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Development of ratiometric fluorescent pH sensors based on chromenoquinoline derivatives with tunable pK_a values for bioimaging

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ABSTRACT

In this work, we first studied the pH-dependent characteristic of chromenoquinoline. Based on this, we then designed and synthesized two novel chromenoquinoline derivatives that can act as fluorescent pH sensors. The pK_a values of two novel chromenoquinoline derivatives can be modulated from 2.32 to 4.38 and 6.27 by introducing EDG on the backbone of chromenoquinoline. Furthermore, we demonstrate that the sensor **4** can be used as a ratiometric fluorescent pH sensor for fluorescence imaging in living cells.

Intracellular pH plays an important role in many essential biological processes including cell growth and apoptosis,[1] enzymatic activity,[2] ion transport,[3] calcium and sodium regulation,[4] and muscle contraction.[5] However, abnormal intracellular pH variations may lead to diseases such as Alzheimer's disease[6] and cancer.[7] Therefore, monitoring pH values inside living cells is very important for investigating physiological and pathological processes. A number of ways have been developed to measure pH values. For instance, microelectrodes,[8] NMR,[9,8b] and absorbance spectroscopy.[10,8b] Compared to these methods, fluorescence spectroscopy has attracted much attention because of its excellent sensitivity, high signal-to-noise ratio, and minimal damage to living samples.[11] Up to date, a large array of pH sensors have been constructed, and some of which are commercially available.[12] However, most of the known fluorescent sensors respond to pH with changes only in fluorescent intensity.[13] A major limitation of intensity-based sensors is that fluorescence measurement is affected by variations in sample environment and sensor distribution. By contrast, ratiometric fluorescent sensors allow the measurement of emission intensities at two different wavelengths, which would provide a built-in correction for environmental effects and also increase the dynamic range of fluorescence measurement.[14]

Chromenoquinoline derivatives are well known for their biological activity as estrogenic agents.[15] However, the fluorescence properties of these derivatives are rarely explored for design of fluorescent sensors.[16]

In this work, we first studied the fluorescence properties of chromenoquinoline, the results showed that the compound had strong fluorescence at 414 nm when excited at 350 nm in ethanol (Fig. 1) with a high quantum yield (Φ_f = 0.80, Table 1). Encouraged by these results, we continued to examine the pH-dependent characteristic of chromenoquinoline (Figs. S1–S5). These studies indicated that chromenoquinoline could act as a ratiometric fluorescent pH sensor with high selectivity and fine reversibility (Figs. S3–S5). However, the p*K*a of chromenoquinoline is too low (pK_a = 2.32, Table 1) to be applicable for fluorescence imaging in living cells.

It is well-known that introducing electron donating groups (EDG) can increase electron density on the nitrogen and hence increase basicity.[17] Therefore we reasoned that introducing EDG on chromenoquinoline at proper positions may increase its pK_a. DFT calculations were then conducted to examine the potential effect of the position of the EDG on the electronegativivty of the quinoline nitrogen. As shown in Table S1, when the EDG is at the 3- and 2′-positions on the chromenoquinoline backbone, the electronegativity of the quinoline nitrogen is minimal, indicating that the EDG at the 3- and 2′-positions may be optimal for the increase in the pK_a of chromenoquinolines. To investigate the effect of EDG,

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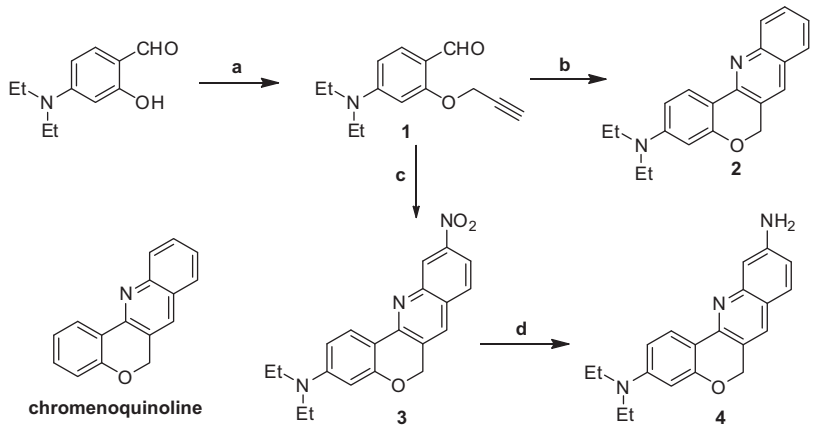


