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ORIGINAL PAPER

Sensitive Spectrofluorimetric Determination of Cytochrome c with Spirocyclic Rhodamine B Hydrazide in Micellar Medium

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Abstract A new spectrofluorimetric method for the determination of cytochrome c using spirocyclic rhodamine B hydrazide (RBH) as fluorogenic reagent in the presence of sodium dodecylbenzene sulfonate (SDBS) surfactant micelles was developed. The method was based on the reaction of cytochrome c with RBH, a colorless, nonfluorescent spirolactam of rhodamine B in SDBS micelles to give highly fluorescent rhodamine B and hence led to a large increase in fluorescence intensity. The dynamic range and detection limit for the determination of cytochrome c are 4.0–120 ng ml^{-1} and 0.87 ng ml^{-1} (3σ), respectively. The optimal conditions for the detection of cytochrome c were evaluated and the possible detection mechanism was also discussed.

Keywords Cytochrome c · Rhodamine B hydrazide · SDBS · Spectrofluorimetry

Introduction

Cytochrome c is a water-soluble heme-containing protein that is localized in the intermembra ne space and loosely attached to the surface of the inner mitochondrial membrane. Cytochrome c is important to the process of creating cellular energy, the main function of mitochondria. It is also an essential component of the mitochondrial respiratory chain. Therefore, measurement of cytochrome c can lead to greater understanding of certain diseases on a cellular level.

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In recent years, several methods for cytochrome c assay have been reported, such as spectrophotometry [1, 2], electrochemistry [3–5], chemiluminescence [6], electrophoresis [7, 8], and high performance liquid chromatography [9, 10].

The rhodamine framework is an ideal mode to construct "off–on" fluorescent probe due to its particular structural property. As is well-known, rhodamine derivatives with spirolactam structure are colorless and nonfluorescent, whereas ring-opening of the spirolactam gives rise to a strong fluorescence emission. To date, several rhodamine-modified chemosensors and probes based on the spirocyclic ring-opening mechanism haven been developed [11–20]. However, most of these kinds of chemosensors or probes are designed for metal ions [11–18], and fluorescent probes for biological important molecules are scarcely reported [19, 20].

In recent studies, we observed that upon adding cytochrome c to RBH (the chemical structure was shown in Fig. 1), a colorless, non-fluorescent spirolactam of rhodamine B in sodium dodecylbenzene sulfonate (SDBS) surfactant micelles, both the pink color and the fluorescence characteristic of rhodamine B appeared instantly. Based on this phenomenon, a simple fluorimetric method for the determination of cytochrome c was developed. Compared with traditional methods for the detection of cytochrome c, the proposed method is simple, fast, selective and highly sensitive. Moreover, the longer excitation wavelength of rhodamine fluorophore makes it possible avoids the background fluorescence caused by biological substances when applied to the cytochrome c determination in biological fluids. Thus we believe the present method can be applicable for cytochrome c assay in a variety of systems.

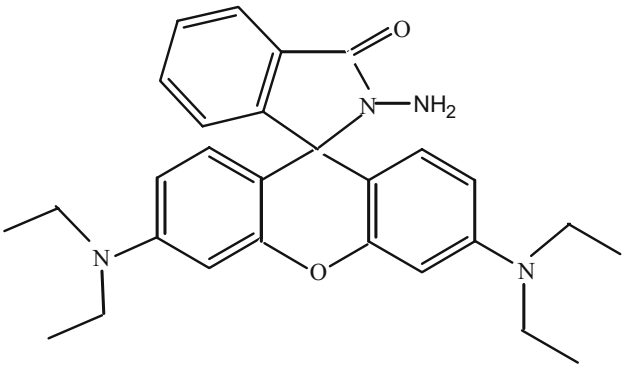

Fig. 1 Chemical structure of RBH

Experimental

Apparatus

The fluorescence spectra and relative fluorescence intensity were measured with a Sanco CRT-970 spectrofluorimeter (Shanghai, China) with a 10 mm quartz cuvette. The excitation and emission wavelength bandpasses were both set at 10 nm. Unless specific noted, the sensitivity of the spectrofluorimeter was set at "2". The absorption spectra were recorded with a Shimadzu UV-1700 spectrophotometer. The pH was measured with a Model pHs-3B meter (Shanghai, China). Mass spectrometry was obtained with AXIMACFR plus MALDI-TOF Mass Spectrometer.

