Rhodamine-Triazine Based Probes for Cu^{2+} in Aqueous Media and Living Cells

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Abstract The performance of a number of rhodamine-triazine derivatives（probe **R1~R4)** which utilize rhodamine as the fluorophore with cyanuric chloride as the molecular platform have been evaluated. Spectroscopic analysis revealed that differing structural substitution patterns of the probe resulted in different sensitivity and selectivity for specific metal ions. The probes **R1** and **R2** were fluorescent/colorimetric probes for Cu^{2+}, whilst **R3** and **R4** were probes for Al^{3+}, Cr^{3+} and Fe^{3+}. The probe **R2** exhibited superior recognition for Cu^{2+} in neutral aqueous medium, and the optical switching behavior of **R2** for Cu^{2+} and S^{2-} could be used to construct a molecular logic gate. In addition, fluorescence imaging of probe **R2** for Cu^{2+} in living cells was demonstrated.

Keywords Rhodamine-triazine derivative, Probe, Cu^{2+}, Cell imaging

1. Introduction

Fluorescent and colorimetric probes of environmental and biologically relevant ions have been actively investigated in recent years [1-4]. Heavy metals and transition metal ions can be toxic at high concentrations and can disrupt normal cell function, and thus have a big impact on the environment as well as on human health [5-7]. In particular, copper plays a significant role in living systems and has an extremely ecotoxicological impact on human health as a catalytic cofactor in a variety of metalloenzymes [8, 9] and can hamper the self-purification capability of the sea and/or rivers and can destroy biological reprocessing systems [10]. Moreover, excessive amounts of copper ions in cellular homeostasis were reported to be connected with serious

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neurodegenerative diseases [11-13]. Due to the Janus-faced properties of copper in organisms, facile techniques for monitoring the Cu^{2+} ion in environmental samples and the ability to visualize cellular distribution in physiological processes are extremely useful. Examples of probes for Cu^{2+} have been reported for biological and environmental uses [14-17], but only a few probes with applications in neutral aqueous media have been reported [18, 19]. In addition, fluorescence bio-imaging technology by virtue of its high sensitivity, high-speed spatial analysis and minimal cell damage is of interest [20, 21]. It is necessary to development probes for analyzing Cu^{2+} in varying types of sample matrix and over a variety of concentration ranges. Rhodamine-based probes are ideal for detection and imaging [22, 23], and studying such probes with a view to enhancing the performance has always been the goal of researchers, and a number of different research strategies are ongoing [24-28]. In our previous research, a series of rhodamine fluorescent probes based on tripodal ligands [29], calixarenes [30] and Schiff bases [31] have been synthesized, and good detection performances were exhibited. There are still challenges and opportunities remaining for developing rhodamine-base probes including systems with structural novelty, molecular diversity, and practical applications in biological systems. Taking advantage of rhodamine as the fluorophore and the excellent reactivity properties of cyanuric chloride [32, 33], a series of rhodamine-triazine derivatives (**R1**~**R4**, Scheme 1) have been synthesized by substitution of the active chlorine of cyanuric chloride via a step-by-step reaction with rhodamine hydrazide or rhodamine ethylenediamine [34]. Herein, the performance of these rhodamine-triazine derivatives as probes has been studied. Specifically, probe **R2** was found to be a Cu^{2+}-selective probe in neutral aqueous media, and was successfully employed in the fluorescence imaging of living cells. Furthermore, the optical switching behavior of **R2** for Cu^{2+} and S^{2-} was investigated; molecular logic gates using fluorescence or color changes of **R2**-Cu^{2+} as outputs and S^{2-} as inputs were designed.

