluorescence titration data provides the pK_a of compound **1e** as 6.5, which is much higher than those of compounds **1a–d** (< 4.0) and other rhodamine derivatives reported (4.85).[7a] This finding confirms the anticipated effect of steric groups on the pK_a values. Similarly, compound **2e** also exhibited a large fluorescence enhanced response with a decrease of pH values from 8.5 to 3.5 (Fig. S12 and Table S2). The pK_a of compound **2e** was calculated to be 5.6, which is smaller than that of compound **1e**, reinforcing the steric effect of methyl group.

The potential interference of biologically relevant species on the fluorescence response of probes **1e** and **2e** was evaluated. As shown in Figures S14–15, high concentrations of K^+, Na^+, Ca^{2+}, Mg^{2+}, Cu^{2+}, Zn^{2+}, Fe^{3+}, Co^{2+}, and other biologically important species (glucose, Ser, Cys, Arg, Val, GSH, vitamin C, H_2O_2) caused no visible effect on the fluorescence signals. In addition, the probes **1e** and **2e** are highly stable after irradiation with green light (centered at 500 nm) for 30 min (Figure S16).

To examine the potential utility of the novel rhodamine-based probes for fluorescence imaging in living cells, probe **1e** (2 μM) was incubated with Hela cells for 30 min at 37 °C, and then the cells were washed in PBS medium of varying pH values with the addition of nigericin (1 μg mL^{-1}) to elicit a rapid exchange of K^+ for H^+ for a fast equilibration of external and internal pH.[10] As shown in Fig. 3b–e–h, the fluorescence in living cells became much brighter when the pH values were decreased from 7.4 to 5.6, indicating that probe **1e** is cell membrane permeable and could be used to monitor attenuation of pH values in living cells with a fluorescence turn-on signal. Furthermore, the MTT assays (Fig. S17 in the Supporting Information) [11] indicate that probe **1e** of concentrations below 100 μM does not display observable cytotoxicity. The results of the nuclear staining of Hela cells with Hoechst 33258 further support that probe **1e** has low cytotoxicity and the cells remained alive during the imaging process (Fig. S18). The nuclear staining also revealed that probe **1e** associates with the cytoplasm of Hela cells (Figure S18).

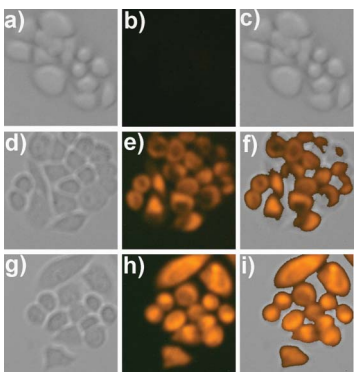


Fig. 3 Images of Hela cells treated with probe **1e** at different pH values. (a) Bright field image of Hela cells incubated with probe **1e** (2 μM) at pH 7.4; (b) Fluorescence image of (a); (c) The overlay image of (a) and (b); (d) Brightfield image of Hela cells incubated with probe **1e** (2 μM) at pH 6.5. (e) Fluorescence image of (d); (f) The overlay image of (d) and (e); (g) Brightfield image of Hela cells incubated with probe **1e** (2.0 μM) at pH 5.6; (h) Fluorescence image of (g); (i) The overlay image of (g) and (h). The fluorescence images were acquired with green light excitation.

In summary, we have described, for the first time, a general approach to systematically tune the pK_a values of rhodamines by incorporating a steric group on the nitrogen atom of the amide moiety. This novel approach was validated by examining the pK_a values of a series of rhodamine 6G derivatives **1a–e** and rhodamine B derivatives **2a–e**. On the basis of this approach, new compound **1e** with a pK_a of 6.5 was judiciously designed. Notably, the pK_a of compound **1e** is higher than that of other rhodamine amide derivatives known, and it is suitable for detecting acidic pH variations in living cells with a fluorescence turn-on signal. This demonstrates the significance of our approach. We anticipate that the simple and effective strategy introduced herein may be widely applicable for the rational design of xanthene-based fluorescent pH probes. Furthermore, the steric effect on the fluorescence turn-on of rhodamines should have profound implications for the judicious design of xanthene-based fluorescent probes for a wide variety of targets including metal ions and biologically relevant molecules. Although the steric group is incorporated on the nitrogen atom of the amide moiety in the approach described herein, we expect that the introduction of steric groups on the xanthene ring may have the similar effect on pH values and perhaps on fluorescence turn-on in general. Work along this line is in progress in our laboratory.

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