Reagents

Stock solution of cytochrome c (0.4 mg ml^{-1}) was prepared by dissolving 10.0 mg of cytochrome c (Sigma) in a 25-ml brown flask. The stock solution was stored in the refrigerator at 4°C. RBH solution (1.0 mmol l^{-1}) was prepared by dissolving 45.6 mg of RBH with 100 ml of DMF. RBH was synthesized according to the method reported in our previous work and was identified by MALDI-TOF mass spectrometry [20]. A SDBS solution (5.0 mmol l^{-1}) was prepared by dissolving 1.74 g of SDBS in 1,000 ml of water. A pH 4.0 of citric acid (0.1 mol l^{-1})–Na_2HPO_4 (0.2 mol l^{-1}) buffer and a pH 8.0 of NaH_2PO_4–Na_2HPO_4 (0.2 mol l^{-1}) were prepared, respectively. The sources of surfactants were as follows: Tween 80, Triton X-100 and sodium dodecyl sulfate (SDS) were obtained from Shanghai Chemical Reagent Co., SDBS and Cetytrimethylammonium bromide (CTAB) was obtained from Xi'an Chemical Reagent Co.

All the chemicals used were of analytical reagent grade and double distilled water was used for the preparation of all solutions and for all measurements.

Procedure

In a set of 10 ml volumetric tubes containing pH 4.0 of citric acid–phosphate buffer solution, 0.8 ml of 40.0 μmol l^{-1} RBH, 0.8 ml of 5.0 mmol l^{-1} SDBS and different concentrations of cytochrome c were added orderly. The reaction solution was kept at room temperature for 10 min, and then 3.0 ml of pH 8.0 phosphate buffer was added and the reaction mixture was diluted to the mark with water. The fluorescence intensity was recorded at $\lambda_{ex}/\lambda_{em}$=567/592 nm. The fluorescence increment of RBH-SDBS system by cytochrome c was represented as $\Delta F = F - F_0$, where F and F_0 are the fluorescence intensity of the system with and without cytochrome c, respectively.

Results and Discussion

Spectral characteristics

In contrast to its parent dye, RBH exists in a closed, spirocyclic form at neutral and basic pH. Therefore RBH was a colorless solution showing no absorption at visible spectra range. Upon incubation with cytochrome c in SDBS micellar medium, a pink color was observed. Figure 2 shows the absorption spectra upon addition of different concentration of cytochrome c to the RBH solution in the presence of SDBS. It can be seen that once the ring-opening reaction take place, a characteristic absorption at about 556 nm was appeared, which was agreed well with that of authentic rhodamine B at the same conditions. This

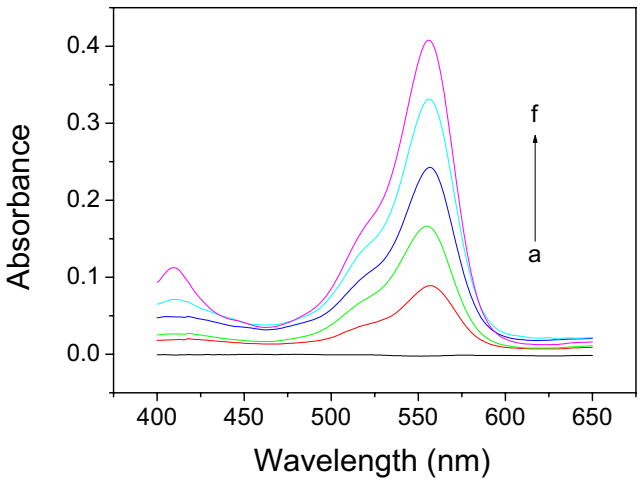

Fig. 2 Absorption spectra of RBH (20.0 μmol l^{-1}) in SBDS micelles (0.4 mmol l^{-1}) in the presence of different concentration of cytochrome c. Cytochrome concentration: (a), 0; (b), 1.9; (c) 3.8; (d) 5.6; (e), 7.5; (f), 15.0 μg ml^{-1}. The mixture of RBH with different concentrations of cytochrome c in SDBS surfactant solution were stand at pH 4.0 of citric acid-phosphate buffer solution for 10 min, and then the spectra were recorded at pH 8.0 of phosphate buffer solution

chromogenic indication reaction allows simple cytochrome c detection by using a conventional spectrophotometer. The fluorescence spectra of RBH in the presence of different concentrations of cytochrome c were measured (Fig. 3), a characteristic fluorescence emission maximum centered at about 589 nm was recorded and the fluorescence intensity was linear with the cytochrome c concentration.

