RSC Advances

PAPER

View Article Online
View Journal | View Issue

Cite this: *RSC Adv.*, 2015, **5**, 20634

A simple yet effective fluorescent probe for detecting and imaging mercury ions in cells†

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Received 12th December 2014
Accepted 6th February 2015

DOI: 10.1039/c4ra16267f

www.rsc.org/advances

We have synthesized rhodol hydrazide (RDH) as a simple fluorescent probe for detecting Hg^{2+}. The probe can be applied in nontoxic solvents (EtOH and H_2O). The probe has high selectivity and sensitivity to Hg^{2+} at pH 6–8. In addition, the probe has a superior capacity to resist interference from other ions. Both fluorescence intensity and absorbance have a linear relationship with the concentration of Hg^{2+}, which ensured the precise detection of Hg^{2+}. Furthermore, we have studied the intracellular Hg^{2+} imaging behavior of the probe on mammalian cells, which indicated that the probe can be applied to monitor Hg^{2+} within biological samples, especially in mammalian cells.

1. Introduction

Mercury is a highly toxic material which is distributed widely in the environment. It can damage brain, kidney, central nervous system, mitosis and endocrine system.[1–5] Seriously, mercury can cause irreversible harm to people because it can easily enter organic cells and cannot be excreted from body *via* metabolism.[6–10] Moreover, mercury can accumulate gradually in organisms *via* the food chain, though it is present in low concentrations in the surrounding.[6,7] Therefore, developing new techniques for the detection of Hg^{2+} is of great significance. Fluorescence probes have attracted considerable interest because they have high selectivity and sensitivity, and low detection limits.[11–14]

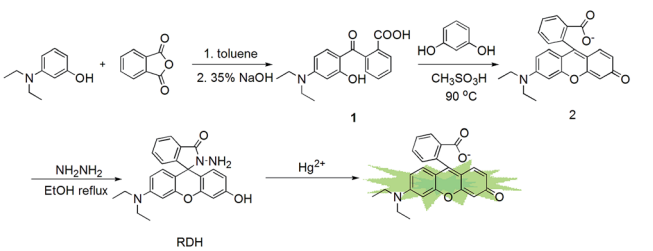
As fluorophores, xanthenes are highly favorable owing to their excellent photochemical properties, including excellent fluorescence intensities, high molar extinction coefficients and tolerance to photobleaching.[15,16] Thus, numerous fluorescent chemosensors based on xanthenes have been designed for the detection of metal ions. Most of these fluorescent probes are based upon the principle of fluorescence enhancement (turn-on) when they encounter specific metal ions.[17–23]

In this work, we designed and synthesized rhodol hydrazide (RDH) for the detection of Hg^{2+}. RDH shows higher selectivity to Hg^{2+} than Cu^{2+} and other ions, although many rhodamine hydrazide derivatives have been reported for the detection of Cu^{2+}.[24–28] In addition, RDH has a superior capacity to resist interference from other ions and displays low toxicity, remarkable membrane permeability and high sensitivity to Hg^{2+} in living cells.

2. Results and discussion

2.1. Synthesis and characterization

RDH was synthesized in three steps from 3-(diethylamino) phenol and *o*-phthalic anhydride (Scheme 1). The structure of RDH was confirmed by ^1H NMR, ^{13}C NMR and HRMS. No absorbance and emission peaks of RDH were observed in the absence of Hg^{2+} because RDH was in a spiro ring-close state. When Hg^{2+} was added to the solution of RDH, the typical absorbance (525 nm) and emission (553 nm) of rhodol acid apparently appeared. This result could be attributed to the hydrolyzation of rhodol hydrazide producing rhodol acid, which was confirmed by HRMS (Fig. S1, ESI†). Rhodol acid was in a spiro ring-open state, forming a conjugated system, and thus the typical absorbance and emission were observed. This detection mechanism is consistent with the rhodamine hydrazide method for the detection of Hg^{2+}.[29]



Scheme 1 Synthesis of RDH and detection mechanism for Hg^{2+}.

