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"On–off–on" Switchable Sensor: A Fluorescent Spiropyran Responds to Extreme pH Conditions and Its Bioimaging Applications

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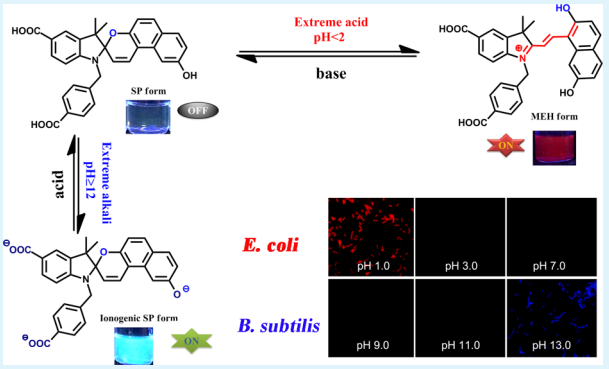
Supporting Information

ABSTRACT: A novel spiropyran that responds to both extreme acid and extreme alkali and has an "on–off–on" switch is reported. Benzoic acid at the indole N-position and carboxyl group at the indole 6-position contribute to the extreme acid response. The ionizations of carboxyl and phenolic hydroxyl groups cause the extreme alkali response. Moreover, the fluorescent imaging in bacterial cells under extreme pH conditions supports the mechanism of pH response.



KEYWORDS: *chromophores, spiropyran, extreme pH sensor, dual color, "on–off–on" switch, bacterial labeling*

Measurements of pH play a key role in biotechnology.[1] An environment with pH changing in vivo often leads to severe diseases. The most common method for measuring pH is the potentiometric method using a glass electrode. In recent years, the optical pH sensing technique based on the absorption or emission of certain dyes has received increasing attention because it offers many advantages, such as rapid response time, electrical safety, and ease of microscopic intracellular pH sensing, over the potentiometric method.[2−11] In contrast, relatively less attention has been paid on the fluorophore that are pH sensitive in the extreme acid and alkali pH ranges.[12−16] Since pH glass electrodes cannot work at extreme pH (pH ≤5.0 and ≥9.0), the development of new extreme pH sensors is important.[14] Some excellent extreme pH sensors have been reported in the literature.[12−16] It has been found that the introduction of a bulky substituent to the responsive group in these extreme pH sensors is desirable. This finding inspires us to design new extreme pH sensors by introducing different bulky substituents.

Spiropyran is well-known for its photochromism. Classical spiropyran turns into the ring-opening merocyanine (**ME**) form under the irradiation of UV light.[17−25] Furthermore, it also undergoes ring opening in the presence of an acid to form merocyanine H^+ (**MEH**) and then converts back to the closed spiropyran form (**SP**) with the addition of a base.[26−29] Because of their facile synthesis, distinct chromic transition, and high fatigue resistance,[30] various excellent sensors based on spiropyran have been developed for pH measurements.[26,27] Because no suitable substituents were introduced, these reported spiropyran-based pH sensors only responded with gradual changes in the pH range from 1.0 to 13.0, showed no sudden fluorescent variation, and lacked an "on–off" switch.[26,27] To the best of our knowledge, dual responses of spiropyran-based sensors with an "on–off" switch to extreme acid and extreme alkali have not been reported.

In this study, we designed a pH-responding spiropyran (**S1**) with an "on–off–on" switch (Scheme 1). Specifically, the pH response was regulated to both extreme acid (pH <2.0) and extreme alkali (pH ≥12.0). In the whole pH range, **S1** presents two "on" signals with different colors. It turns red ("on") under extreme acidic conditions, turns "off" at intermediate pH, and turns blue green ("on") under extreme alkaline conditions. On the basis of NMR and optical spectral analyses, the response mechanisms are proposed: The transformation from **SP** to **MEH** makes **S1** pH responsive under acidic conditions. Moreover, the introduction of the bulky substituent benzoic acid at the indole N-position and the electron-withdrawing carboxyl group at the indole 6-position causes a response to extreme acidic conditions (pH <2.0). In the meantime, this design brings the interesting result that spiropyran transforms to the **MEH** form only under extreme acidic conditions. The ionizations of carboxyl and phenolic hydroxyl groups play an essential role in the pH response under alkaline conditions. Four additional sensors (**S2**–**S5**, Scheme 2) were designed with

