

Multiple Factors Regulate the Spirocyclization Equilibrium of Si-Rhodamines

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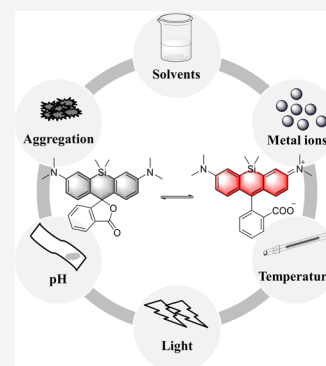
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ABSTRACT: Si-rhodamine has been extensively used in super-resolution fluorescence imaging in recent years. Its equilibrium between ring-closed nonfluorescent spirolactones and ring-opened fluorescent zwitterions endows Si-rhodamine with excellent fluorogenicity, membrane permeability, and photostability. In this paper, the equilibrium of Si-rhodamine between lactones and zwitterions was revealed to be greatly affected by various environmental factors, including molecular aggregation, solvent polarity, pH, metal ions, irradiation, and temperature. These environmental sensitivities make Si-rhodamine useful as a hydrochromic material, a fluorescent sensor array for metal ions or solvents, and a photoactivatable switch. Importantly, these results indicate that using Si-rhodamine as a fluorogenic probe or a blinking fluorophore in single-molecule localization super-resolution microscopy requires caution on possible false signals caused by its environmental sensitivity.



1. INTRODUCTION

New fluorescence imaging techniques and biolabeling methods have given ancient organic dyes a new development opportunity in biological and biomedical research.^{1–3} Notably, super-resolution and in vivo fluorescence imaging require high brightness and photostability, as well as near-infrared emission and accurate labeling of biological targets.^{4–6} These requirements are relatively easy to meet through structural modifications of organic dyes (than genetic engineering of fluorescent proteins).^{7–9} For example, inhibiting twisted intramolecular charge transfer significantly improves fluorescence brightness and photostability,^{10–12} and the introduction of protein tags or bio-orthogonal reactive groups allows accurate labeling of biological targets.^{13–15} At the same time, fluorescence is affected by various environmental factors that result in changes in fluorescence intensity, emission wavelengths, and/or lifetime. Based on this environmental sensitivity, fluorescent probes convert molecular recognition into emission signals. A comprehensive understanding of the environmental sensitivity of fluorescence is essential for removing spurious signals, improving the accuracy of the fluorescent detections, and developing new applications of dyes.^{15–17} To this end, a representative example is the environment-dependent fluorescent switches which enable super-resolution fluorescence imaging based on single-molecule localization.^{6,18–20}

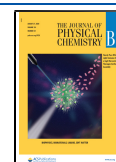
Si-rhodamine is a new type of fluorophore that has attracted much attention in recent years.^{21–23} It has excellent near-infrared emission, high fluorescence intensity and photostability, and favorable fluorogenicity. These photophysical and photochemical properties are believed to be the result of the

spirocyclization equilibrium between the ring-closed non-fluorescent spirolactones (L) and the ring-opened fluorescent zwitterions (Z) in Si-rhodamines.^{23–27} As first discovered by Johnsson et al., this spirocyclization equilibrium shifts from L to Z when a Si-rhodamine binds a biomolecule target via a ligand.²⁴ The resulting large fluorescence enhancement makes Si-rhodamine an excellent fluorogenic probe for imaging biomolecules,^{23–27} and owing to the excellent photostability, Si-rhodamine has been successfully applied in stimulated emission depletion super-resolution fluorescence imaging.²⁸ This shift of the spirocyclization equilibrium was initially thought to be caused by the disaggregation of spirolactones as they bind biomolecules.²⁴ However, after studying the fluorescence changes of Si-rhodamine in the presence of different concentrations of anionic surfactant sodium dodecyl sulfate (SDS), Lavis et al. proposed that the transition to zwitterions was due to the surface anions of SDS micelles stabilizing rhodamine cations.²⁶ Indeed, it is well known that the equilibrium between lactones and zwitterions of conventional rhodamine is affected by a variety of environmental factors,²⁹ including dye aggregation,³⁰ pH,³¹ polarity,³² metal ions,³³ light stimulation,³⁴ and so forth. However, for Si-rhodamine, except dye aggregation, the influence of these environmental factors on L–Z switching is still poorly