Scheme 1. The structures of the probes (**R1**~ **R4**)

2. Experimental methods

2.1. Apparatus and reagents

Fluorescence spectroscopy measurements were performed on a Cary Eclipse fluorescence

spectrophotometer (Varian) equipped with a xenon discharge lamp using a 1 cm quartz cell. UV-vis spectra were recorded on a UV-1800 spectrophotometer (shimadzu). IR spectra were obtained using a Vertex 70 FT-IR spectrometer (Bruker). ^{1}H NMR spectra were measured with Nova-400 NMR spectrometers (Varian) using TMS as an internal standard. The cell imaging test was carried out with a Nikon eclipse Ti-U inverted fluorescence microscopy. All of the above measurements were operated at room temperature (298K).

The solutions of the metal ions were prepared from their nitrate salts (Aldrich and Alfa Aesar Chemical Co., Ltd.). All of the chemicals are of analytical grade and were used without further purification. Doubly-distilled water was used in the experiments.

Stock solutions (1.00×10^{-3} M) of the probes were prepared in a 10 mL volumetric flask with 6.05 mg probe **R1** or (7.43 mg, **R2**, 6.34 mg **R3**, 7.71 mg **R4**) dissolved in CH_3CN, respectively, then diluting to the mark with CH_3CN-H_2O (v/v, 1/1, for **R1** and **R3**, 3/7, for **R2** and **R4**, 20 *m*M Tris-HCl, pH 7.0)

A Cu^{2+} stock solution (2.00×10^{-3} M) was prepared in a 100 mL volumetric flask by dissolving 48.4 mg $Cu(NO_3)_2\cdot3H_2O$ in 20 mL water, and then diluted to the mark with water. The ^{1}H NMR experiment was conducted using copper perchlorate hexa-hydrate $Cu(ClO_4)_2\cdot6H_2O$ dissolved in CD_3CN. Solutions of other metal ions were likewise prepared at 2.00×10^{-3} M in water.

Tris-HCl buffer stock solution of differing pH (20 *m*M) were prepared from 40 mM Tris and the appropriate amount of HCl, under adjustment by a pH meter.

2.2 Analytical procedure

Stock solutions of probes (1.00×10^{-3} M) were further diluted to prepare 1.00×10^{-4} M solutions respectively in CH_3CN/H_2O (v/v, 1/1, for **R1** and **R3**, 3/7, for **R2** and **R4**, 20 *m*M Tris-HCl, pH 7). To 1.0 mL aliquots of the diluted probe solutions in 10 mL volumetric flasks were respectively added 9.0 mL buffered CH_3CN/H_2O containing different concentrations of metal ions, affording an overall probe concentration of 10 μM for each experiment. The solutions were mixed at room temperature (298K) for 2 h, after which 3.0 mL of each solution was transferred to a 1 cm quartz cell for the required measurement using either a UV-vis or fluorescence spectrophotometer. For the determination of Cu^{2+} at low concentrations, Cu^{2+} stock solutions of 1.00×10^{-4} M and 1.00×10^{-5} M were prepared by diluting the original stock solution, and the required amount of

these solutions was used in the analytical procedure.

2.3 Application of R2 for Cu^{2+} analysis in water samples

Samples of tap water and river water were respectively analyzed by the fluorescence and absorption methods reported in this study. To control the pH value in the detection, 9 mL of Tris-HCl buffered CH_3CN/H_2O (3/7, v/v) containing **R2** was added to 1 mL of the water samples to keep the pH at about 7, and then the fluorescence intensity and absorption changes were determined.

2.4 Cell incubation and imaging

Hela cells were grown at the Roswell Park Memorial Institute (RPMI-1640), the medium supplemented with 10 % bovine serum, 100 $U \cdot mL^{-1}$ penicillin and 100 $\mu g \cdot mL^{-1}$ streptomycin at 310 K and 5 % CO_2. Before staining, the cells were washed twice with fresh RPMI-1640, and subsequently exposed to the 10 μM **R2** solution (900 μL RPMI-1640 added with 100 μL of a 100 μM **R2** in DMSO) for 60 min at ambient temperature. After washing twice with fresh RPMI-1640, the cells were immersed for 30 min with 50 μM Cu^{2+} solution (900 μL RPMI-1640 added with 100 μL of a 500 μM Cu^{2+} in H_2O), the RPMI-1640 was removed, and the cells were washed twice with fresh RPMI-1640 and imaged.