Effect of reaction time

For better understanding of the proposed fluorogenic reaction, the time-dependent fluorescence intensity changes of RBH with cytochrome c in SDBS micellar medium was studied and the results were shown in Fig. 4. It can be observed that fluorescence signal of the system increased for a few minutes, and leveled off as the time continues, while the blank solution showed almost unchanged at the same conditions. The fluorescence intensity of the reaction solution reached its maximum value after 6 min, after which the fluorescence intensity of the detection system remained almost constant. Therefore, a 10-min reaction time was selected in subsequent experiments.

Effect of surfactants

The incorporations of various surfactants in the chromogenic or fluorogenic reaction system offered many advantages in comparison with those systems in the absence of surfactant, such as high sensitivity and high reaction speed [21, 22]. Accordingly, the effect of various surfactants was systematically investigated in the fluorogenic reaction

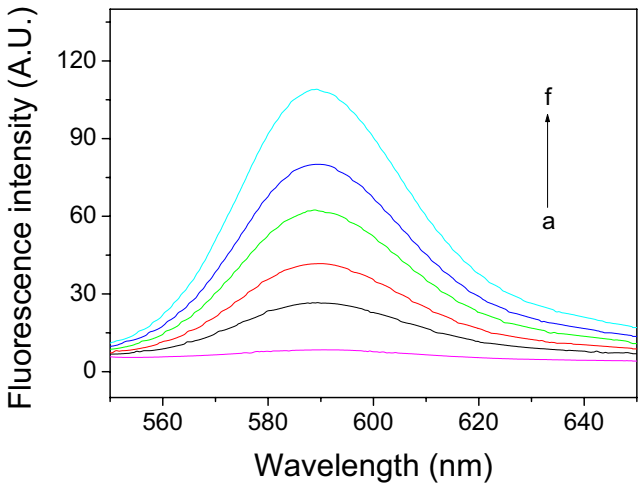

Fig. 3 Fluorescence spectra of RBH (3.2 μmol l^{-1}) in SBDS micelles (0.4 mmol l^{-1}) in the presence of different concentration of cytochrome c. Cytochrome c concentration: (a), 0; (b), 20; (c) 40; (d) 60; (e), 80; (f), 100 ng ml^{-1}. The mixture of RBH with different concentrations of cytochrome c in SDBS surfactant solution were stand at pH 4.0 of citric acid–phosphate buffer solution for 10 min, and then the spectra were recorded at pH 8.0 of phosphate buffer solution with excitation wavelength set at 520 nm

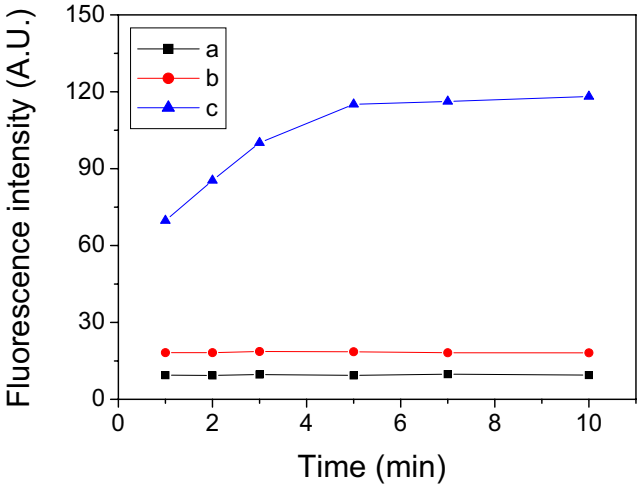

Fig. 4 Kinetic behavior of the systems. a, RBH+cytochrome c; b, RBH+SDBS; c, RBH+SDBS+cytochrome c. RBH, 3.2 μmol l^{-1}; SDBS, 0.4 mmol l^{-1}; Cytochrome c, 20 ng ml^{-1}. The fluorescence development reaction was carried out in pH 4.0 of citric acid-phosphate buffer solution for different time, then pH 8.0 of phosphate buffer solution was added and the fluorescence intensity of the system was measured

between RBH and cytochrome c. As can be seen from Table 1, SDBS as an anionic surfactant was most effective for the fluorogeinc reaction.

The effect of SDBS concentration on fluorescence increment (ΔF) of the reaction system was studied and the result was shown in Fig. 5. It could be seen that ΔF increased with an increase in SDBS at low concentration, it remained almost constant when SDBS concentration was in the range of 0.3–0.6 mmol l^{-1}, and then decreased when the concentration of SDBS was above 0.6 mmol l^{-1}. Thus, the SDBS concentration of 0.4 mmol l^{-1} was chosen for further research. The optimal SDBS concentration was close to the critical micelle concentration (CMC) of SDBS (CMC= 0.63 mmol l^{-1}) [23], indicating that micellar medium of SDBS was critical to the present fluorogenic reaction.