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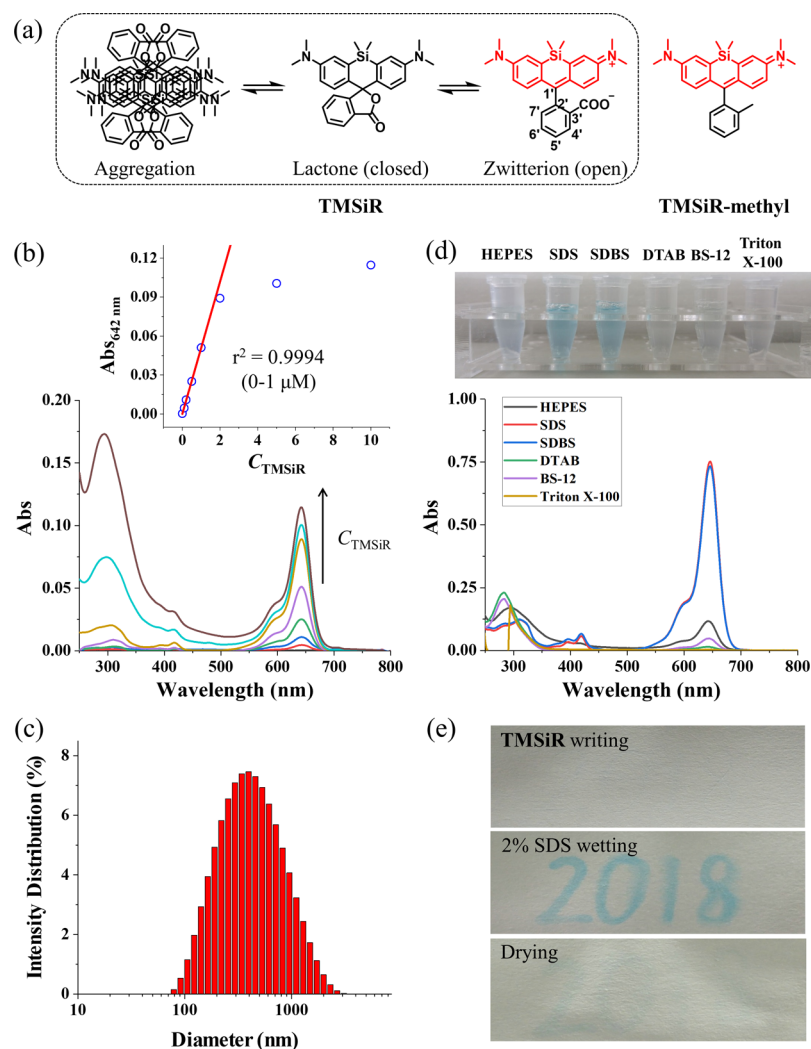


Figure 1. (a) Molecular structures of Si-rhodamines used in this research and the equilibrium of **TMSiR** between aggregation, lactone, and zwitterion forms. (b) UV–vis absorption spectra of **TMSiR** of different concentrations in 20 mM *N*-(2-hydroxyethyl)piperazine-*N'*-ethanesulfonic acid (HEPES) buffer solution (pH = 7.4). Inset: maximum absorbance at 641 nm as a function of **TMSiR** concentration. (c) DLS analysis of 10 μ M **TMSiR** (20 mM HEPES, pH = 7.4). (d) Absorption changes of 10 μ M **TMSiR** in 20 mM HEPES buffer solution (pH = 7.4) with the addition of various surfactants (final surfactant concentration: 20 mM). Inset: color changes after the addition of surfactants. (e) Photos of **TMSiR**-doped paper with a text of “2018” written via SDS solution.

understood.^{24,26,35} This environmental sensitivity, nevertheless, may cause Si-rhodamine to generate false signals as they are deployed as a fluorogenic probe.