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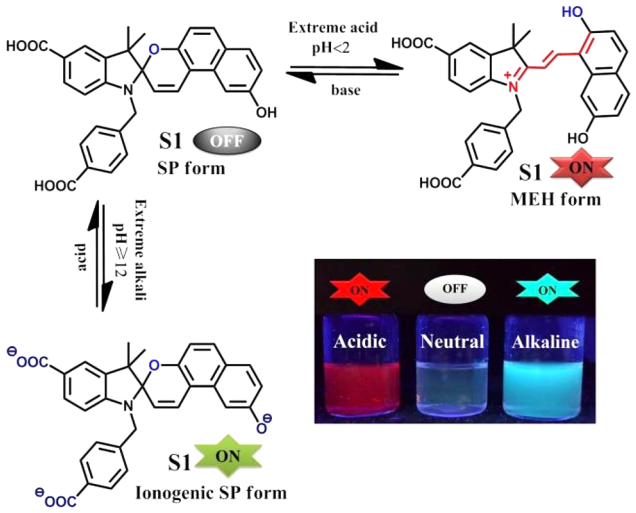
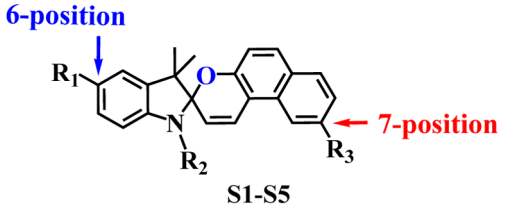
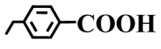
Scheme 1. "On−off−on" Switching Mechanism of S1 Stimulated by Extreme Acid (pH <2.0) and Extreme Alkali (pH ≥12.0)

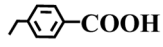


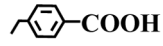
Scheme 2. Chemical Structures of S1, S2, S3, S4, and S5



S1. R_1= COOH R_2=  R_3= OH

S2. R_1= COOH R_2= $-CH_3$ R_3= OH

S3. R_1= H R_2=  R_3= OH

S4. R_1= COOH R_2=  R_3= H

S5. R_1= H R_2=  R_3= H

different substituents to confirm the extreme pH-response mechanism of **S1**. Moreover, **S1** can be used as a pH sensor labeling the bacterial cells while maintaining its dual "on−off" switch in the extreme acid and extreme alkali pH ranges. This study opens a way to design spiropyran-based fluorophores for pH sensing and bioimaging.

The synthesis of **S1** started with 2,7-dimethoxynaphthalene-1-aldehyde, which was obtained according to a standard Vilsmeier−Haack reaction. Further deprotection with BBr_3 afforded the key intermediate 2,7-dihydroxy-1-naphthaldehyde. Condensation of indolium **3a** with 2,7-dihydroxy-1-naphthaldehyde yielded **S1**. The synthesis and structural characterization of **S1** are presented in the Supporting Information (Scheme S1).

As expected, **S1** has rapid and distinct response in the extreme acid and extreme alkali pH ranges. From pH 3.0 to pH 12.0, **S1** exists in the colorless **SP** form (Scheme 1), which shows no absorption above 400 nm (Figure 1A, B). At pH <2.0, a peak centered at 520 nm evolves. Particularly, a sharp variation of the absorption peak centered at 520 nm is clearly observed between pH 1.0 and 2.0. Under extreme acidic conditions (pH <2.0), **S1** exhibits two absorption bands centered at 300 and 520 nm (Figure 1A), which are assigned to