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† Electronic supplementary information (ESI) available: Characterization and some spectra of probe. See DOI: 10.1039/c4ra16267f

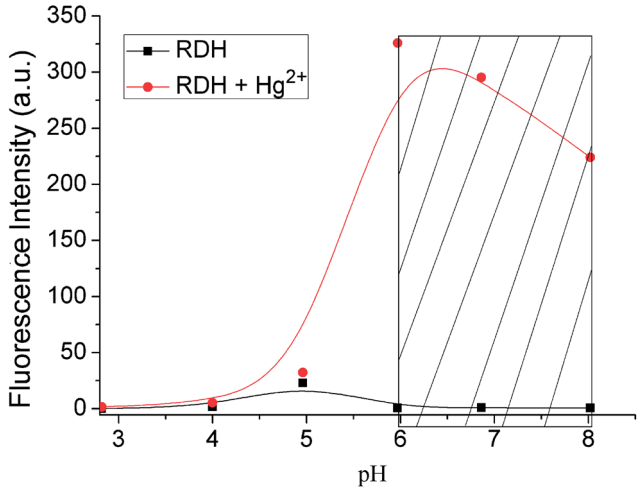

Fig. 1 Effect of pH on the fluorescence intensity of RDH. Probe: 10 μM, ethanol/HEPES buffer (1 : 9, v/v, pH = 7.4), λ_{ex}: 525 nm.

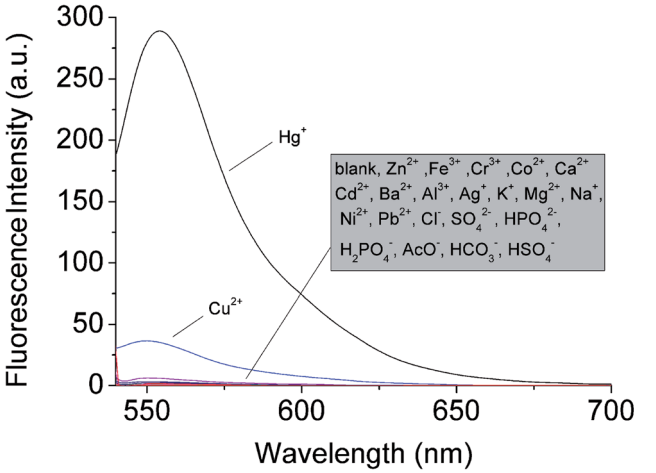

Fig. 2 Fluorescence intensity of the probe (10 μM) in ethanol/HEPES buffer (1 : 9, v/v, pH = 7.4) with representative cations and anions (100 μM), including Hg^{2+}, Zn^{2+}, Fe^{3+}, Cr^{3+}, Co^{2+}, Ca^{2+}, Cd^{2+}, Ba^{2+}, Al^{3+}, Ag^{+}, K^{+}, Mg^{2+}, Na^{+}, Ni^{2+}, Pb^{2+}, Cu^{2+}, Cl^{-}, HPO_4^{2-}, SO_4^{2-}, $H_2PO_4^{-}$, AcO^{-}, HCO_3^{-}, and HSO_4^{-} (λ_{em}: 553 nm).

2.2. Effect of pH and response time

To evaluate the effect of pH on the fluorescence of RDH, we investigated the fluorescence spectra of RDH at various pH values from 3 to 8. As shown in Fig. 1, no fluorescence of the free probe is observed in the pH range of 3–8. With the addition of Hg^{2+}, the fluorescence intensity increased dramatically in biological conditions (pH 6–8). This result indicates that RDH is suitable for the imaging of Hg^{2+} in biological systems. A time course of the fluorescence of RDH (10 μM) was investigated upon the addition of Hg^{2+} (10 equiv.) (Fig. S2, ESI†). The fluorescence intensity reached equilibrium in about 2 h.