In this paper, we chose tetramethyl Si-rhodamine (**TMSiR**, Figure 1a) as a model molecule and found that the L–Z equilibrium of Si-rhodamines was also collectively affected by multiple environmental factors, such as dye aggregation, pH, polarity, metal ions, light stimulation, and temperature. These properties make Si-rhodamine useful as a hydrochromic material, a fluorescent sensor array for metal ions or solvents, and a photoactivatable switch. Importantly, our results show that the environment sensitivity of the L–Z equilibrium in Si-rhodamine probes causes excessive or nonspecific binding of probes at a high density prone to imaging artifacts. Our results have important implications for the correct usage and interpretation of fluorescent signals from Si-rhodamines.

2. RESULTS AND DISCUSSION

2.1. Solvents Regulate the L–Z Equilibrium. We first investigated the influence of solvent polarity on the

spirocyclization equilibrium in 14 solvents. As shown in Figure S1 and Table S1, **TMSiR** formed colorless lactones in all solvents except in the aqueous solution. This suggested that the zwitterion form of **TMSiR** could be stabilized by the high polarity and the hydrogen bonding in H₂O. On the contrary, organic solvents including alcohols could hardly stabilize the zwitterion form.

2.2. Aggregation Regulates the L–Z Equilibrium. It is worth noting that the ultraviolet–visible (UV–vis) absorption peak for lactones around 290 nm in aqueous solution was reported to be attributed to the dye aggregation.²⁴ With the **TMSiR** concentration decreased, the characteristic ring-opening zwitterion absorption peak around 640 nm decreased much more slowly than the ring-closed lactone peak around 290 nm (Figure 1b). This indicated the relative shift of the spirocyclization equilibrium toward the zwitterion form. Besides, an excellent linear correlation between Abs_{642nm} (or Flu_{661nm}) and **TMSiR** concentration below 1 μ M implied that the aggregation appeared only in high **TMSiR** concentrations (>1 μ M) (Figures 1b and S2). Dynamic light scattering (DLS)

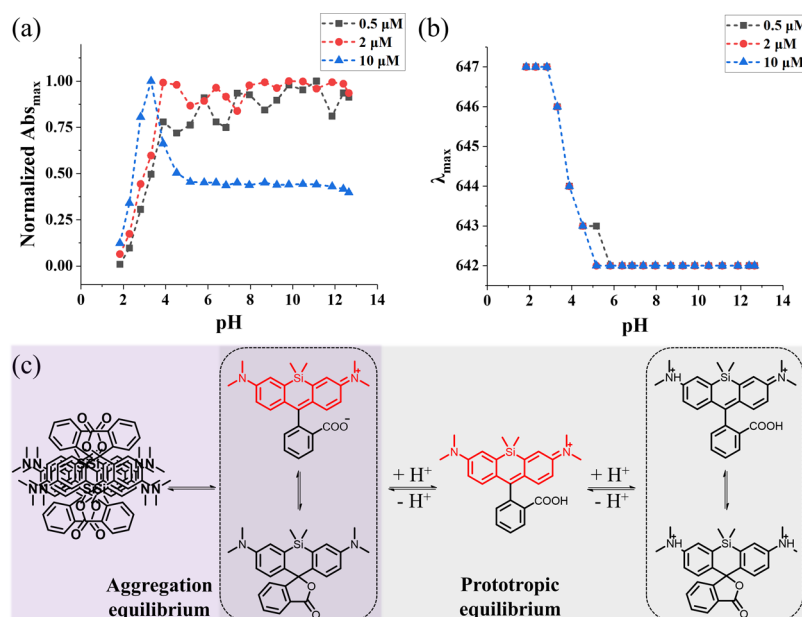


Figure 2. (a) Normalized Abs_{max} of TMSiR at different pH values. (b) Wavelength of the maximum absorption of open-ring TMSiR at different pH values. (c) Equilibrium of TMSiR with pH changes.

analysis implied that the diameter of the aggregation particles in 10 μM TMSiR solution (pH = 7.4) was 274 nm (Figure 1c).