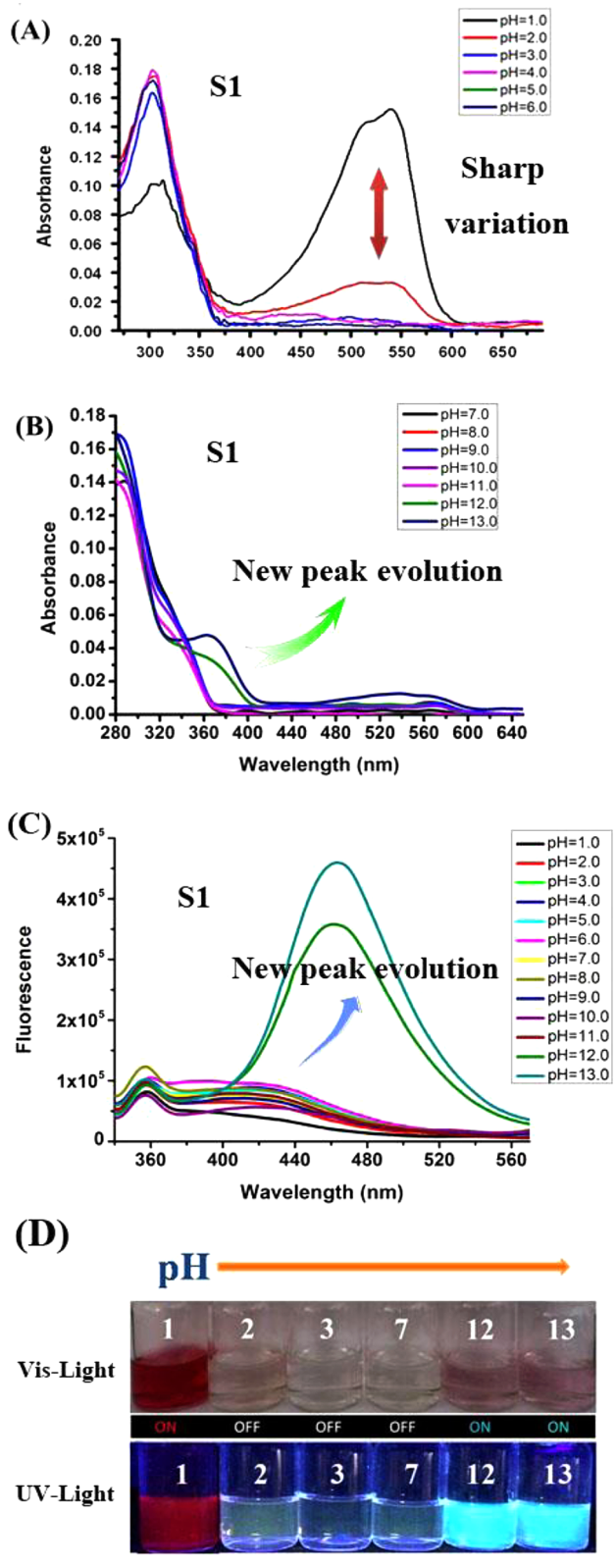


Figure 1. Variation in absorption spectrum of **S1** with pH: (A) pH 1.0−6.0, (B) pH 7.0−13.0. (C) Variation in emission spectrum of **S1** with pH (λ_{ex} = 320 nm) (buffer/MeOH mixture (1/1 v/v) solution, 5 × 10^{-6} M, room temperature). (D) pH-dependent colors of **S1** solution under visible light and UV light.

the **SP** form and **MEH** form, respectively. In other words, **S1** exists in its **MEH** form only at pH <2.0. Under extreme alkaline