Scheme 1. Structure of chromenoquinoline and synthesis of compounds **2** and **4**. Reagents and conditions: (a) 3-bromoprop-1-yne, DMF, K_2CO_3, rt, 10 h; (b) aniline, DMF, CuCl, 110 °C, 5 h; (c) 3-nitroaniline, DMF, CuCl, 110 °C, 5 h; (d) EtOH, $SnCl_2{\cdot}2H_2O$, reflux 3 h.

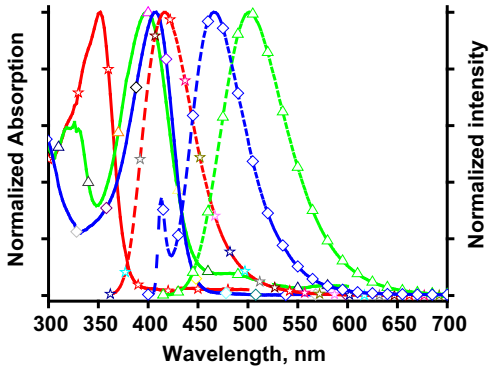


Figure 1. Absorption (solid line) and emission spectra (dash line) of compounds chromenoquinoline (open star), **2** (open triangle), and **4** (open square).

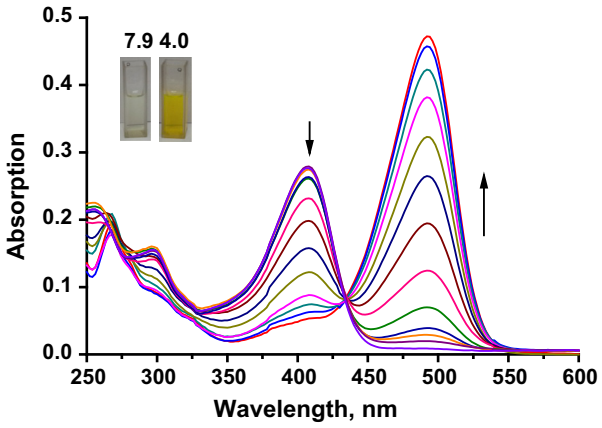


Figure 2. pH-dependence of the absorption spectra of sensor **4** (5 μM) in aqueous solution (containing 40% EtOH as a co-solvent). The arrows indicate the change of the absorption with pH decrease from 7.9 to 4.0. Inset: the change of color with pH at 7.9 and 4.0.

Table 1
Photophysical data of chromenoquinoline, compounds **2** and **4** in EtOH

Compound	λ_{abs}/nm[a]	λ_{em}/nm[b]	ε_{max}/M^{-1} cm^{-1}	Φ_f[c]	pK_a[d]
chromenoquinoline	352	414	24717	0.80	2.32
2	396	483	15765	0.81	4.38
4	406	465	18751	0.88	6.27

[a] The maximal absorption of the dye.
[b] The maximal emission of the dye.
[c] Φ_f is the relative fluorescence quantum yield estimated by using rhodamine 6G (Φ_f = 0.95 in H_2O) as a fluorescence standard.[19]
[d] The pK_a was calculated by using the Henderson–Hasselbach-type mass action equation ($\log[(F_{max} - F)/(F - F_{min})] = pK_a - pH$, where F is the fluorescence emission intensity at 414, 483 or 465 nm).[20]

we further designed two novel chromenoquinoline derivatives (2 and 4, Scheme 1). The new types of compounds have EDG at the 3- or 3 and 2′-positions, which may have an impact on the pK_a of the chromenoquinolines. Thus, we envisioned that these new compounds should have appropriate pK_a values and can act as ratiometric fluorescent pH sensors for fluorescence imaging in living cells.