3. Results and discussion

3.1 Fluorescence and UV-vis spectral behavior of R1~R4

In the different aqueous/acetonitrile medium (CH_3CN/H_2O, v/v, 1/1 for **R1** and **R3**, 3/7 for **R2** and **R4**) using a pH of about 7 via Tris-HCl (20 *m*M) buffer solution, the four probes **R1**~**R4** (10 μM) were colorless and exhibited no fluorescence-emission. Addition of Cu^{2+} led **R1** (Figure 1a, 1b) and **R2** (Figure 1c, 1d) to fluorescence and absorbance with remarkable enhancements. Meanwhile, orange fluorescence was observed under ultraviolet light (Figure 1a, 1c inset), and the solution turned to pink (Figure 1b, 1d inset), whereas there was no change in the presence of other common metal ions. The probe **R1** exhibited a similar performance except that the absorption and fluorescence observed were weaker upon addition of Fe^{3+} and Al^{3+}. **R3** and **R4** exhibited varying degrees of response to Al^{3+}, Cr^{3+} and Fe^{3+} ions (Figure 1e-1h), accompanied by different color changes under ultraviolet or visible light (Figure 1e~h, inset). From the above, it is clear that

R1~**R4** can recognize specific metal ions, whilst the optical changes (Figure S1, Supporting Information) of **R2** displayed a somewhat superior selectivity and sensitivity than did the other probes. **R1** and **R2** were obtained through rhodamine B hydrazide substitution of an active chlorine atom in the triazine rings, whilst **R3** and **R4** were obtained via rhodamine B ethylenediamine; the steric hindrance arising from connecting the two groups perhaps led to differences in interaction with the divalent metal ion Cu^{2+} and the trivalent Al^{3+}, Cr^{3+} and Fe^{3+} ions, leading to these metal-specific probes. In contrast to **R1** and **R3**, in the molecular structures of **R2** and **R4**, the two active chlorine atoms in the triazine rings were substituted by diethanolamine. The enhanced solubility in water was due to the introduction of hydroxyl groups. The probes of different molecular structure displayed different responsive behavior in the presence of different metal ions. In the above described probes, **R2** exhibited high sensitivity and selectivity for Cu^{2+}, as well as good solubility in water.

Figure 1. Fluorescence and absorption spectra of the probes (10 μM) **R1** (a, c), **R2** (b, d), **R3** (e, f) and **R4** (g, h) in the presence of various metal ions (200 μM). 20 *m*M Tris-HCl buffer solution, pH 7, **R1**: CH_3CN/H_2O, 1/1 , v/v, $\lambda_{ex}/\lambda_{em}$ = 557/583 nm, λ_{max} = 557 nm; **R2**: CH_3CN/H_2O, 3/7, v/v, $\lambda_{ex}/\lambda_{em}$ = 553/580 nm, λ_{max} = 553 nm; **R3**: CH_3CN/H_2O, 1/1, v/v, $\lambda_{ex}/\lambda_{em}$ = 557/578 nm, λ_{max} = 557nm; **R4**: CH_3CN/H_2O, 3/7, v/v, $\lambda_{ex}/\lambda_{em}$ = 563/585 nm, λ_{max} = 563nm.

3.2. Spectral characteristics of probe R2

The stability of a probe under physiological conditions is a prerequisite for application, especially for live cell imaging. The effect of the water fraction and the pH on the spectral responses of **R2** was therefore evaluated. The fluorescence and absorbance of the **R2**-Cu^{2+} solution exhibited stable and considerably higher intensity over the range 20-80 % H_2O in CH_3CN/H_2O mixed solvent (Figure S2, Supporting Information). The acid titration experiments revealed that the fluorescence and absorbance of **R2**-Cu^{2+} remained unaffected over the range 3-7.5 pH (Figure S3, Supporting Information) and was stable under near physiological conditions.