2.3. Selectivity and interference

Selectivity is a crucial parameter to evaluate the properties of fluorescent probes. Therefore, we measured the selectivity of RDH towards various metal ions (namely, Hg^{2+}, Zn^{2+}, Fe^{3+}, Cr^{3+}, Co^{2+}, Ca^{2+}, Cd^{2+}, Ba^{2+}, Al^{3+}, Ag^{+}, K^{+}, Mg^{2+}, Na^{+}, Ni^{2+}, Pb^{2+}, and Cu^{2+}) and ordinary anions (namely, Cl^{-}, Br^{-}, HPO_4^{2-}, $H_2PO_4^{-}$, AcO^{-}, HCO_3^{-}, and HSO_4^{-}) by fluorescence and UV-vis absorption spectroscopy. The fluorescence data indicate that the RDH probe has a higher selectivity to Hg^{2+} over other ions (Fig. 2). In particular, the RDH probe shows insignificant response towards Cu^{2+}, although many rhodamine hydrazide analogues have been reported as Cu^{2+} fluorescence probes.[24–28] The UV-vis spectra (Fig. S3, ESI†) showed that a new absorption at 525 nm was observed in the presence of Hg^{2+}. In contrast, other ions did not cause this obvious spectral change. Therefore, Hg^{2+} can be distinguished from other ions by UV-vis absorption spectroscopy. Moreover, the fluorescence change of the probe towards Hg^{2+} was negligible in the presence of coexisting ions. The interference from other common ions was expressed with the ratio of $I_{RDH+Hg^{2+}+ion}/I_{RDH+Hg^{2+}}$ that varied from 0.79 to 1.11 in a finite range (Fig. 3). The abovementioned results suggest that the probe not only has an excellent selectivity but also can resist interference from other ions.

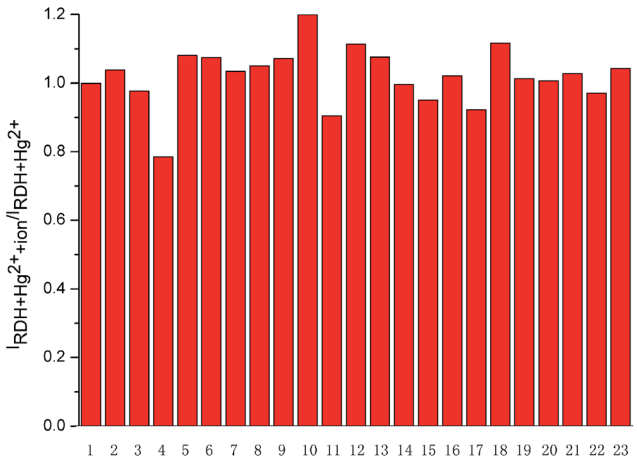

Fig. 3 The relative fluorescence intensity profiles (F/F_0) of the probe (10 μM) in ethanol/HEPES buffer (1 : 9, v/v, pH = 7.4) in the presence of 100 μM Hg^{2+} and interfering ions ((1) blank; (2) Ag^{+}; (3) Al^{3+}; (4) Ba^{2+}; (5) Ca^{2+}; (6) Cd^{2+}; (7) Co^{2+}; (8) Cr^{3+}; (9) Cu^{2+}; (10) Fe^{3+}; (11) Ni^{2+}; (12) Pb^{2+}; (13) K^{+}; (14) Mg^{2+}; (15) Na^{+}; (16) Zn^{2+}; (17) AcO^{-}; (18) Cl^{-}; (19) $H_2PO_4^{-}$; (20) HCO_3^{-}; (21) HPO_4^{2-}; (22) HSO_4^{2-}; (23) SO_4^{2-}) (λ_{ex}: 525 nm).

