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Communication

Quantitative assessment of rhodamine spectra

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ABSTRACT

With the development of single-molecule detection and super-resolution fluorescence imaging, rhodamine dyes gain new life. Through the modification of the *N*-substituents and the replacement of the oxygen atom in xanthene, the wavelength and brightness can be effectively changed. However, the spectra of rhodamine, especially due to the balance between ring-closed non-fluorescent lactone and ring-opened fluorescent zwitterion/cation, are sensitive to interference from various environmental factors. In this way, the spectral data of various rhodamines reported by different research groups under different test conditions lacked comparability, sometimes even lacked accuracy. In order to meet the requirements for the accuracy and uniformity of spectral data in the research of single molecule imaging and dye structure-fluorescence relationship study, we have tested the spectra of fifteen rhodamine dyes that cover the visible and near-infrared regions under exactly the same conditions. By studying the dependence of the spectra on dye concentrations, it was confirmed that 1 μmol/L was ideal for detection less from the interference of dye molecule aggregation. We provide comprehensive and reliable spectral data of these fifteen dyes, which are expected to be used as references for future research. And the direct comparison of different rhodamine spectra would help to understand the structure-fluorescence relationship of rhodamines.

Fluorescence imaging and sensing reveal the temporal and spatial position, function and interactions of biomolecules, playing an increasingly important role in life science and medical diagnosis and treatment [1–4]. Correspondingly, fluorescent dyes have ushered in new and rapid development opportunities [5–7]. Rhodamine is one of the dyes that have received the most attention [8–10]. This is mainly because rhodamine dyes have high fluorescence intensity and photo-stability, and the spectra can cover the entire visible and near-infrared region.

Rhodamine derivation and structure diversity helped reveal some typical structure-fluorescence relationships. Rhodamine dyes are derivatives of xanthene (Fig. 1). The increase in the electron donating ability of amino substituents will cause a red shift of absorption and fluorescence wavelengths. Conversely, the decrease in electron donating ability will cause a blue shift of absorption and fluorescence wavelengths [11]. The replacement of the oxygen atom of xanthene by other heteroatoms can effectively increase the wavelength. The most representative one is that the introduction of silicon atom makes the emission wavelength of rhodamine enter the near-infrared region [12]. The dialkylamino substituent in the excited state will undergo intramolecular twist to form a non-emissive TICT state. The structural modification to suppress the TICT excited state can effectively improve the quantum yield of rhodamine dyes [13]. For example, Lavis *et al.* changed *N,N*-dialkylamino substituents to four-membered azetidine ring to suppress TICT and obtain rhodamine fluorophores with high brightness [14]. Xiao and Guo *et al.* introduced electron withdrawing groups to reduce the generation of TICT [15,16]. Rhodamine dyes, especially the balance between ring-closed non-fluorescent lactones and ring-opened fluorescent zwitterions/cations, are sensitive to the effects of aggregation at high concentrations and other environmental factors, which can cause spectral changes [17,18]. Due to the different test conditions such as concentration (other factors include polarity and pH, *etc.*), the spectral data of different structures of rhodamines can often not be reasonably compared. Even for rhodamine dyes of the same structure, the spectral properties reported by different research groups will be very different. For example, the quantum yield of

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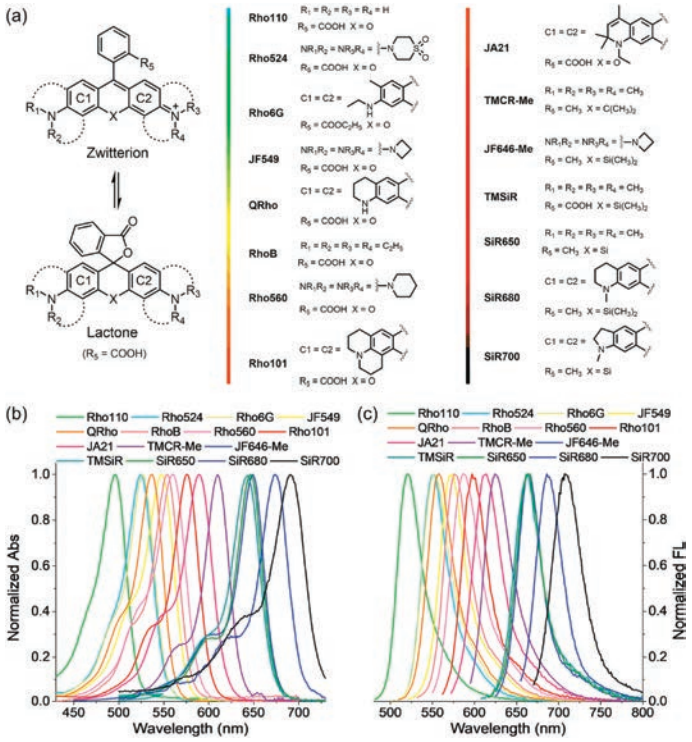