The spectral characteristics of **R2** in acetonitrile/aqueous solution (CH_3CN/H_2O, 3/7, v/v, 20 *m*M Tris-HCl, and pH 7) are illustrated herein (Figure 1c and 1d). The solution of **R2** emitted no fluorescent or absorbance due to the five-membered spirolactam structure [35], however upon addition of Cu^{2+}, a drastic fluorescence enhancement (about 360-fold, at 580 nm) and significant absorbance enhancement (about 445-fold, at 553 nm) were observed. The fluorescence response

of **R2** was in agreement with the ring opening mechanism, which was attributed to the Cu^{2+} induced opening of the rhodamine ring. By contrast, the other tested metal ions caused neither a color nor a spectral change under identical conditions. This unique property enables Cu^{2+} to be distinguished easily from the other tested ions either directly by the naked eye or by spectral measurements.

The fluorescence titration of probe **R2** was investigated, and it was found that the addition of amounts of Cu^{2+} caused an increase in the band at 580 nm, which reached a steady state after adding 10 equivalents of Cu^{2+} (Figure S4a, Supporting Information). Similarly, upon the gradual addition of up to 20 equivalents of Cu^{2+}, a new absorption band, centered at 553 nm, appeared with increasing absorbance (Figure S4b Supporting Information), and this is attributed to the spirolactam ring-opening process of rhodamine B unit in probe **R2**.

The effect of co-existing ions was studied including the abundant cellular cations (Na^{+}, K^{+}, Mg^{2+} and Ca^{2+}), the essential transition metal ions (Fe^{3+}, Al^{3+}, Zn^{2+}, Mn^{2+}, Co^{2+}, and Ni^{2+}), and the environmentally relevant metal ions (Ag^{+}, Sr^{2+}, Ba^{2+}, Hg^{2+}, Pb^{2+}, Cr^{3+}, and Cd^{2+}). The spectral responses of probe **R2** (10 μM) to the above metal ions are shown in (Figure S5, Supporting Information); only Cu^{2+} (100 μM) induced a significant enhancement in the fluorescence and absorption, whilst the other mentioned metal ions (200 μM) did not cause any discernible changes to **R2**. There were minor variations in the fluorescence intensity or absorbance of the **R2**-Cu^{2+} mixture when adding the above metal ions; the relative standard deviations were less than 5 %. These results illustrate that the binding of the Cu^{2+} ion by the probe was not significantly influenced by the presence of the co-existing ions. Given this, the selectivity of **R2** for the Cu^{2+} ion can be said to be remarkably high and meets the requirements for biomedical and environmental applications.

3.3 The recognition behavior of probe R2 for Cu^{2+}

To investigate the complexation of probe **R2** with Cu^{2+}, the method of continuous variations (Job's plot) was used for **R2**-Cu^{2+} in CH_3CN/H_2O solution. The formation of a 1:1 stoichiometry between **R2** and Cu^{2+} was clearly suggested by both fluorescence and absorption spectrometric methods (Figure S6, Supporting Information). The binding constant for the **R2**-Cu^{2+} complex was estimated to be 3.83×10^4 M^{-1} and 3.71×10^4 M^{-1} by the Benesi-Hildebrand method [36],