2.4. Fluorescence and UV-absorption titration

The fluorescence (Fig. 4) and UV-absorption (Fig. S4, ESI†) titrations were performed in a mixed solution of HEPES and EtOH (9 : 1, v/v, pH = 7.4). The free probe showed no fluorescent emission upon excitation at 525 nm. The characteristic fluorescent emission at 553 nm of rhodol was observed in the presence of Hg^{2+}, and the fluorescence intensity increased dramatically upon the addition of Hg^{2+}, from 0 to 130 μM. The fluorescence intensity peaked at 110 μM (Hg^{2+}) and enhanced by about 550 fold compared to that of the free probe.

Moreover, the RDH probe showed an excellent linearity between fluorescence intensity and the concentration of Hg^{2+}

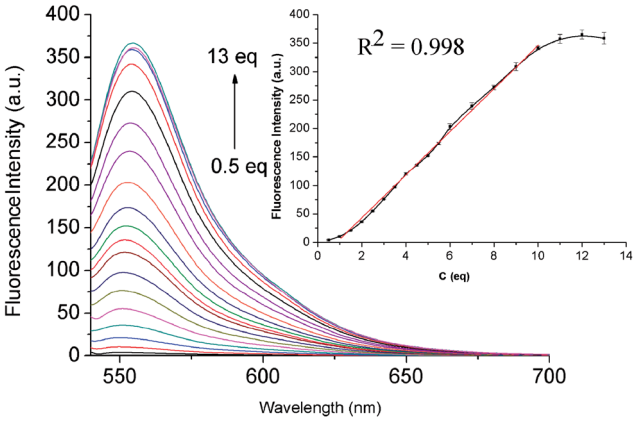

Fig. 4 Fluorescence spectra of reaction solution of the probe (10 μM) in ethanol/HEPES buffer (1 : 9, v/v, pH = 7.4) with different concentrations of Hg^{2+} (λ_{em}: 553 nm). (Inset-left) Fluorescence intensity at 553 nm upon the addition of Hg^{2+} (from 0 to 13 eq); (inset-right) linearity of the fluorescence intensity and the concentration of Hg^{2+} from 0 to 10 eq.

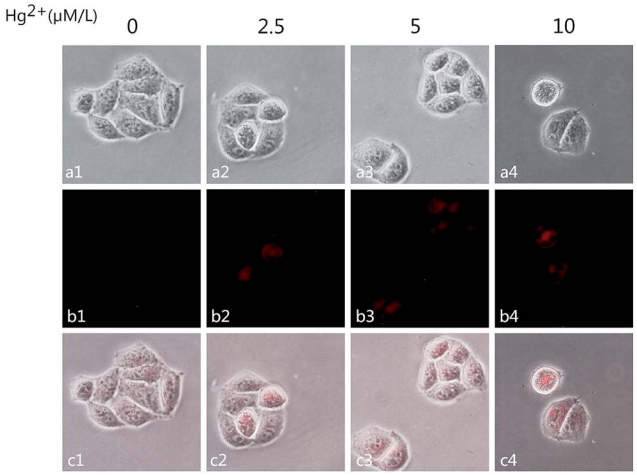

Fig. 5 Fluorescence images of the application of the probe to detect Hg^{2+} in living HeLa cells. (a1–4) Bright-field view; (b1–4) fluorescent; (c1–4) overlay image of (a1–4) and (b1–4). (1) HeLa cells incubated with 2 μM of the probe for 30 min at 37 °C. (2–4) HeLa cells were pretreated with Hg^{2+} at the indicated concentrations (2 : 2.5 μM; 3 : 5 μM; 4 : 10 μM) for 30 min at 37 °C before incubating with the 2 μM probe under the same conditions.

from 15 to 100 μM. The detection limit for Hg^{2+} was calculated to be 3.14×10^{-8} M according to the formula $3\sigma/k$ (σ is the standard deviation of ten blank solutions without Hg^{2+} and k is the slope of fluorescence titration calibration equation). The results indicated that RDH exhibited high sensitivity to Hg^{2+} and can quantitatively detect Hg^{2+} in a wide range of concentrations. As shown in Fig. S4 (ESI†), the absorbance increased with the addition of Hg^{2+}, and there was linearity between the absorbance of RDH and the concentration of Hg^{2+} from 15 to 100 μM. The two linear relationships of fluorescence and UV-absorbance ensure the precise detection of Hg^{2+}.