Effect of pH

The effect of pH on the fluorogenic reaction was studied over the range of 2.2–7.0. The results are shown in Fig. 6. It

Table 1 Effect of surfactant on the fluorescence signal of the system, where F and F_0 are the fluorescence signal of the system in the presence and absence of 20.0 ng ml^{-1} of cytochrome c, respectively

Surfactants	Concentration	F_0	F	$\Delta F = F - F_0$
–	–	7.2	11.3	4.1
SDBS	0.4 mmol l^{-1}	21.7	130.7	109
SDS	8.0 mmol l^{-1}	19.0	41.2	22.2
Triton X-100	0.24 mmol l^{-1}	27.8	34.1	6.3
CTAB	0.85 mmol l^{-1}	7.0	7.5	0.5
Tween 80	0.016 mg ml^{-1}	13.2	15.0	1.8

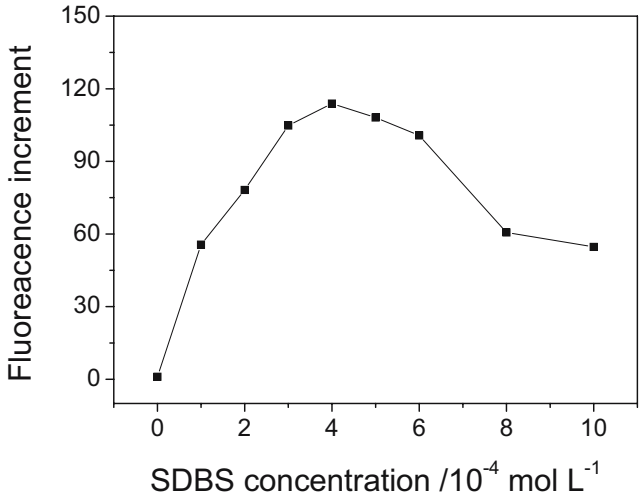

Fig. 5 Effect of SDBS concentration on the fluorescence intensity of the system. RBH, 3.2 μmol l^{-1}; Cytochrome c (20 ng ml^{-1}). The fluorogenic reaction was carried out in pH of 4.0 citric acid–phosphate buffer solution for 10 min, and then the fluorescence intensity was recorded at pH 8.0 of phosphate buffer solution

can be seen that ΔF increased with increasing pH up to 4.0, and above 4.0 a sharp decrease of fluorescence intensity was observed. Hence, pH 4.0 was chosen for the fluorogenic reaction in subsequent experiments.

The effect of pH on the fluorescence background of the detection system was also examined. As it has an amino group, RBH exists in its different forms over different pH range, therefore, changing pH will change its dominant present form in solution, which in turn changing the fluorescence background of the system. The effect of pH on the fluorescence background was examined, and the results were shown in Fig. 7. From Fig. 7, it can be seen that the minimum fluorescence background of the system

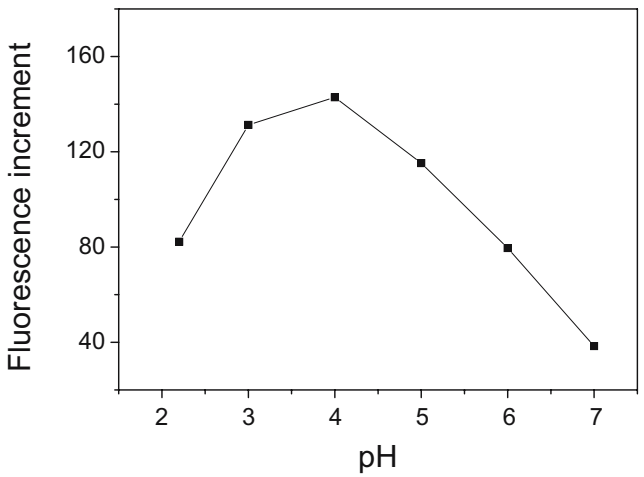

Fig. 6 Effect of pH on the fluorogenic reaction of the system. RBH, 3.2 μmol l^{-1}; SDBS, 0.4 mmol l^{-1}; Cytochrome c, 20 ng ml^{-1}. The fluorogenic reaction was carried out in different pH of citric acid–phosphate buffer solution for 10 min, and then the fluorescence intensity was recorded at pH 8.0 of phosphate buffer solution

was obtained at basic pH conditions. On the other hand, the fluorescent product generated in the reaction mixture has proved to be rhodamine B (see the following discussion) and its fluorescence intensity is stable above pH 4.0 [24]. Therefore, to obtain a low fluorescence background and an optimal condition for fluorescence measurement of rhodamine B generated in the reaction mixture at the same time, the detection pH of the reaction mixture was selected at 8.0.