To further understand the dye aggregations, we added different types of surfactants (Table S2) into 10 μM TMSiR solutions. The addition of anionic surfactants (SDS and sodium dodecylbenzenesulfonate) induced the increase of absorption and fluorescence more than 6-fold (Figures 1d and S3), indicating that the disaggregation of TMSiR shifted the equilibrium from L to Z. Instead, other types of surfactants (cationic: dodecyltrimethylammonium bromide; zwitterionic: BS-12; nonionic: Triton X-100) decreased the absorption and fluorescence of TMSiR. This should be because the hydrophobic environment of the surfactant shifts the equilibrium toward the L form. Only anionic surfactants can cause the shift to the Z form, indicating that the anion stabilizes rhodamine cations, and this stabilization plays a decisive role in mediating the L–Z equilibrium.²⁶ It also suggested that TMSiR could be used as a turn-on fluorescent probe for anionic surfactants. These “aggregation-monomer” and “L–Z” double equilibria were affected by the dye concentration and the ion concentrations in the surrounding. Accordingly, special attention is required in the ratio between the dye concentration and the composition of the culture solution during cell staining.

Moreover, the hydrochromic property made TMSiR useful as a water-switchable material.³⁶ We evenly deposited TMSiR adsorbed on a piece of paper. This paper turned blue after encountering water and became colorless after the water evaporated. Water containing SDS resulted in a darker color. This type of paper can be used repeatedly with water as the ink (Figures 1e and S4).

2.3. pH Regulates the L–Z Equilibrium. We then examined the absorption and fluorescence response of TMSiR at different pH values. It is known that the L–Z equilibrium of rhodamines is pH-sensitive.³¹ As shown in Figures S5–S7, the concentration of TMSiR greatly influences the optical properties of TMSiR in different pH solutions. With a high concentration of TMSiR (10 μM), the zwitterion absorbance

increased as pH decreased from 6 to 3 (Figure 2a). During this course, the wavelength of maximum absorption also gradually shifted from 642– to 647 nm (Figure 2b). This red shift was attributed to the protonation of the carboxyl anion. When pH further decreased, the absorbance decreased sharply, indicating that another proton was added to the cation (most likely at one of the amine nitrogens) and caused the ring-closing reaction. For lower concentration of TMSiR (2 μM or 0.5 μM), the pH-dependent λ_{max} curves were similar to that in 10 μM TMSiR. However, the trends of zwitterion absorbance were totally different. We could hardly observe the increase of absorbance as pH decreased from 6 to 3, especially in 0.5 μM TMSiR.

This concentration-dependent absorbance response to pH was due to the aggregation of the dye at high concentrations. The increased concentration of proton leads to the disaggregation of the dyes and shifted the L–Z equilibrium toward the Z form. This hypothesis was confirmed by the DLS analysis of 10 μM TMSiR (Figure S8). Compared to 274 nm in pH 7.4, the diameter of TMSiR aggregates increased to 580 nm as pH decreased to 5. On further decreasing pH to 3, the TMSiR aggregate dissolved gradually until undetectable. These results indicated that the disaggregation of TMSiR might include loosening the aggregates and dissolving them. Besides, one must consider both the “aggregation-monomer” equilibrium and the protonation equilibria when referring to pH variations (Figure 2c). In high TMSiR concentrations, the disaggregation of TMSiR was used to compensate for the protonation equilibrium shift toward the cationic lactone structure induced by the protonation of the amine nitrogens in low pH. These results also suggested that TMSiR aggregates in solution could act as a buffer against pH changes.