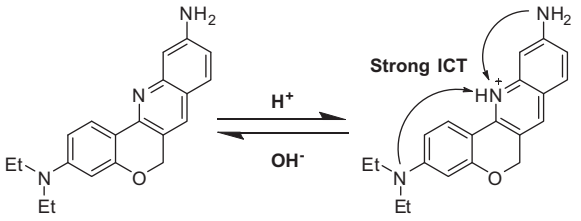
Compounds **2** and **4** were synthesized in a facile route as shown in Scheme 1. Alkylation of 4-(diethylamino)-2-hydroxybenzaldehyde afforded compound **1**. Conversion of compound **1** to compound **2** or **3** was conducted according to the literature procedures.[18] Reduction of compound **3** provided amine **4**. All of the new compounds were fully characterized by ^{1}H NMR, ^{13}C NMR, and HRMS.

With compounds **2** and **4** in hand, we first investigated their photophysical properties. Compound **2** displayed strong fluorescence at 483 nm when excited at 396 nm in ethanol (Fig. 1) with a high quantum yield (Φ_f = 0.81 in ethanol, Table 1). The emission profiles of **4** (Fig. 1) are similar to those of **2** with a maximum at 465 nm (Φ_f = 0.88, Table 1).

The standard pH titrations on compounds **2** and **4** (5 μM) were performed in aqueous solution (containing 40% EtOH as a co-solvent). With the decrease in pH values from 7.9 to 4.0, the absorption peak of sensor **4** at around 408 nm significantly diminished, and simultaneously a new red-shifted absorption band at around 492 nm was formed (Fig. 2). There is a well-defined isosbestic point at 435 nm in the absorption spectrum, suggesting the presence of two species in equilibrium. Consistently, a notable color change from pea green to yellow with pH decrease was observed (inset in Fig. 2).

The red-shift in the absorption spectra with the decrease of pH can be explained due to the protonation of the quinoline moiety (Scheme 2) in the sensor, which elicits a strong intramolecular charge transfer (ICT) process. The pH-dependent absorption spectra of sensor **2** (Fig. S6) exhibit a similar trend to those of sensor **4** with the decrease in pH values from 5.8 to 2.5.

The fluorescence emission spectrum of sensor **4** at pH 7.9 displayed an emission band with a maximum at 485 nm (Fig. 3).



Scheme 2. Mechanism of pH response by protonation and deprotonation of the sensor **4**.

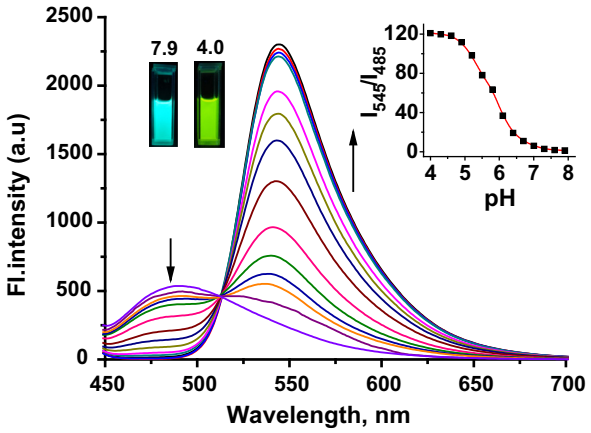


Figure 3. pH-dependence of the emission spectra of sensor **4** (5 μM) in aqueous solution (containing 40% EtOH as a co-solvent). The arrows indicate the change of the emission intensities with pH decrease from 7.9 to 4.0. Inset 1: the change of fluorescence with pH at 7.9 and 4.0. Inset 2: sigmoidal fitting of the pH-dependent ratios of fluorescence intensity (I_{545}/I_{485}).