Effect of RBH concentration

The effect of RBH concentration on ΔF of the system was investigated and the results were shown in Fig. 8. It can be seen that ΔF of the detection system was increased with increasing the concentration of RBH in the range of 0.8–8 μmol l^{-1} and remained almost unchanged when the concentration of RBH was above 3.2 μmol l^{-1}, therefore, 3.2 μmol l^{-1} of RBH was selected for the subsequent experiment.

Possible detection mechanism

The possible mechanism of the present method for the determination of cytochrome c was studied in detail. First of all, comparison of the fluorescence excitation and emission spectra of the reaction solution with that of authentic rhodamine B showed that they were identical, both having the excitation maximum at 564 nm and emission maximum at 589 nm, indicating that rhodamine B might be generared in the reaction solution. Secondly, the reaction solution was analyzed by mass spectrometry, and the MALDI-TOF mass spectrometry of the reaction mixture confirmed the suggested rhodamine B was produced by showing a prominent peak at m/z=442.92 for rhodamine B (calculated for $[M–Cl]^+$, 443.52). Based on the aforemen-

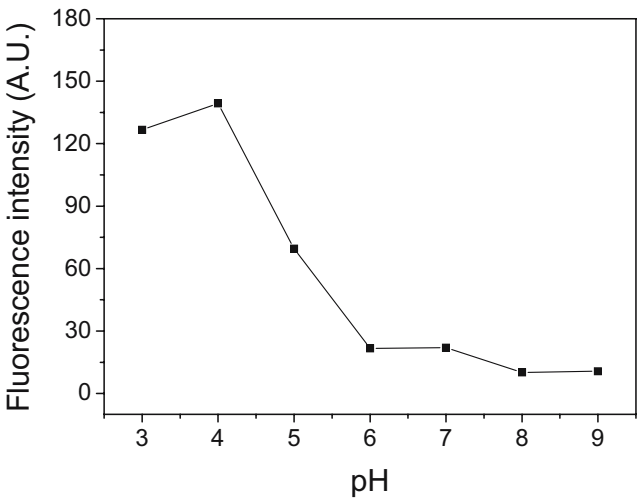

Fig. 7 Effect of pH on the fluorescence background of the system. RBH, 3.2 μmol l^{-1}

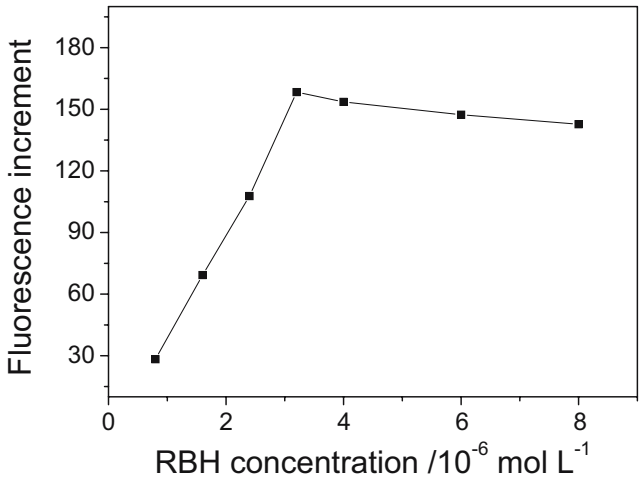

Fig. 8 Effect of RBH concentration on the fluorescence increment (ΔF) of the system. SDBS (0.4 mmol l^{-1}); Cytochrome c (20 ng ml^{-1}). The fluorogenic reaction was carried out in pH 4.0 of citric acid–phosphate buffer solution for 10 min, and then the fluorescence intensity was recorded at pH 8.0 of phosphate buffer solution

tioned experimental results, we believe that the highly fluorescent product generated in the reaction mixture is rhodamine B.