Fig. 1. (a) Rhodamine dyes studies in this work. (b) Normalized absorption and (c) emission spectra of these rhodamine dyes.

rhodamine 6G (**Rho6G**) was reported by different authors as 0.95 and 0.48, respectively [19,20], while that of **Rho560** (Fig. 1) was reported to be 0.1 or 0.06 [14,16]. Rapid evolution of fluorescence imaging techniques in recent years demands fluorescent dyes with enhanced brightness and photostability. And at the same time, the accurate spectral data, including absorbance, quantum yield, absorption and emission wavelength, *etc*., are required to ensure the performance of single-molecule positioning, the accuracy of single-molecule level fluorescence sensing, the feasibility of multi-color imaging, the comparability of different rhodamine spectral data, and the study of structure-fluorescence relationship of rhodamine fluorophore [21,22].

In order to obtain accurate and comparable data, here we tested the spectra of fifteen rhodamine dyes under the same test conditions as shown in Fig. 1. The emission wavelengths of these dyes covered the visible and near-infrared regions and the HPLC

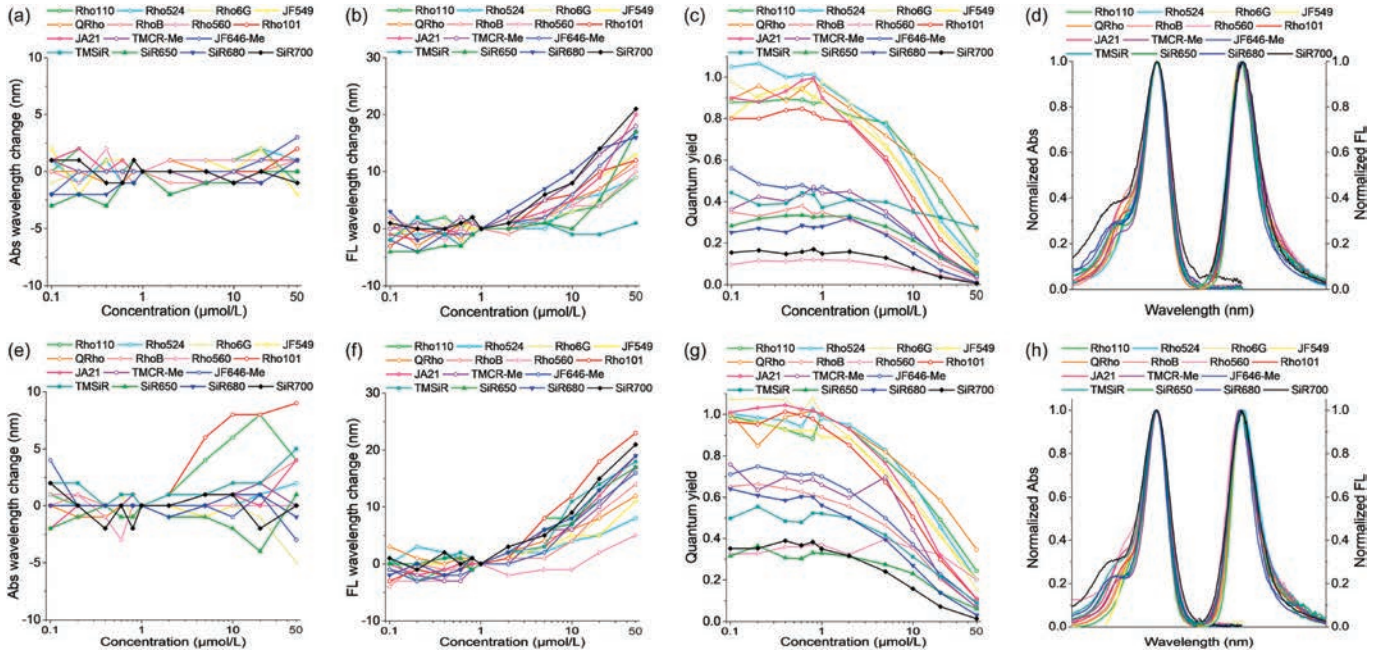

Fig. 2. The concentration dependence of the spectra of 15 rhodamine dyes in PBS (a-d) and ethanol solutions (e-h). (a, e) Absorption wavelength changes with different dye concentrations ranging from 0.1 μmol/L to 50 μmol/L. (b, f) Emission wavelength changes with different dye concentrations ranging from 0.1 μmol/L to 50 μmol/L. (c, g) Quantum yield changes with different dye concentrations ranging from 0.1 μmol/L to 50 μmol/L. (d, h) Normalized absorption and emission profile of these 15 dyes.

