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New Near-infrared Rhodamine Dyes with Large Stokes Shifts for Sensitive Sensing of Intracellular pH Changes and Fluctuations

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Abstract

New near-infrared rhodamine dyes with large Stokes shifts were developed, and applied for sensitive detection of cellular pH changes and fluctuations by incorporating an additional amine group with fused rings to rhodamine dyes to enhance the electron donating ability of amine groups, and improve the spectroscopic properties of the dyes.

For TOC Only

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Conflicts of interest

There are not conflicts to declare.

Traditional rhodamine dyes such as rhodamines 6G, B and 101 have been widely used to develop fluorescent probes by introducing a variety of functional groups to form closed spirolactam on-off switches. This results in outstanding photophysical properties such as high molar extinction coefficients, excellent fluorescence quantum yields, great photostability and water solubility.^{1–7} The disadvantages of traditional rhodamine dyes include short fluorescence wavelengths with less than 600 nm absorptions and small Stokes shifts less than 35 nm, which may cause excitation wavelength interference, cellular damage and limit their biological applications.^{1–7} There have been recent reports on the tuning of traditional rhodamine dyes to the deep-red and near-infrared regions by extending π -conjugation of the xanthene ring, replacing the central oxygen atom by other elements such as N, C, S, Se, P, Si, Ge, Sn and Te in order to take advantage of NIR imaging features such as minimal tissue absorption, low background autofluorescence from biological samples, and deep tissue penetration.^{8–22} Si-rhodamines and Ge-rhodamines and P-rhodamines show far-red and NIR emission while retaining the main advantages of the traditional rhodamine dyes such as high fluorescence quantum yields, excellent photostability and sufficient water solubility for biological imaging applications.^{8–11, 13–16} Another very effective approach is to introduce the spirolactam fluorescence on-off switches to near-infrared hemicyanine dyes, which have shown some biological imaging applications.^{11, 23–25} It is still very important to develop near-infrared rhodamine dyes with large Stokes shifts, high

photostability and increased fluorescence to overcome excitation light interference for practical bioimaging applications.

In this communication, we tuned rhodamine emission to the near-infrared region by incorporating an additional amine group with fused rings to rhodamine dyes to enhance the electron donating ability of amine groups and improve the spectroscopic properties of the dyes. Rhodamine dyes **A** and **B** show near-infrared emission with large Stokes shifts, high molar absorptivity, excellent photostability, good hydrophilicity, and stable fluorescence under different pH conditions. Rhodamine dye **C** obtained by introducing o-phenylenediamine to rhodamine dye **A** shows fast and highly sensitive responses to cellular pH changes with significantly higher pK_a of 5.4 related to spirolactam ring opening^{27–31} because of larger steric hindrance between the o-phenylenediamine residue and xanethene moiety compared to traditional rhodamine dyes. Rhodamine dye **C** was successfully used to detect lysosomal pH changes, and pH fluctuations under oxidative stress and drug treatment.

Near-infrared rhodamine dyes **A** and **B** were prepared by a condensation reaction of 5-methyl-1,2,3,3a,4,5-hexahydropyrrolo[1,2-a]quinoxalin-8-ol (**4**) with either 2-(4-diethylamino-2-hydroxybenzoyl)benzoic acid (**5**) for **A**, or 2-[(2,3,6,7-tetrahydro-10-hydroxy-1H,5H-benzo[*ij*]quinolizin-9-yl)carbonyl]-benzoic acid (**6**) for **B** in trifluoroacetic acid under reflux (Scheme 2). Compound **4** was prepared according to a reported procedure.²⁶ Rhodamine dye **C** was prepared by introducing o-phenylenediamine to rhodamine dye **A** to form a non-fluorescent closed spirolactam ring structure through the condensation reaction of one amine group from o-phenylenediamine and the carboxylic acid on rhodamine dye **A** (Scheme 2).

Rhodamine dye **A** spectra contain absorption and emission peaks at 575 and 648 nm respectively corresponding to a large Stokes shift of 73 nm (Figure 1). The absorption has a high molar absorptivity of $3.1 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$, and a high fluorescence quantum yield of 22.6% in a pH 7.4 buffer containing 10% ethanol was measured. Rhodamine dye **B** spectra exhibit an absorption peak at 586 nm and an emission peak 644 nm with a slightly smaller Stokes shift of 58 nm compared to rhodamine **A** (Figure 2). Dye **B** displays a high molar absorptivity of $3.4 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$, and, a slightly higher fluorescence quantum yield to dye **A** of 25.1% under identical conditions. The fluorescence intensities of rhodamine dyes **A** and **B** increase with increases in the ratio of ethanol to water in solutions of ethanol and water (Figure S7-S8). Increases in the ethanol percentage reduce dye aggregation and fluorescence quenching and result in enhancement of dye fluorescence intensity. The fluorescence intensity and absorbance of rhodamine dyes **A** and **B** do not change much in different pH buffer solutions (Figure 1).