2.4. Metal Ions Regulate the L–Z Equilibrium. Next, we studied the effect of metal ions on the spirocyclization equilibrium. We speculated that metal ions could stabilize the TMSiR zwitterion, even in organic solvents; once stabilized, the L–Z equilibrium might shift toward the zwitterion form, as in conventional rhodamine lactam interactions. Based on this

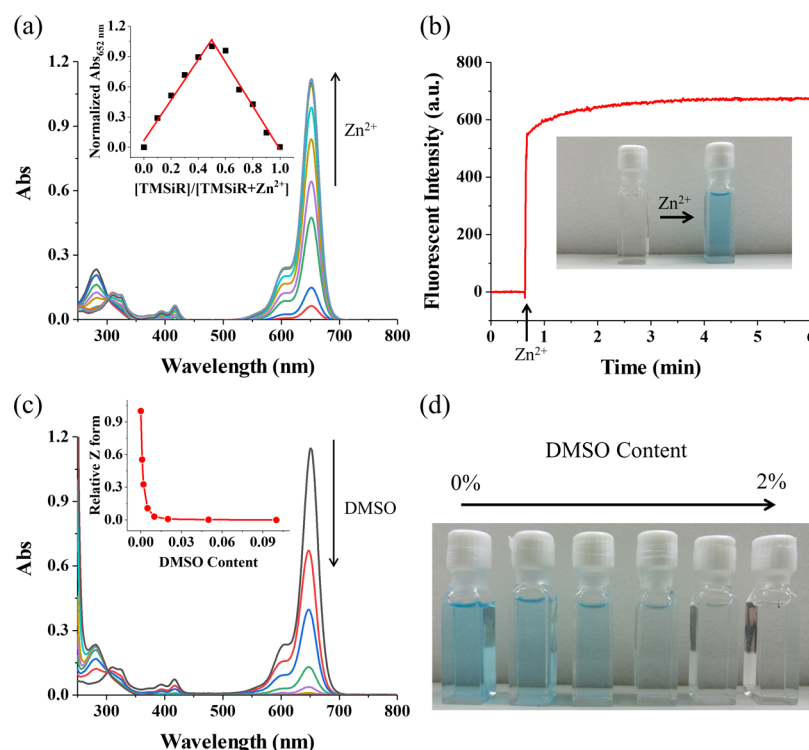


Figure 3. (a) Absorption spectra of **TMSiR** (10 μM in DCM containing 1% acetonitrile) in the presence of different concentrations of Zn^{2+} (1 to 50 μM). Inset: Job's plot evaluated from the absorbance at 652 nm with a total concentration of 10 μM . (b) Time-dependent fluorescence enhancement of **TMSiR** (10 μM in DCM containing 1% acetonitrile) upon addition of Zn^{2+} (50 μM , final concentration). Excitation: 610 nm; emission: 670 nm; recording interval: 0.2 s. Inset: Color changes after the addition of Zn^{2+} . (c) Absorption spectra of 10 μM **TMSiR** and 50 μM Zn^{2+} with different fractions of DCM–DMSO solvent mixtures. Inset: the ratio of **TMSiR** zwitterions as a function of DMSO volume ratio in DCM–DMSO mixtures. (d) Color changes of 10 μM **TMSiR** and 50 μM Zn^{2+} in DCM–DMSO solvent mixtures of different volume fractions.

hypothesis, we chose several commonly used solvents and metal ions to evaluate their impacts on the equilibrium between L and Z. In dichloromethane (DCM), the colorless **TMSiR** lactone turned blue immediately with the addition of Zn^{2+} . The UV–vis absorption and fluorescence emission peaked at 652 and 669 nm, respectively, indicating the formation of the fluorescent zwitterionic structure (Figures 3a and S9). Kinetic analysis of the Zn^{2+} -promoted **TMSiR** ring-opening reaction was carried out by monitoring the fluorescent intensity change. The observed half-life ($t_{1/2}$) of the reaction was less than 1 s (Figure 3b). To demonstrate that the **TMSiR** zwitterion was stabilized by Zn^{2+} , we subsequently added a rhodamine-based Zn^{2+} chelator to displace Zn^{2+} from **TMSiR**. As shown in Figure S10, when Zn^{2+} was chelated by the added sensor (indicated by absorption at 543 nm), the characteristic absorption peak of the **TMSiR** zwitterion at 652 nm disappeared and the lactone peak at 282 nm appeared. The Job's plot further indicated that the **TMSiR**/ Zn^{2+} complexes had 1:1 stoichiometry (Figure 3a inset).