2.5. Imaging of intracellular Hg^{2+} and evaluation of cytotoxicity

To verify the potential biological applications of RDH, we studied intracellular Hg^{2+} imaging with the probe in mammalian cells (Fig. 5). Incubating HeLa cells with 2 μM of RDH probe for 30 min at 37 °C showed very weak fluorescence that could be negligible. When HeLa cells were pretreated with 2.5 μM of Hg^{2+} with the same treatment of the probe, fluorescence was visible. It provided visual evidence that the probe can permeate cells and inform the intracellular existence of Hg^{2+}. Moreover, the fluorescence intensity revealed a remarkable enhancement with increasing concentration of Hg^{2+} (5, 10 μM). It suggested that the probe RDH can be applied to monitor Hg^{2+} within biological samples, especially in mammalian cells.

We used an SRB assay to detect the viability of HeLa cells incubated with different concentrations of RDH probe for 6 h (Fig. 6) and found no distinct decline compared to controls. Furthermore, even when RDH probe concentration was as high as 5 μM, which is more than 2 times higher than the normal concentration (2 μM) used in cell imaging, the viability of HeLa cells remained at a normal value. Therefore, the RDH is non-toxic to cells and could be used for clinical applications in living organisms.

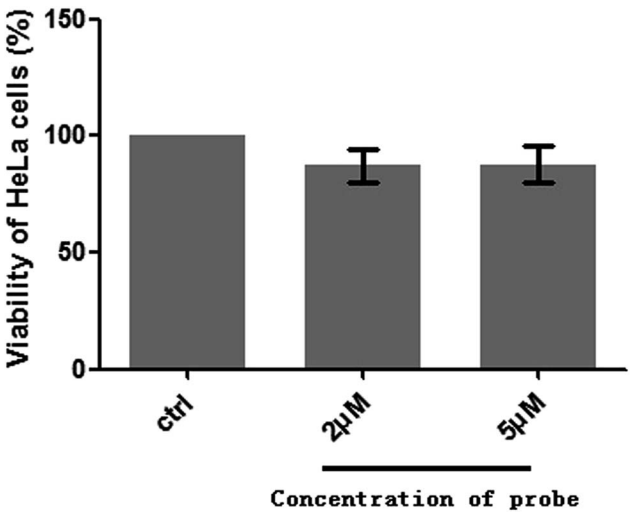

Fig. 6 The viability of HeLa cells after incubation with different concentrations of probe (2 and 5 μM) or with DMSO 0.1% (v/v) (control) for 6 h measured by SRB assay and illustrated in the labelled column. Results are presented as mean ± SE (n = 3).

3. Materials and methods

3.1. Instruments and materials

HRMS spectra were recorded on a Q-TOF6510 spectrograph (Agilent). ^{1}H NMR (300 MHz) and ^{13}C NMR (75 MHz) spectra were recorded on a Bruker Avance 300 spectrometer using DMSO as the solvent and tetramethylsilane (TMS) as an internal standard. Fluorescent measurements were recorded on an F-7000 (Hitachi) luminescence spectrophotometer, and UV-vis spectra were recorded on a U-4100 Spectrometer (Hitachi).

The pH was measured by the use of a PHS-3C digital pH-meter (YouKe, Shanghai). Images of HeLa cells were captured with a fluorescent microscope (Nikon ECLIPSE TE 2000-U, Japan).