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conditions (pH ≥12.0), a new absorption band centered at 370 nm arises with a slight decrease at 300 nm (Figure 1B). Correspondingly, a new emission peak centered at 460 nm evolves in the fluorescence spectra of **S1** at pH ≥ 12.0 (Figure 1C). A visual representation for **S1** shows two distinct fluorescence colors in the whole pH range. The colors of the **S1** solution under UV light are red, colorless, and blue green at the pH values of 1.0, 7.0, and 12.0, respectively. In other words, **S1** exhibits an "on−off−on" effect: it "turns on" in extreme acid, "turns off" in a range of pH 3.0−11.0, and "turns on" again in extreme alkali. The bulky substituent benzoic acid at the indole N-position and the electron-withdrawing carboxyl group at the indole **6**-position stabilize the **SP** form of **S1**, making the ring opening of **S1** occur only in the extreme acid range and leading to the "on−off" optical effect. Additionally, the ionizations of three ionogens (one benzoic acid, one carboxylic acid, and one hydroxyl group) cause the response of **S1** in the extreme alkali range, leading to an "on−off" switch. Two fluorescence colors (red in the extreme acid range, blue green in the extreme alkali range, and nonfluorescence in the intermediate pH range and rapid responses in the extreme acid and extreme alkali pH ranges with an "on−off−on" switch make **S1** an excellent fluorophore sensor responding at extreme pH values.

To explore the mechanism for the responses to extreme pH, we investigated the variation of the ^{1}H NMR spectrum of **S1** with pH in MeOD/D_2O mixture by adding an acid or a base into the solution. As shown in Figure 2B, at pH <2.0, the peaks

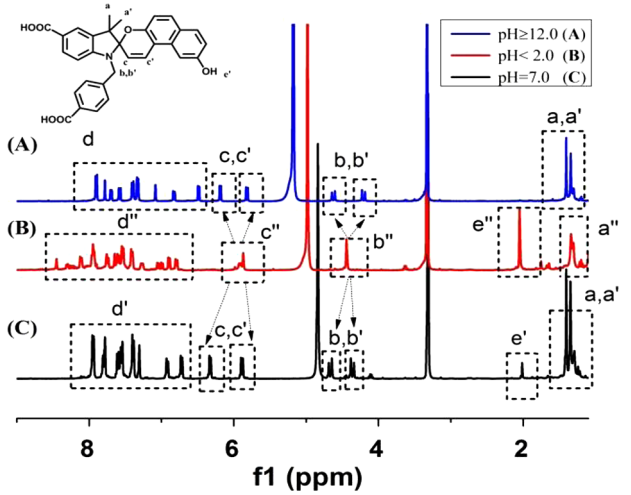

Figure 2. ^{1}H NMR spectra of **S1** in 0.5 mL of MeOD/D_2O (4:1 v/v): (A) under extreme alkaline conditions (pH ≥12.0), (B) in neutral solution (pH 7.0), and (C) under extreme acidic conditions (pH <2.0). The pH values of solution are tuned by the addition of DCl and NaOD.

a and a′, assigned to the methyl protons on the *N*-heterocycle, b and b′, assigned to the benzyl protons, and c and c′ assigned to the alkene protons, merge into the single groups a″, b″, and c″, respectively, because of the breakage of the ether linkage in **S1** (**MEH** form in Scheme 1). The protons of the aromatic rings shift to the low field. Meanwhile, the above merging of doublets into singlets indicates that **S1** undergoes a traditional ring-opening process under extreme acidic conditions. The characteristic peaks of **S1** under extreme alkaline conditions are totally different from those under extreme acidic conditions.

Figure 2A, C shows the ^{1}H NMR spectra of **S1** at pH 12.0 and pH 7.0, respectively. The characteristic symmetric peaks a and a′, b and b′, and c and c′ remain doublets, demonstrating that no ring opening occurs at pH 12.0 and pH 7.0. The characteristic peak e′ assigned to the phenolic hydroxyl group disappears under alkaline conditions (Figure 2A). In addition, some protons in the aromatic region split. Two conclusions can be reached: First, the pH response of **S1** to extreme acid is induced by ring opening; second, the pH response to extreme alkali is attributed to the substituents attached to the aromatic rings because ring opening cannot occur under alkaline conditions.[31]