purity of these dyes was all above 97% (Figs. S1–S14 in Supporting information). The absorption and emission spectra of 11 different concentrations ranging from 0.1 μmol/L to 50 μmol/L were detected for each dye. Absolute fluorescence quantum yields for all dyes were measured using a Quantaurus-QY spectrometer. Considering the balance between ring-closed non-fluorescent lactones and ring-opened fluorescent zwitterions/cations in rhodamine, we added 1‰ trifluoroacetic acid (v/v) to the ethanol solution to get zwitterionic rhodamines. In the PBS buffer solutions (pH 7.4), rhodamines mainly exist as zwitterions.

Fig. 2 and Figs. S15–S46 (Supporting information) showed the concentration dependence of the spectra of 15 rhodamine dyes in PBS and ethanol solutions. The emission wavelength of all rhodamine dyes gradually red-shifted as the concentration increased (Figs. 2b and f), while the absorption wavelength remained unchanged (Figs. 2a and e). As shown in Figs. 2b and f, the better the planarity of the fluorophore, the more obvious the red shift of the emission wavelength, such as **Rho101**, **JA21**, **SiR680** and **SiR700**. The fluorescence quantum yields were most affected by the dye concentration (Figs. 2c and g). As the concentration increased, the quantum yield of each dye dropped sharply. All the above results should be ascribed to the inner filter effect (IFE) and excimer formation of rhodamine with the increase of concentration, which both red-shifted and quenched the emission. We changed the light absorption path length form 1 cm to 0.25 cm, and found that the rate of decrease in quantum yield had slowed which proved the existence of IFE (Fig. S47 in Supporting information). However, the shortening of the light path did not change the tendency of wavelength redshift (Fig. S48 in Supporting information), and there was still a strong decrease in quantum yield even at a concentration as low as 2 μmol/L. Considering that there was no change in the absorption wavelength of rhodamine at different concentrations, we guessed that rhodamine in the excited state, due to the increase in charge separation, would interact with rhodamine molecules in the ground state. That may be the generation of excimer. Many crystal structures had proved that rhodamine molecules can interact as a dimer through the π-π interaction between xanthene planes, while the two meso-substituted benzoic acids were oriented in the opposite direction. Most importantly, we found that these 15 rhodamine dyes all have relatively large changes in their spectra when the concentration was greater than 1 μmol/L, while the spectra remained the same when the concentration was less than 1 μmol/L. Therefore, the formation of rhodamine excimer at 1 μmol/L was considered negligible, so 1 μmol/L was recommended to be a reasonable concentration for detecting rhodamine optical spectra. It deserved to mention that the absorbance of 0.05 to 0.10 was the guideline to avoid IFE. And it happens that 1 μmol/L was a good choice for rhodamines. For other compounds with different molar extinction coefficients, other concentrations might be a better choice. In addition, it should be pointed out that the sensitivity of fluorescence detection is higher than that of absorption detection. In order to reduce the interaction between fluorescent molecules and IFE, while ensuring that absorption can follow Beer Lambert's law, fluorescence detection can be performed at a relatively lower concentration, while the absorption detection can be sought at higher concentrations. For rhodamines, fluorescence detection is recommended to be carried out at 1 μmol/L or less, and absorption can be performed at higher concentrations.

Table 1 showed the spectral data of these 15 rhodamine dyes at a concentration of 1 μmol/L in PBS (pH 7.4) and ethanol solutions. Spectral data in the literature was also shown [14,16,19,20,23–28]. Since the detection conditions were exactly the same, the comparison between these data would have better reliability,

Table 1
Optical properties of these 15 rhodamine dyes.