Resorcinol, 3-(diethylamino)phenol, *o*-phthalic anhydride, methanesulfonic acid, hydrazine hydrate, and all the reagents were purchased from commercial suppliers and used without further purification. The solutions of metal ions were prepared from KNO_3, $NaNO_3$, $Ca(NO_3)_2 \cdot 4H_2O$, $Mg(NO_3)_2 \cdot 6H_2O$, $Al(NO_3)_3 \cdot 9H_2O$, $Ba(NO_3)_2$, $Cr(NO_3)_3 \cdot 9H_2O$, $Ni(NO_3)_2 \cdot 6H_2O$, $Cd(NO_3)_2 \cdot 4H_2O$, $Fe(NO_3)_3 \cdot 9H_2O$, $AgNO_3$, $Co(NO_3)_2 \cdot 6H_2O$, $Pb(NO_3)_2$, $Cu(NO_3)_2 \cdot 3H_2O$, $Zn(NO_3)_2 \cdot 6H_2O$ and $HgCl_2$ with deionized water. Deionized water was used throughout the absorption and fluorescence determinations. All the samples were prepared at room temperature, shaken for 10 s and allowed to stand for 5 h before UV-vis and fluorescence determination. HEPES buffer solutions (pH 7.4) were prepared with 20 mM HEPES and adjusted with aqueous sodium hydroxide.

3.2. Synthesis of probe RDH

2-(4-Diethylamino-2-hydroxy-benzoyl)-benzoic acid (compound 1). Compound **1** was synthesized according to a previous report.[30]

3′-(Diethylamino)-6′-hydroxy-3*H*-spiro[isobenzofuran-1,9′-xanthen]-3-one (compound 2). Compound **1** (1.2 g, 3.83 mmol) and benzene-1,3-diol (0.43 g, 3.39 mmol) were dissolved in 30 mL methanesulfonic acid. The mixture solution was stirred at 90 °C for 9 h. The reaction mixture was poured into 200 mL of ice-water and adjusted to neutral with saturated sodium carbonate solution. The abovementioned solution was extracted with dichloromethane (100 mL × 3). The organic layers were combined, washed with distilled water (50 mL × 3), dried over anhydrous magnesium sulfate, and then filtered. The dichloromethane solvent was removed under reduced pressure. The residue was purified by silica gel column chromatography (PE : EA = 2 : 1) to obtain 460 mg of red solid (yield: 28.4%).

2-Amino-3′-(diethylamino)-6′-hydroxyspiro[isoindoline-1,9′-xanthen]-3-one (RDH). Compound **2** (400 mg, 1.07 mmol) was dissolved in EtOH (30 mL) and $NH_2NH_2 \cdot H_2O$ (100 mL). The reaction solution was heated and kept under reflux for 4 h. The ethanol was removed under reduced pressure and the aqueous layer was extracted with dichloromethane (100 mL × 3). The organic solution was washed with distilled water (50 mL × 3), dried over anhydrous magnesium sulfate and then filtered. The solvent was removed under reduced pressure and the crude product was purified by column chromatography on silica gel (dichloromethane : methanol = 5 : 1) to obtain 350 mg of white solid (yield: 87.5%). ^{1}H NMR (300 MHz, DMSO) δ 9.75 (s, 1H), 7.82–7.70 (m, 1H), 7.54–7.39 (m, 2H), 7.02–6.93 (m, 1H), 6.59 (d, J = 2.1 Hz, 1H), 6.45–6.29 (m, 5H), 4.31 (s, 2H), 3.36–3.29 (m, 4H), 1.08 (t, J = 6.9 Hz, 6H). ^{13}C NMR (75 MHz, DMSO) δ 165.32, 158.07, 152.74, 152.69, 151.66, 148.19, 132.44, 129.47, 128.21, 127.92, 127.62, 123.42, 122.21, 111.67, 110.12, 108.05, 105.21, 102.29, 97.44, 64.66, 43.62, 40.35, 40.07, 39.79, 39.52, 39.24, 38.96, 38.68, 12.41. HRMS: m/z $[M + H]^+$ calcd for $C_{24}H_{23}N_3O_3$: 402.1817, found: 402.1815.