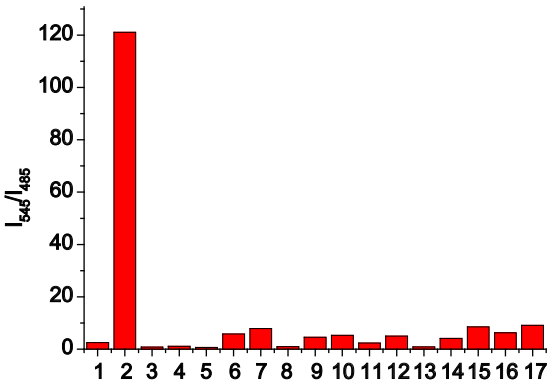


Figure 4. Ratios of fluorescence intensity (I_{545}/I_{485}) of sensor **4** (5 μM) in the presence of various analytes in aqueous solution (pH 7.9, containing 40% EtOH as a co-solvent) 1, blank; 2, H^+;(pH 4.0) 3, Na^+ (150 mM); 4, K^+ (150 mM); 5, Ca^{2+} (10 mM); 6, Mg^{2+} (2 mM); 7, Fe^{3+} (2 mM); 8, Ni^{2+} (0.5 mM); 9, Cd^{2+} (0.5 mM); 10, Zn^{2+} (0.2 mM); 11, Cu^{2+} (0.2 mM); 12, Mn^{2+} (0.2 mM); 13, Hg^{2+} (0.2 mM); 14, Ag^+ (0.2 mM); 15, Pb^{2+} (0.2 mM); 16, Al^{3+} (0.2 mM); 17, Fe^{2+} (0.2 mM).

Protonation of **4** increased the electron accepting ability of the quinoline ring (Scheme 2) and therefore resulted in a ca. 60 nm red-shift in the emission spectra from 485 to 545 nm (Fig. 3). The pH-dependent emission spectra of **2** (Fig. S7) displayed a similar trend to those of **4** with a red-shift in the emission spectra from 502 to 562 nm.

The analysis of emission intensities changes at 562 nm in **2** and 545 nm in **4** as a function of pH by using the Henderson–Hasselbach-type mass action equation[20] yielded a pK_a of 4.38 and 6.27, respectively (Table 1). The ratios of fluorescence intensities at 545 and 485 nm (I_{545}/I_{485}) of **4** showed a dramatic change from 0.49 at pH 7.9 to 121.1 at pH 4.0 (inset 2 in Fig. 3), a nearly 248-fold variation in the emission ratios. It is important to note that such a huge change of signal ratios at two wavelengths is highly desirable for ratiometric fluorescent sensors, as the sensitivity and the dynamic range of ratiometric sensors are controlled by the ratios.[14a,b,21] The large emission shift (60 nm) and relatively resolved emission peaks of **4** are highly desirable for ratiometric fluorescence detection of pH variations. By contrast, the emission ratios of compound **2** (I_{562}/I_{502}) exhibited about a 16-fold change (inset 2 in Fig. S7).

The selectivity and competitive experiments of sensor **4** to H^+ over metal ions were further investigated. When some physiologically important metal ions, such as Na^+, K^+, Ca^{2+}, Mg^{2+}, and Fe^{3+}, were added to the solution of sensor **4**, only moderate emission ratio changes were observed (Fig. 4 and Fig. S11). By contrast, H^+ induced a very modest ratiometric response with I_{545}/I_{485} = 248, and negligible changes in the emission ratio (I_{545}/I_{485} < 10) were noted upon addition of excess of other heavy and transition metal ions (0.2 mM), indicating that the sensor **4** has a moderate selectivity for H^+ over other analytes. In addition, sensor **4** exhibited a fine sigmoidal fitting of the pH-dependent ratios of fluorescence intensity (I_{545}/I_{485}) at 545 and 485 nm (inset 2 in Fig. 3), and displayed a reversible pH response (Fig. S13). Similarly, the sensor **2** also had high selectivity and exhibited a reversible pH response (Figs. S8–S10).

The prominent spectral features of sensor **4** prompted us to test its potential applications. Sensor **4** was first employed to determine the pH values of water samples from different sources (Table S2). The pH Meter gave almost the same value (Table S2). Thus, these results demonstrate that sensor **4** is potentially useful for detecting the pH values of the water samples.