Dye	Solvent	λ_{abs} (nm)		λ_{em} (nm)		ε (L mol^{-1} cm^{-1})		Φ		Ref.
		exp	ref	exp	ref	exp	ref	exp	ref	
Rho110	PBS	495	497	520	520	78,000	76,000	0.88	0.88	[14]
	EtOH[a]	501	–	522	–	82,000	–	1.00	–	
Rho524	PBS	524	524	550	555	90,000	90,000	0.97	0.99	[16]
	EtOH[a]	533	–	555	–	100,000	–	0.98	–	
Rho6G	PBS	526	531	552	548	90,000	106,000	0.95	0.48	[20]
	EtOH	530	–	551	–	112,000	–	1.00	0.91	[28]
JF549	PBS	549	549	570	571	99,000	101,000	0.88	0.88	[14]
	EtOH[a]	538	–	561	–	115,000	–	0.89	–	
QRho	PBS	536	538	557	559	99,000	84,000	0.94	0.95	[26]
	EtOH[a]	533	–	552	–	115,000	–	1.00	–	
RhoB	PBS	554	553	577	580	111,000	105,000	0.35	0.31	[16]
	EtOH[a]	543	–	566	–	115,000	–	0.60	–	
Rho560	PBS	559	560	587	586	97,000	80,000	0.12	0.10	[14]
	EtOH[a]	549	–	577	–	132,000	–	0.37	–	
Rho101	PBS	576	580	597	600	108,000	116,000	0.80	0.30	[20]
	EtOH[a]	565	–	587	–	105,000	–	0.94	0.92	[28]
JA21	PBS	589	599	613	623	104,000	–	0.90	–	[25]
	EtOH[a]	577	–	600	–	118,000	–	1.00	–	
TMCR-Me	PBS	610	612	624	–	105,000	–	0.44	–	
	EtOH	612	–	629	–	118,000	–	0.66	–	
JF646-Me	PBS	649	649	664	663	119,000	–	0.47	0.47	[23]
	EtOH	653	–	668	–	142,000	–	0.70	–	
TMSiR	PBS	649	643	664	662	–[b]	118,000	0.41	0.41	[14]
	EtOH[a]	643	–	666	–	138,000	141,000	0.52	–	
SiR650	PBS	648	646	664	660	104,000	110,000	0.33	0.31	[24]
	EtOH	651	–	665	–	116,000	–	0.54	–	
SiR680	PBS	674	674	686	689	122,000	130,000	0.28	0.35	[24]
	EtOH	676	–	689	–	127,000	–	0.56	–	
SiR700	PBS	691	691	707	712	93,000	100,000	0.15	0.12	[24]
	EtOH	694	–	710	–	115,000	–	0.35	–	

Solvent: PBS (10 mmol/L, pH 7.4) and EtOH. [Dye] = 1 μmol/L. Temperature: 25 ± 0.2 °C.
[a] Add 1‰ trifluoroacetic acid (v/v).
[b] TMSiR existed in lactone form (over 90%) in PBS (pH 7.4), so ε was not given.

which could help to further understand the structure-fluorescence relationship of rhodamine dyes. By comparison, it can be clearly seen that TICT would strongly quench the fluorescence. **RhoB**, **Rho560**, **TMCR-Me** and **TMSiR** that can form an excited TICT state have a yield of less than 0.35. For near-infrared dyes, even if the formation of TICT was suppressed, the quantum yield can only be improved appropriately. For example, the quantum yield of **JF646-Me** was 0.47, but the quantum yield of short-wavelength dyes will not increase to about 0.9 (0.88 for **JF549**). The longer the fluorescence wavelength of the dye, the lower the quantum yield. These dyes have higher absorbance and fluorescence quantum yield in ethanol than in water, which is probably due to the solubility of the dye in different solvents and the hydrogen bonding between water and dye.

We further normalized the absorption and emission spectra of these 15 rhodamine dyes, and found that the absorption and emission profiles of these dyes completely overlap, except that **RhoB** and **SiR700** have higher shoulder absorption (Figs. 2d and h). This peak absorption was previously thought to be caused by H-aggregation of the dye. However, through the comparison and analysis of the spectra of different dyes above, we suspected that this peak absorption was likely to be caused by the influence of different substituents in the conjugated system.

In conclusion, the absorption and fluorescence spectra of 15 rhodamine dyes covering the visible to near-infrared regions were tested under exactly the same conditions, and the concentration-dependent spectral changes were obtained. It was further determined that 1 μmol/L was reasonable concentration for detecting rhodamine spectroscopy to exclude molecular aggregation interference. We hope that the data obtained in this paper can be used as reference for rhodamine spectra (especially quantum yield), and provide a reference for understanding the structure-fluorescence relationship of rhodamine and developing rhodamine dyes with better performance.

Declaration of competing interest

The authors report no declarations of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.cclet.2021.02.003.

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