3.3. Preparation of solution for detection

Stock solutions (10^{-2} M) of the ions (including Hg^{2+}, Zn^{2+}, Fe^{3+}, Cr^{3+}, Co^{2+}, Ca^{2+}, Cd^{2+}, Ba^{2+}, Al^{3+}, Ag^+, K^+, Mg^{2+}, Na^+, Ni^{2+}, Pb^{2+}, Cl^-, Br^-, HPO_4^{2-}, $H_2PO_4^-$, AcO^-, HCO_3^-, and HSO_4^-) were prepared for detection by dissolving them in deionized water. The probe was dissolved in ethanol as stock solution (10^{-3} M). The solvent was composed of ethanol and HEPES buffer (v/v, 1 : 9, pH = 7.4). The excitation slit was 3 nm and the excitation wavelength was 525 nm. The emission slit was 13 nm and the emission wavelength was 553 nm.

3.4. Cell culture and imaging

HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) containing 10% calf bovine serum (HyClone) in dishes maintained at 37 °C in humidified air with 5% CO_2. For fluorescence imaging, cells at the desired cell concentration were seeded into 24-well plates, and then incubated with fresh DMEM with different concentrations of Hg^{2+} (0, 2.5, 5, 10 μM) for 30 min. The cells were washed twice with PBS buffer before staining experiments. Then, the cells were incubated with DMEM containing 2 μM of the RDH probe dissolved in DMSO (DMSO : DMEM < 2 : 1000, v/v) for 30 min. After washing twice with PBS, the cells were imaged under a fluorescence microscope (Nikon ECLIPSE TE 2000-U, Japan).

3.5. Cytotoxicity

The RDH probe was dissolved in DMSO as a stock solution. HeLa cells were cultured in DMEM (Gibco) containing 10% calf bovine serum in dishes maintained at 37 °C in humidified air with 5% CO_2. Cells were harvested with 0.25% trypsin (Sangon Biotech) in PBS and seeded in 96-well plates. When cells covered 60–70% of the plate surface, the culture was replaced with fresh DMEM containing 0.1% DMSO (as control) or the RDH probe at different concentrations (2 and 5 μM). Cell viability was evaluated by a sulforhodamine B (SRB) assay. After incubation for 6 h, cells were fixed with 10% cold trichloroacetic acid (TCA) at 4 °C for 1 h. The stationary liquid was then removed and washed five times with deionized water. Then, the air-dried plates were stained with SRB solution for more than 10 min in a shaker (QILINBEIER Orbital Shaker TS-100, China) at room temperature. The superfluous SRB was washed away with 1% acetum and plates were air-dried and subsequently 100 μL of 10 mM unbuffered Tris base (pH 10.5) was added to every well with HeLa cells to dissolve the bound dye and mixed for 5 min on a microtiter plate shaker. Optical density (OD) was measured by the use of a microplate reader (TECAN Nano-Quant Infinite M200PRO, Austria) at a wavelength of 540 nm after plates were shaken at room temperature for 10 min. The OD values of probe-incubated wells were compared and normalized to those of controls treated with 0.1% DMSO to calculate the relative cell viability. We used 3 parallel wells in every measurement and the results are expressed as the average of triplicate assays.

4. Conclusions

A simple yet effective fluorescent probe (RDH) was developed for the detection of Hg^{2+}. The probe exhibited an excellent

selectivity to Hg^{2+} in friendly solvents (EtOH and H_2O). Both fluorescence intensity and absorbance have linear relationships with the concentration of Hg^{2+}. Moreover, the probe can be applied to monitor Hg^{2+} in mammalian cells with remarkable membrane permeability and non-toxicity to the cells.

Acknowledgements

This study was supported by the National Basic Research Program of China (2010CB933504).

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