The stabilization effects were also verified in other solvents. To facilitate comparison, we chose **TMSiR-methyl** as an open-form reference compound. As shown in Figures 4, S12, and Table S1, Zn^{2+} -induced **TMSiR** ring-opening occurred in most moderately polar solvents (except tetrahydrofuran and acetonitrile) with a varied amount of zwitterion formations. As the solvent polarity further increases, the L–Z equilibrium of **TMSiR** alone shifts to Z; however, the interaction between Zn^{2+} and solvents also becomes stronger, thus inhibiting the binding of Zn^{2+} with the carboxyl anion. Consequently, only in

medium polar solvents, Zn^{2+} can effectively induce the equilibrium shift to the Z form.

The zwitterion population of **TMSiR** in other solvents was smaller than that in DCM, indicating that solvent properties had a great impact on the **TMSiR** L–Z equilibrium constant (in the presence of Zn^{2+}). In particular, DCM and dimethyl sulfoxide (DMSO) have opposite effects on the **TMSiR** ring-opening reaction. We thus mixed DCM and DMSO in different ratios and studied the impact on the spirocyclization equilibrium. As illustrated in Figure 3c,d, the zwitterion population decreased sharply with the addition of DMSO into DCM. When the volume ratio of DMSO reached 2%, the UV–vis absorbance of the zwitterions drops to ~ 0 . We made a diagram based on the zwitterion population at different DMSO ratios, and found that it fitted the function of ExpAssoc

$$y = 0.9916 - 0.5552 \times (1 - e^{0.000366 - x/0.00129}) - 0.4344 \times (1 - e^{-0.00235 - x/0.00462})$$

with a correlation coefficient of 0.9999 (see Figure 3c inset, x and y represent the DMSO content and the relative Z form, respectively). These results show that the DCM–DMSO solvent mixtures could be utilized to regulate the L–Z equilibrium constant.

In addition to Zn^{2+} , other 14 metal ions were also used to study the **TMSiR** L–Z equilibrium in different solvents. The orthogonal results in Figures 4 and S13–S26 suggest that most transition metal ions, especially, Cr^{3+} , Fe^{3+} , and Cu^{2+} , had good stabilization effects on the carboxyl anion. These results might be attributed to the partially filled d subshell in transition metal