As for SDBS micellar medium, we propose that it has two functions on the detection system. First, an ionic associate was formed in the micellar solution, which was beneficial to increase the effective absorption cross-section of the fluorophore and resulted in the increase of molar absorption coefficient. However, this is not its leading role in the reaction system, because fluorescence measurement of rhodamine B in SDBS micellar medium shows about a twofold increase in its intensity. The proposed fluorogenic reaction cannot occur in the absence of SDBS, hence we believe that the leading role of SDBS lies in its advantageous micro-environment for the fluorogenic reaction.

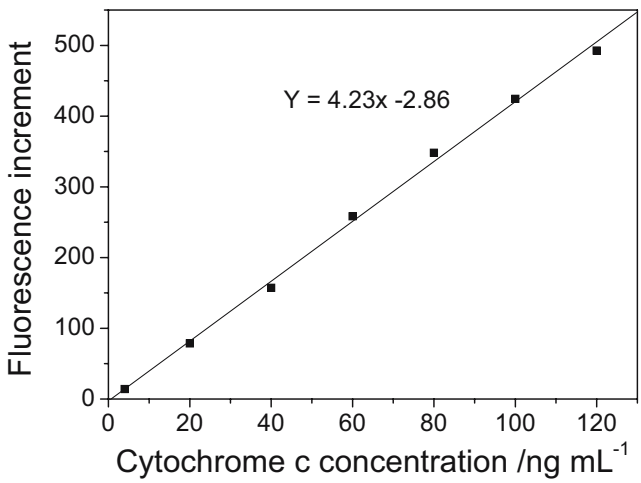

Fig. 9 Calibration graph for cytochrome c with RBH-SBDS system. The reaction and detection conditions were the same as those described in the procedure

Based on the above facts, a possible detection mechanism was given as follows: Cytochrome c catalyzed the decomposition of RBH in SDBS micellar medium, yielding highly fluorescent rhodamine B, and hence leading to the fluorescence increment of the system. The reaction was as follows:

$$RBH \xrightarrow{Cytochrome\ c} Rhodamine\,B + Other\ products$$

Interference study

The effect of foreign substances was evaluated by analyzing a standard solution of cytochrome c (1.8 nmol l^{-1}) to which interference species was added. The tolerance amount and the value (%) of relative error are shown in Table 2. The criterion for interference is fixed at a ±10% relative error calculated for the established level of cytochrome c. From Table 2 it can be seen that most coexisting substances show no effects on the determination of cytochrome c.

Limit of detection, linear calibration range and precision

Under the selected conditions given above, the fluorescence increment showed a linear relationship with cytochrome c concentration in the range of 4.0–120 ng ml^{-1} (r=0.9988, n=7), as shown in Fig. 9. The detection limit was 0.87 ng ml^{-1}

Table 2 Interference of foreign substances with the determination of 1.8 nmol l^{-1} of cytochrome c

Foreign substances	Amount added (molar ratio)	Relative error (%)[a]
CO_3^{2-}	1,000	6.4%
Al^{3+}	1,000	6.8%
NO_3^-	1,000	−6%
Cl^-	1,000	−0.9%
SO_4^{2-}	1,000	1.3%
Fe^{2+}	20	8.2%
I^-	1,000	−5.2
NH_4^+	1,000	−7%
Mg^{2+}	1,000	3.3%
Mn^{2+}	1,000	9.4%
Urea	1,000	5.4%
Ascorbic acid	20	7.5%
$C_2O_4^{2-}$	500	−7%
L-glutamic acid	1,000	1.7%
L-threonine	1,000	3.4%
L-methionine	1,000	3.4%
L-valine	1,000	1.8%
HAS	20.0 μg ml^{-1}	5.6%

[a] The value (%) of relative error was calculated by $(\Delta F_2-\Delta F_1)/\Delta F_1\times 100$, where ΔF_1 is the fluorescence increment for 1.8 nmol l^{-1} of cytochrome c, ΔF_2 is the fluorescence increment for 1.8 nmol l^{-1} of cytochrome c+foreign substances

(3σ). The relative standard deviation was 2.1% (n=8) for 20.0 ng ml^{-1} of cytochrome c.

Conclusions

In summary, we report a fluorescent turn-on detection for cytochrome c in aqueous solution with high sensitivity and selectivity using the ring-opening process of spirolactam of rhodamine B. The method is based on the reaction of spirocyclic RBH with cytochrome c in SDBS micellar medium, yielding highly fluorescent rhodamine B and hence leading the dramatic increase in fluorescence intensity of the reaction solution. The method is proved to be simple, selective and highly sensitive and can be broadly applicable to detection of cytochrome c in a variety of systems.

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