Rhodamine dye **C** displays no absorption peak from 500 nm to 800 nm under neutral and basic pH conditions as the dye maintains a closed spirolactam ring structure. However, a stepwise decrease in pH value from 7.6 to 2.0 leads to a new absorption peak at 587 nm with corresponding increases in the intensity of this peak because of pH-activated spirolactam ring opening (Scheme 1).^{27–31} Rhodamine dye **C** is non-fluorescent with the closed spirolactam ring form under basic and neutral conditions. However, a new fluorescence peak at 655 nm, corresponding to the aforementioned peak at 587 nm, which also increases with

decreases in pH from 7.6 to 2.0 presumably due to significantly enhanced π -conjugation as the spirolactam ring opens under acidic conditions,^{27–31} is obtained (Figure 2). A large Stokes shift of 68 nm for a pH 3.2 buffer is obtained. In addition, it displays a higher pK_a value of 5.4 related to spirolactam ring opening,^{27–31} obtained using the Henderson–Hasselbach-type mass action equation (Figure S10).^{27–30} This unusually higher pK_a value and very fast response to pH changes is due to the steric hindrance between the spirolactam moiety and the amine ring on the rhodamine backbone. A fluorescence quantum yield of 19.8 % with a molar absorptivity of $3.8 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ in a 10 mM pH 3.2 buffer containing 10% ethanol is obtained. Rhodamine dye **C** is highly selective to reactions with protons at pH 3.2 compared to 50 μM solutions of different anions, cations, and amino acids according to selectivity experiments (Figures S12–S13).

In order to confirm the nature of the electronic transitions obtained for rhodamine dyes **A–D**, theoretical studies (optimization, frequency and TD-DFT calculations) at the APFD^{32/6–311+G(2d, p)}³³ level were conducted. Electronic transition maxima were calculated (expt.) at 526 (575), 531 (586), 322 and 527 (587) nm for dyes **A–D** respectively (Figures S16, S22, S27 and S33) and these values are within an expected error range of 0.20–0.25 eV.³⁴ Full details are available as supporting information (Figure S14–S35). The results are best summarized in the form of difference density illustrations in Figure 3, which consists of subtracting the total density cube for the HOMO from that of the LUMO orbital that contributes the most to the UV-Vis transition as verified from the calculations.^{34, 35} If both the HOMO and LUMO orbitals of a molecule are delocalized, there would not be great differences in color observed in these plots. The illustrations in Figure 3 reveal the movement of electron density towards the positively charged N atom (see Scheme 1) in dyes **A**, **B** and **D**, and clearly indicate that dye **C** has a greater difference in color than the other dyes. Finally, delocalization is restored in dye **D**, judging by the similar appear to the illustration for dyes **A** and **B**.

Rhodamine dyes **A**, **B** and **C** show excellent photostability (Figures S11–S12) and very low cytotoxicity with cell viability of more than 80% at dye concentrations of 20 μM according to a standard MTS assay through 48 h incubation of HeLa cells with the rhodamine dyes (Figure S36). Lysosomes are the intracellular acidic organelles with pH values from 4.0 to 5.5. In order to test whether rhodamine **C** can target lysosomes in live cells, we co-stained HeLa cells with rhodamine dye **C** and lysotracker green, a lysosome specific dye. Confocal microscopic co-localization analysis of rhodamine dye **C** and lysotracker green gave the Pearson's coefficient value of 0.89 or higher, indicating that rhodamine dye **C** accumulates and becomes activated to engender fluorescence inside lysosomes in live cells (Figures 4 and S37). In addition, fluorescence of rhodamine dye **C** becomes brighter with increases of the dye concentrations (Figure 4). Next, we investigated whether rhodamine dye **C** could monitor intracellular pH changes by using H^+/K^+ ionophore nigericin to equilibrate the intracellular and extracellular pH values of HeLa and MCF7 human breast cells in media containing 5 $\mu\text{g/mL}$ H^+/K^+ ionophore nigericin with different pH values from 3.0 to 7.5 (Figures 5 and S38).^{27–30} Cellular fluorescence intensity of rhodamine dye **C** becomes brighter when pH decreases from 7.5 to 3.0 (Figure 5). It shows the same fluorescence responses to pH changes in buffer solutions and live cells with a higher pK_a value of 5.4

related to spirolactam ring opening^{27–31} since its fluorescence can be observed at pH 6.5 in live cells (Figures 2, 5 and S38). The unusual pK_a value arises from considerable steric hindrance between o-phenylenediamine residue and bulky xanethene moiety compared with traditional rhodamine dyes (Scheme 1).