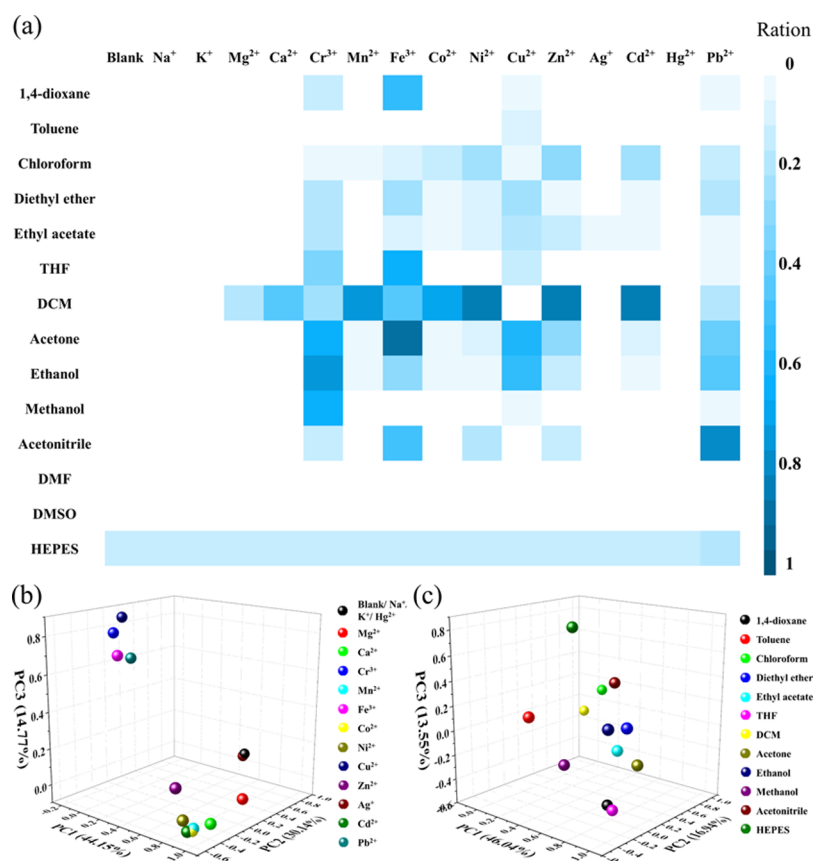


Figure 4. (a) Relative zwitterion ratio of TMSiR after adding 50 μM metal ions in different solvents (containing 1% acetonitrile); (b) 3-D canonical score plot of the fluorescence response patterns obtained by TMSiR in different solvents against metal ions; (c) 3-D canonical score plot of the fluorescence response patterns obtained by TMSiR with different metal ions against solvents.

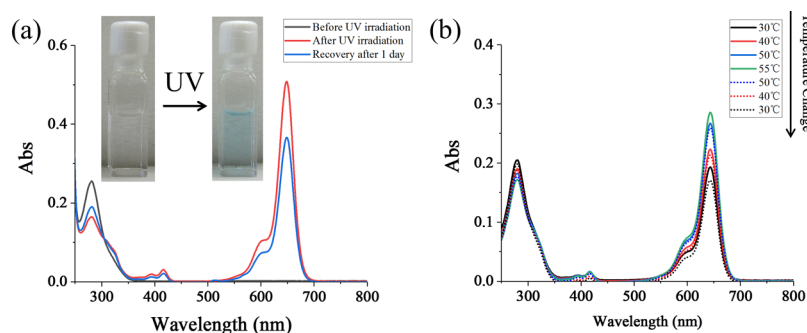


Figure 5. (a) UV-vis absorption spectra of 10 μM TMSiR and 50 μM Ca^{2+} in DCM-1% DMSO solvent mixtures after 254 nm irradiation. Radiant intensity: 500 $\mu\text{W}/\text{cm}^2$; exposure time: 30 s. Inset: color change of this system after irradiation. (b) UV-vis absorption spectra of 10 μM TMSiR and 50 μM Zn^{2+} in ethanol as a function of temperature.

ions, which facilitate the coordination of the carboxyl anion. It is worth noting that TMSiR lactone directly coordinated with some metal ions (especially Cu^{2+}) to display a new absorption peak around 500 nm in some solvents (Figures S15–S17, and S19). These binding modes were also stable but had no contributions to ring-opening reactions.²² Overall, DCM, acetone, and ethanol were the recommended solvents in this ring-opening reaction promoted by metal ions.

Traditional “lock-key” sensors for metal ions were developed based on selective chelators. Although some ligands have a stronger binding affinity for certain metal ions after the structural screening, such as aza crown ether for alkali metals or di-2-picolyamine for zinc ions, it is almost impossible for arbitrary ligands to achieve specific selective binding to a

certain metal ion. Fluorescent sensor arrays based on diverse cross-reactive dyes probe the chemical reactivity of analytes and provide impressive discrimination among very similar analytes and exquisite fingerprinting of extremely similar mixtures over a wide range of analyte types.³⁷ Solutions of TMSiR in different solvents comprise the sensor array for these metal ions. The polarity and metal binding force were the main factors affecting the ring-opening reaction of TMSiR in response to the addition of different metal ions. As shown in Figure 4b, except alkali metal ions, all other 12 metal ions were well discriminated. In the same way, solutions of TMSiR with different metal ions comprise the sensor array for solvents. As shown in Figure 4c, all 12 tested solvents were well discriminated.