respectively (Figure S7, Supporting Information). The IR spectra of **R2** and **R2**-Cu^{2+} mixtures were measured in acetonitrile medium, and revealed that the amide carbonyl peak (1700 cm^{-1}) and the C=N peak (1615 cm^{-1}) of **R2** had disappeared on combination with Cu^{2+}, indicating the participation of the carbonyl and C=N moieties in the complexation (Figure S9, Supporting Information). The ^1H NMR spectra of **R2** upon complexation with Cu^{2+} were measured (Figure 2). Upon addition of Cu^{2+}, the proton peak shapes of **R2** significantly broadened, and the protons on the rhodamine moiety shifted downfield (δ 0.04-0.13 ppm), Ha, Hb, Hc, Hd, He, Hf Hg and Hh (δ 0.11, 0.1, 0.13, 0.051, 0.072, 0.038, 0.04, and 0.08 ppm, respectively). This was due to the decrease in the electron density of the rhodamine moiety upon coordination of the Cu^{2+} ions to the amide carbonyl group of **R2**, similar to reports on the ion-induction rhodamine based fluorescent probes [31, 37]. The ESI mass spectra of **R2** and **R2**-Cu^{2+} were obtained (Supporting Information, Figure S10). The peak at m/z=807.2 (calcd=807.05) corresponded to [**R**2+Cu]$^+$, and 743.2 (calcd=743.5) corresponded to [**R2**]$^+$ and were clearly observed when Cu^{2+} was added to **R2**. The results clearly indicate the 1:1 complexation between **R2** and Cu^{2+} complex. The proposed bound structure is illustrated (Scheme 2).

Figure 2. Partial ^1H NMR spectra of **R2** and **R2**-Cu^{2+} measured in $CDCl_3/CD_3CN$ (1/4, v/v). (a) **R2**, (b) **R2** with Cu^{2+}.

Scheme 2. The proposed bound structure of **R2**-Cu^{2+}

3.4 Logic gate

Since the pioneering work reported by de Silva *et al* [38], remarkable progress has been made in the development of molecular logic gates. Various molecular logic gates have been explored by utilizing fluorescent signals as outputs and chemically encoded information (such as pH, temperature, light, and ion) as inputs [39-41]. The reversibility of probe **R2** was investigated by the introduction of S^{2-} as a reagent. Upon addition of excess amounts of S^{2-} to the **R2**-Cu^{2+} complex solution, the fluorescent emission intensity was quenched (Figure 3) and the color of the latter changed from pink to colorless (Figure 3, inset), indicating that S^{2-} replaced **R2** to coordinate with Cu^{2+} to form the more stable CuS. This behavior could be analyzed with a combinational logic circuit using Cu^{2+} and S^{2-} in response to a particular input, designated as input In1 (Cu^{2+}) and

In2 (S^{2-}), respectively, and considered as "1" when they are present and "0" if they are absent. Binary digits (1 or 0) can be used to represent the two states "on or off" for each signal. The output signals were emission of strong fluorescent signal or color changes. Input 1 led to fluorescence or absorbance of the probe **R2** (enhancement in its occupied state), equivalent to a YES operation. The interaction of input 2 with **R2** led to fluorescence quenching or an absorbance decrease, thereby implementing the NOT gate. **R2** acts in parallel on the spectrum output signals, which implements the required AND function. In the presence of both inputs, the quenching (by input 2) overrides the fluorescence or absorbance enhancement by input 1, in accordance with the truth table (Figure 4 Table), and this constitutes an example of a flexible functional integrated INHIBIT logic at the molecular level.

Figure 3. Fluorescence spectrum of probe **R2** (10 μM) in the presence of Cu^{2+} (50 μM) and S^{2-} (200 μM) in CH_3CN/H_2O (3/7, v/v, 20 mM Tris-HCl buffer solution, pH 7), truth table, and logic scheme (Inset, A: **R2**-Cu^{2+}. B: **R2**-Cu^{2+}-S^{2-}), λ_{ex} /λ_{em} = 553/580 nm

3.5 Detection and imaging in living cells

According to the fluorescence and UV-vis titration experiments on Cu^{2+} with probe **R2**, the intensity enhancements were found to be proportional to the concentration of Cu^{2+} ion over the ranges 4.0×10^{-6} ~ 5.0×10^{-5} M and 6.0×10^{-6} ~ 7.0×10^{-5} M, respectively (Figure S8, Supporting information). The detection limits for Cu^{2+} were determined to be 8.4×10^{-7} M and 2.45×10^{-6} M respectively in the fluorescence and UV-vis spectra [42]. The fluorescence quantum yield (Φ, 0.56) was calculated using rhodamine-B (Φ, 0.89) as a reference [43].