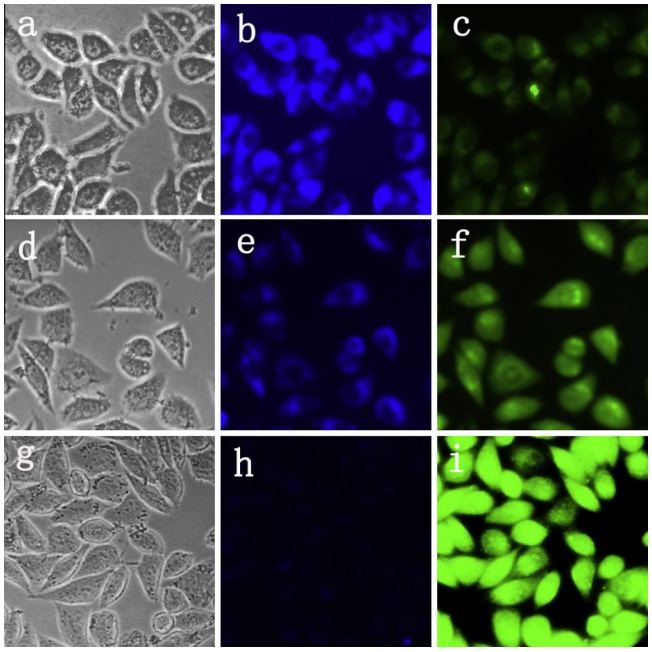


Figure 5. Images of Hela cells treated with sensor **4** at different pH values. (a) Bright field image of Hela cells incubated with sensor **4** (2 μM) at pH 7.4; (b) fluorescence image of (a) from blue channel; (c) fluorescence image of (a) from green channel; (d) bright field image of Hela cells incubated with sensor **4** (2 μM) at pH 6.4; (e) fluorescence image of (d) from blue channel; (f) fluorescence image of (a) from green channel; (g) bright field image of Hela cells incubated with sensor **4** (2 μM) at pH 5.5; (h) fluorescence image of (g) from blue channel; (i) fluorescence image of (g) from green channel.

Fluorescence sensors have the advantage for in situ detection for living biosystems. In addition, the sensor **4** has an appropriate pK_a, which encouraged us to test its potential applications for ratiometric fluorescence imaging in living cells. HeLa cells were incubated with sensor **4** (2 μM) at 37 °C for 30 min, and then the cells were washed in PBS medium of varying pH values with the addition of nigericin (1 μg/mL) to elicit a rapid exchange of K^+ for H^+ for a fast equilibration of external and internal pH.[12a,22] As shown in Figure 5, with the decrease in pH values from 7.4 to 5.5, the fluorescence in the green channel became much brighter, while the fluorescence from green channel were weaker. As can be seen from Figure 5c, we can still observe that the cells still had green fluorescence at pH 7.4, which may attribute due to sensor **4** entering into acidic organelles (i.e. lysosome, pH 4.7).[23] Thus, the results indicate that sensor **4** is cell membrane permeable and have potential applications for dual-emission ratiometric fluorescence imaging of pH fluctuations in the living cells.

In conclusion, we have successfully synthesized two chromenoquinoline derivatives and studied their photophysical properties. We conclude that introducing EDG on chromenoquinoline at 3- and 2′-positions can modulate the pK_a values of chromenoquinoline derivatives. Compounds **2** and **4** with EDG (**2** with a diethylamino at 3-position, **4** with an amino at 2′-position and a diethylamino at 3-position) have pK_a values at 4.38 and 6.27, respectively. The favorable features of the sensor **4** include large emission ratios, high selectivity, and good reversibility. Significantly, the sensor **4** can be employed as a ratiometric fluorescent pH sensor for monitoring pH variations from neutral to acidic conditions in living cells.

Acknowledgments

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Supplementary data

Supplementary data (experimental procedure and charecterization data) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tetlet.2013.10.130.

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