To find out why **S1** exists in the opened **MEH** form only under extreme acidic conditions, we designed and synthesized spiropyrans **S2** and **S3** bearing different R_1 and R_2 substituents, but containing the same R_3 group (see Scheme 2 and the Supporting Information). As reported in the literature, the site-specific attachment of benzoic acid to the N-position of indole plays an essential role in the pH response under extreme acidic conditions.[14] To evaluate the effect of benzoic acid on pH response, we designed **S2** without a substituent benzoic acid group. As shown in Figure S1 in the Supporting Information, the absorption bands centered at 500 nm remain in the spectra of **S2** from pH 1.0 to 6.0. Thus, unlike **S1**, **S2** shows no pH response and exists in the **MEH** form at pH 1.0−6.0. This result indicates that the substituent benzoic acid attached to the indole N-position affects the pH response to extreme acid. The benzoic acid provides steric hindrance and can stabilize the original **SP** form. Therefore, spiropyran requires strong external stimuli to reach the **MEH** form. We also designed **S3** without a carboxyl group at the indole 6-position. Figure S1 in the Supporting Information shows that the **MEH** characteristic peak of **S3** gradually decreases with increasing pH and disappears at pH 6.0, far from the extreme acidic pH of 1.0. It is well-known that a pH-dependent, electron-withdrawing group attached to the indole 6-position, such as the carboxyl group, can enhance the stability of the closed **SP** form.[26] Obviously, both the benzoic acid and the carboxyl group at the indole N- and 6-position stabilize the **SP** form; thus, only an extreme acid can induce the opening of the pyran ring. Finally the synergistic effect of the benzoic acid and the carboxyl groups contributes to the pH response of **S1** to extreme acid.

The pH-dependent fluorescence of **S1** at pH 1.0 to 13.0 is presented in Figure 1C. Consistent with the absorption spectra, the fluorescence spectra of **S1** at pH ≥12.0 all have a new emission peak evolved at 460 nm. The ionogens directly connected to the conjugated framework can induce a variation of emission peak.[32−34] Therefore, we assume that the new absorption and fluorescence peaks of **S1** appearing at pH ≥12.0 may be due to the ionogenic product of the **SP** form. **S1** contains three ionogenic groups: two carboxyl groups at the indole N- and 6-position and one phenolic hydroxyl group group at the naphthalene 7-position. In order to investigate the cause for this extreme alkali response, we designed sensors **S4** and **S5** to validate the ionization effect by excluding the carboxyl and phenolic hydroxyl groups. **S5** is a fluorophor without any carboxyl or phenolic hydroxyl group and thus totally avoids the effect of ionization. The fluorescence spectra of **S5** at different pH values all show a single and sharp peak at 400 nm, which remains unchanged in the range of pH assignable to the **SP** form (see Figure S2C in the Supporting Information). This singlet of **S5** is different from the doublet of **S1**, indicating that the ionizations cause the evolution of a new

peak in the fluorescence spectra of **S1**. Besides, this new peak is characterized by the red shift of the peak for the **SP** form. To further explore the extreme alkali response of **S1**, we designed **S4**, which contains only one carboxyl group at the indole 6-position and one benzoic acid group at the indole N-position. Compared with **S1**, **S4** has a reduced ionization effect due to the absence of the phenolic hydroxyl group at the naphthalene 7-position. Different from the emission spectra of **S1** and **S5** (see Figure S2B in the Supporting Information), the emission spectra of **S4** has a new peak evolving at about 430 nm, and this new peak changes with increasing pH value. Although ionization also occurs with **S4**, the variation in the emission spectra of **S4** under 500 nm, which is caused by ionization, has not presented a sharp change in extreme alkali (see Figure S2B in the Supporting Information). In other words, no "on−off" effect is observed in the solutions of **S4** and **S5**. These results corroborate the mechanism for the response of **S1** under extreme alkali conditions. The ionizations cause the evolution of an absorption peak of **S1** at 370 nm under alkaline conditions, which is characterized by the red-shifted peak of the **SP** form. Moreover, the ionizations of the three ionogenic groups cause **S1**'s alkali response, which occurs only under extreme alkaline conditions.