The practical applications of **R2** were firstly evaluated by detection of Cu^{2+} in water samples (tap and river water) under the optimized conditions (10 μM **R2** in CH_3CN/H_2O, 3/7, v/v, buffered by 20 *m*M Tris-HCl at pH 7). All the measurements were performed three times. The results are summarized (Table 1, Supporting information), which showed satisfactory recovery and R.S.D. values for all of the samples. These results demonstrated that the proposed probe **R2** meets the monitoring sensitivity as well as selectivity requirements necessary for environmental water samples.

To demonstrate the membrane permeability of the probe and its feasibility to specifically detect Cu^{2+} ions in living cells, the behavior of probe **R2** toward Cu^{2+} in living HeLa cells was

investigated by fluorescence microscopy. HeLa cells were incubated with **R2** (10 μM) for 60 min at 310 K followed by the addition of Cu^{2+} (50 μM) and incubated for 30 min. The cells were washed with phosphate-buffered saline (PBS) solution, and their fluorescence images were recorded before and after the addition of Cu^{2+}. The brightfield image of HeLa cells labelled with probe **R2 (**10 μM) after 60 min of incubation at 310 K (Figure 4a), as determined by fluorescence microscopy, revealed that these cells exhibited no detectable fluorescence signal (Figure 4b). In contrast, when the cells were incubated with probe **R2** and further treated with Cu^{2+} (50 μM) in the growth medium for 30 min under the same conditions, bright fluorescence from the intracellular region was detected (Figure 4c). Fluorescence imaging of the **R2**-labelled cell displayed good biological characteristics, demonstrating that **R2** was cell membrane permeable. The results clearly established that probe **R2** might be an effective tool for visualizing Cu^{2+} in living cells.

Figure 4. (a) Bright-field image of HeLa cells labelled with probe **R2** (10 μM) after 60 min of incubation, (b) Fluorescence image of cells after 60 min treatment with probe **R2**, (c) Fluorescence image of cells that are further incubated with 50 μM Cu^{2+} for 30 min.

4. Conclusions

The performance of the rhodamine-triazine based systems **R1**~**R4**, which possess subtle variations in their molecular structure, as probes was evaluated. The length of the carbon chain which connects the rhodamine and the triazine groups leads to different responses to specific ions. The sensitivity and selectivity depends mostly on the chain length of the linking groups and the amine-modified aromatic ring of the probe molecules. Complexation of the probe with metal ions could be monitored through fluorescence enhancement, as well as color changes, which facilitates "naked-eye" detection. Unlike probes **R3** and **R4** that are affected by interference from competing metal ions, the probe **R1** and **R2** showed remarkably specific discrimination for Cu^{2+} over the other metal ions in neutral aqueous medium; the water-solubility of **R2** is slightly better than that of **R1**. This allows for the accurate quantification of trace Cu^{2+} in environmental water samples. The properties of **R2** and others literature probes derived from rhodamine are listed in table S2 (see Supporting Information). When compared to the previously studied probes, **R2** has better water-solubility, higher sensitivity and fluorescence quantum yields, lower detection limits and a

wider linear range. Also, **R2** can be used as a molecular switch via fluorescence emission/ color change controlled by cationic (Cu^{2+}) and anionic (S^{2-}) sequential input. Living cell imaging suggested that **R2** was membrane-permeable and primarily non-toxic to cell culture, which demonstrated its applicability for the determination of Cu^{2+} in living cells. These observations on the rhodamine-triazine probe using cyanuric chloride suggest that such systems can be developed further for the construction of novel probes for transition metal ions.

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