We further studied whether rhodamine dye **C** could be used to monitor lysosomal pH changes in HeLa cells. After incubation of HeLa cells with rhodamine dye **C**, the cells show near-infrared fluorescence (Figure 6). 10 mM NH₄Cl treatment of HeLa cells causes a decrease of cellular fluorescence intensity because ammonium chloride treatment results in quick alkalification of the lysosomes, causing a pH increase (Figure 6). Treatment of HeLa cells with 100 μM hydrogen peroxide and N-ethylmaleimide also cause cellular fluorescence intensity decreases (Figure 6), indicating that lysosomal pH in live cells under oxidative stress increase because the oxidative stress under hydrogen peroxide redistributes H⁺ ions from acidified lysosomes to cytosolic compartments by weakening proton ATPase, an enzyme that imports H⁺ ions through ATP hydrolysis.^{36–38} Treatment of HeLa with different concentrations of chloroquine also makes cellular fluorescence intensity decrease (Figure 7) because chloroquine functions as a lysosomotropic base to neutralize some proton ions in lysosomes in live cells. As a result, increases in chloroquine concentration results in gradual fluorescence decreases (Figure 7). These results convincingly demonstrate that rhodamine dye **C** can accurately detect pH fluctuations in lysosomes.

Conclusions

We developed new near-infrared rhodamine dyes with large Stokes shifts, excellent photostability and fluorescence brightness by integrating an additional amine group with fused rings to rhodamine dyes to increase the electron donating ability of amine groups. In addition, fluorescent probe (rhodamine dye **C**) with a higher pK_a value of 5.4 was prepared as a useful molecular fluorescent tool in the study of pH-relevant biological processes for fast and sensitive detection of lysosomal pH changes and fluctuations under oxidative stress.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.
Absorption and fluorescence spectra of rhodamine dyes A and B in 10 mM buffers with excitation at 550 nm

Figure 2.

Absorption (left) and fluorescence (right) spectra of rhodamine dye **C** in 10 mM buffers with excitation at 550 nm.

Figure 3. Difference density illustrations as iso-surfaces of rhodamine dyes **A** (top left), **B** (top right), **C** (bottom left) and **D** (bottom right). Red areas indicate values for the different density of -8.613e^{-5} and blue are for 8.613e^{+5} , see scale on top of illustration.

Figure 4.

Acidity-activated turn-on cellular fluorescence of rhodamine dye **C** in lysosomes in HeLa cells. HeLa cells were cultured in media containing 1 $\mu\text{g/ml}$ Hoechst stain, 1 μM Lysosensor green and rhodamine dye **C** with concentrations from 1 μM to 15 μM . The sixth row is the enlarged image of 15 μM rhodamine dye **C**. The images were obtained by confocal fluorescence microscopy with a scale bar of 20 μm . The filter sets used to image dye **C**, Hoechst and Lyso-green were excitation 559 nm and emission 675/50 nm, excitation 405 nm and emission 450/50 nm and excitation 488 nm and emission 550/50 nm, respectively.

Figure 5.

Cellular fluorescence intensities of 10 μM rhodamine dye **C** inside HeLa cells with different intracellular pH values, which was adjusted by using 5 $\mu\text{g/mL}$ H^+/K^+ ionophore nigericin to equilibrate the intracellular and extracellular pH in media with different pH values. Confocal fluorescence microscopy was employed to collect the images with a scale bar of 50 μm . The filter sets used to image dye **C** was excitation 559 nm and emission 675/50 nm.

Figure 6. Cellular fluorescence intensities of 10 μM rhodamine dye **C** inside HeLa cells. Intact cells, and cells treated with 10 mM NH_4Cl , 100 μM NEM and 100 μM HClO , respectively. Confocal fluorescence microscopy was employed to collect the images with a scale bar of 50 μm . The filter sets used to image dye **C** was excitation 559 nm and emission 675/50 nm.

Figure 7. Cellular fluorescence intensities of 10 μM rhodamine dye **C** inside HeLa cells with different concentrations of chloroquine as a stimulant. Confocal fluorescence microscopy was used to collect the images with a scale bar of 50 μm . The filter sets used to image dye **C** was excitation 559 nm and emission 675/50 nm.

Scheme 1.

Chemical structures of near-infrared rhodamine dyes (**A**, **B**, and **C**).