Although extreme pH values are very harsh conditions, some organisms can still overcome them. For example, a large number of microorganisms including acidophile, *Helicobacter pylori*, *Escherichia coli* (*E. coli*) and *Bacillus subtilis* (*B. subtilis*) can survive under such harsh conditions.[13,35] Some of them are harmful to human beings, for example, by inducing stomach diseases. However, few pH sensors can detect them under extreme pH conditions. Thus, there is great value to develop **S1** as an extreme pH sensor for such harsh conditions. We chose *E. coli* and *B. subtilis* as model organisms for the subsequent assays because *E. coli* and *B. subtilis* can survive under extreme acidic and extreme alkaline conditions, respectively. To determine whether **S1** works in bacterial cells under such extreme pH conditions, we controlled the pH values of the culture media by using various Tris-HCl buffers. *E. coli* and *B. subtilis* were each incubated with **S1** for 12 h at 37 °C in a series of pH media.[36−39] The red fluorescence of **S1** can be observed in *E. coli* cells at pH 1.0 (Figure 3A), but not at pH 3.0 and 7.0 (Figure 3B, C). Meanwhile, blue fluorescence appears only at pH ≥12 in *B. subtilis* cells (Figure 3(D-F)), indicating that the fluorescence of **S1** only works under extreme pH conditions.

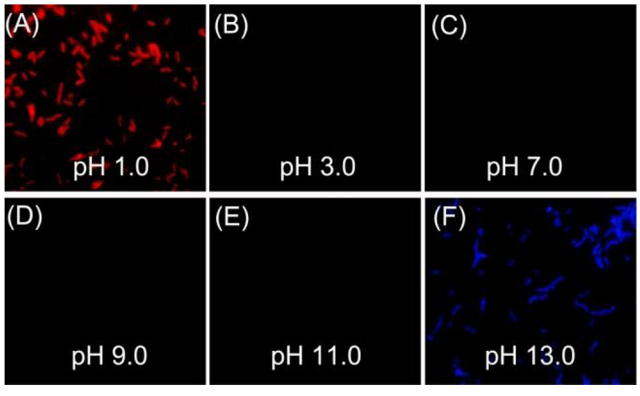

Figure 3. Fluorescence images of bacterials cells incubated with 0.1 M **S1** for 30 min at different pH values: (A−C) *E. coli* cells and (D−F) *B. subtilis* cells.

These results are well-consistent with the results of spectral assays in solution. The pH conditional cell labeling demonstrates that **S1** fluorescence can sensitively turn on in cells with extreme pH. This advantage makes it worthwhile to further develop **S1** as an extreme pH sensor in biological applications.

In summary, we have presented a new acid-alkali discoloration spiropyran **S1** that "turns on" only in the extreme acid or extreme alkali pH ranges. The introduction of the bulky benzoic acid group at the indole N-position and the electron-withdrawing carboxyl group at the indole 6-position contributes to the response to extreme acid. It is interesting that the **MEH** form is formed only under extreme acidic conditions, which is rarely reported in literature. The pH response to extreme alkali is attributed to the ionizations of the carboxyl and phenolic hydroxyl groups. **S1** can be viewed as an excellent extreme pH sensor due to its two-color fluorescence, i.e., red in extreme acid, blue green in extreme alkali, and nonfluorescence at intermediate pH. In vivo experiments showed that **S1** efficiently labeled the bacterial cells at extreme pH with the same effect as in solution. To the best of our knowledge, **S1** is the first vis−NIR spiropyran-based fluorophor that can label cells in both extreme acid and extreme alkali. The "on−off−on" mechanism of pH response to extreme acid and alkali provides an avenue to design better acid-chromic spiropyran-based monitors for pH sensing and bioimaging.

■ ASSOCIATED CONTENT

Supporting Information

Experimental procedures, characterization of the macromolecules, and supporting figures and text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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