2.5. Light Stimulation Regulates the L–Z Equilibrium.

Rhodamine derivatives have been reported as photoactivatable fluorophores.^{38,39} Herein, we applied light as a stimulus to facilitate TMSiR ring-opening reactions. Photoactivation wavelength of rhodamine spirocyclic derivatives was controlled by their absorption spectra.⁴⁰ The absorption of TMSiR lactone was in the UV region, with a wavelength peak around 290 nm. We then chose 254 nm light as the activation source, which was commonly accessible. Unfortunately, we did not observe zwitterion absorption at ~650 nm in all organic solvents referred above after 254 nm irradiation. This observation was attributed to the unstable zwitterion form in organic solvents. Because metal ions could stabilize zwitterions, we repeated the same experiments with the addition of Zn²⁺. In the presence of Zn²⁺, the absorption of TMSiR at ~650 nm increased slightly in some solvents after 254 nm irradiation (Figure S27). Given that the polarity of solvents also affected the ring-opening dynamics, we mixed DCM with DMSO in different volume ratios. The best ring-opening result occurred in DCM containing 1% DMSO. As shown in Figure S28, after irradiating with 254 nm light, the absorption around 650 nm increased sharply from 0.04 to 0.84. The thermal fading of zwitterions to lactones was very slow. After 1 day, only 10% Z form was converted to the L form. Other metal ions also showed similar results. When Zn²⁺ was replaced by Ca²⁺, the absorption increased from 0 to 0.5 (Figure 5a). These results indicated that the photochromic properties could be judiciously tuned by varying the metal ions and solvents.

2.6. Temperature Regulates the L–Z Equilibrium.

Finally, the influence of temperature was also considered. The TMSiR–Zn²⁺–ethanol system was chosen as an example. When the temperature was increased from 30 to 55 °C, the zwitterion absorption at 643 nm increased, in conjunction with a decrease of the lactone absorption at 280 nm, and vice versa (Figure 5b). The absorption intensities of peaks both at 643 and 280 nm obtained during the cooling cycle were always lower than those obtained during the heating cycle at the same temperature. This might be attributed to the decomposition of TMSiR at high temperatures. Further experiments in the TMSiR–methyl–ethanol system supported this conjecture (Figure S29). The absorbance at 643 nm decreased continuously when heated, and the decreased absorbance no longer recovered even with the temperature returned. Nevertheless, these results demonstrated that increasing temperature shifts the L–Z equilibrium of TMSiR toward Z as rising thermal energy facilitates the conversion from L to Z.

3. CONCLUSIONS

In summary, we have revealed that the equilibrium of TMSiR between lactone and zwitterion was significantly affected by environmental effects. Hydrogen bonding in aqueous solution and metal ions stabilizes the carboxyl anion to shift the equilibrium from lactones to zwitterions. However, the stabilization of carboxyl anion was greatly influenced by dye aggregation and solvent polarities, which inhibits the interactions between carboxyl anions and hydrogen bonds/cations. Specifically, moderately polar solvents (e.g., DCM, acetone, and ethanol) and transition metal ions (e.g., Cr³⁺, Fe³⁺, and Cu²⁺) were screened as favorable conditions in which metal ions greatly assisted the ring-opening reaction. Besides, light stimulation and heating can also promote metal-facilitated ring-opening reactions. The equilibrium of TMSiR could be finely tuned with the rational combination of these environ-

mental factors. This work proved that the equilibrium of Si-rhodamines between lactones and zwitterions as a spectral response signal can be used in many applications. At the same time, it is also critical to interpreting the signals with a comprehensive consideration of many influential factors. We hope that this work will kindle the attention to the accurate interpretations of the fluorescence signals of Si-rhodamines. Resistance to these environmentally factors will make a truly reliable fluorogenic probe, based on the lactone-zwitterion switch of Si-rhodamine. Interestingly, we noted that Wombacher and Hertel et al. introduced a tetrazine moiety to serve as a reactive group for the inverse electron demand Diels–Alder reaction and a fluorescence quencher for HMSiR, resulting in an excellent fluorogenic feature.⁴¹

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.jpcb.0c05642>.

Additional computational details and results (PDF)

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Notes

The authors declare no competing financial interest.

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