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Highly sensitive and selective fluorescence detection of Hg(II) ions based on R-phycoerythrin from *Porphyra yezoensis*

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R-Phycoerythrin (R-PE) is a kind of natural fluorescent protein from marine *Porphyra yezoensis*. A highly sensitive and selective fluorimetric method has been developed to detect Hg^{2+} ions utilizing R-PE as a fluorescent probe. R-PE could respond selectively to Hg^{2+} ions among a series of metal ions. The reaction mechanism was investigated through UV-vis, fluorescence microscope imaging and fluorescence measurements. It was demonstrated that the interaction between the R-PE fluorescent probe and Hg^{2+} ions would cause fluorescence quenching. Besides, there was a linear relationship between R-PE fluorescence intensities and Hg^{2+} concentrations. A novel method was thus explored to detect Hg^{2+} ions with high sensitivity, selectivity, and a broad linear range of 0.0010–25.0 μM, which could allow for Hg^{2+} ions down to 0.0130 μM with a relative standard deviation of about 1.60%. Additionally, the method was successfully applied to detect Hg^{2+} ions in diverse water samples, showing the appropriate rates of recovery between 92.0% and 108.0%. This highly sensitive and selective natural green fluorescent probe of R-PE should have huge potential applications for detecting Hg^{2+} ions in diverse environments.

1 Introduction

There has been an increasing awareness of heavy metal pollution throughout the world because of the negative effects on the environment. Among the heavy metals of concern, mercury has long been recognized as a global pollutant because it can remain in the atmosphere, food and water. It can bioaccumulate in the human body *via* the food chain to severely damage the human brain, the nervous system, the endocrine system and other biological systems, resulting in brain damage, kidney failure and various cognitive and motion disorders.[1]

Up to now, many analysis technologies have been developed to detect heavy metal ions including Hg^{2+} ions, such as atomic absorption spectrometry,[2] inductively coupled plasma mass spectrometry (ICP-MS),[3] cold vapor atomic absorption spectrometry (CV-AAS),[4] electrochemical sensing methods,[5] colorimetric assays,[6] and fluorescence detection methods.[7] All of these methods have their own advantages and have made great contributions for the detection of Hg^{2+} ions. Among these analysis methods, fluorescence detection methods have prominent advantages including simplicity, sensitivity, short reaction time, whose detection performances are dependent on the used fluorescent probes to some extent.

Many kinds of synthesized fluorescence materials have been extensively applied as fluorescent probes. But the harsh and complicated synthesis procedures with high cost might limit their further applications. Therefore, a variety of novel fluorescent proteins have been rapidly developed and widely used in marine biosciences, biotechnology, and proteomics.[8] Up to date, many green fluorescent proteins (GFPs) have been separated from *Aequorea*. Particularly, blue fluorescent protein (BFP), cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) were gained and applied. For example, scientists had applied fluorescent proteins for the super-resolution imaging techniques.[9,10] Besides, photo-switchable fluorescent probes with distinct colors were also introduced for the multicolor.[11]

Recently, phycoerythrin (PE), as a natural fluorescent protein, has become a valuable candidate in flow cytometry and immunofluorescence microscopy.[12] In addition, it was applied not only as natural colorants for use in food and cosmetics, but also as fluorescent dye. As one of the most important type of PE, R-PE has been applied to make the R-PE-labeled IgG, which can be applied in fluorescence immunoassays as secondary antibody.[13] In our previous work, many synthetic fluorescent probes were prepared and extensively applied. For example, bimetallic gold–silver nanoclusters was successfully synthesized to analyze

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mercury and copper levels in blood.[14] Silver nanoclusters (AgNCs) were synthesized and upon the GSH passivation, the specific recognition of the AgNCs was modulated from Hg^{2+} ions to Cu^{2+} ions which can apply for ion sensing and biological imaging in the complicated media like blood.[15] Currently, many natural and nontoxic fluorescent probes were in high demanded. In the present report, R-PE extracted from marine *Porphyra yezoensis* was utilized as natural and nontoxic fluorescent probe for detecting Hg^{2+} ions combining with fluorescent method in diverse water environments with high selectivity and sensitivity.

2 Experimental

2.1 Materials and instrumentation

Porphyra yezoensis was purchased from Yantai, Shandong Province, China. $(NH_4)_2SO_4$, $NaHCO_3$, Na_2HPO_4, NaH_2PO_4, hydroxyapatite (HA), ethylenediaminetetraacetic acid (EDTA), HCl, Coomassie Blue R-250, NaCl, $FeSO_4$, $MgSO_4 \cdot 7H_2O$, $CuSO_4$, $Ca(NO_3)_2$, $ZnSO_4$, $NiSO_4$, KNO_3, $BaCl_2$, $CrCl_3$, $CoCl_2$, $HgCl_2$, $Pb(NO_3)_2$, $AgNO_3$, $CdCl_2$, KI, Na_2CO_3, KBr, KCl, $CaCl_2$, Na_2SO_4 and $NaHCO_3$ were purchased from Sinopharm Chemical Reagent Co., Ltd (Beijing, China). All of these reagents were of analytical grade.

Fluorescence spectra were obtained using a fluorescence spectrometer (F-2700, Hitachi, Japan). The sample solutions were excited at 495 nm, with slits for excitation and emission both of 10.0 nm. The characteristic peak was obtained by UV-vis spectrophotometer (UNICO, UV-2000). The photographs were obtained by ultraviolet analyzer (Lichen, ZF-1, China) at 365 nm. The morphological characteristics were obtained by microscope (Olympus, BX53, Japan).

2.2 R-PE extraction, isolation and purification

R-PE was extracted and purified according to the reported literature method with slight modification.[16] *Porphyra yezoensis* was collected and washed in phosphate buffer solution (PBS, 10 mM, pH 6.8). The alga-buffer mixture suffered from freeze–thaw cycles of −25 °C and 4 °C for four times. The resulting slurry was filtrated by gauze, then centrifuged (7500 g, 20 min, 4 °C). The crude extraction was precipitated with ammonium sulfate at concentrations of 10% and 50%, discarding the supernatant. The final precipitant, dissolved in PBS (10 mM, pH 6.8), was dialyzed in PBS (10 mM, pH 6.8). After centrifuged at 7500 g for 20 min, the crude extract was further purified with HA column. After using different concentrations PBS for gradient eluting, the eluent containing R-PE was collected. The R-PE was examined by SDS-PAGE (17% separating and 3% stacking gel). The gel was stained with Coomassie Blue R-250 (0.1%) and destained with double distilled water. The protein content of R-PE was determined using the Bradford method. The absorption peak of R-PE was measured on a spectrophotometer.

2.3 Fluorimetric analysis of metal ions

A series of 1.0 μM metal ions (Zn^{2+}, Cr^{3+}, Cu^{2+}, Ba^{2+}, Co^{2+}, Fe^{2+}, Pb^{2+}, Cd^{2+}, Mg^{2+}, Ag^{+}, Ni^{2+}, Hg^{2+}, K^{+}, Na^{+} and Ca^{2+}) were separately added to R-PE solution for the fluorescent selective assay. Then the morphological characteristics of R-PE with Hg^{2+} ions were observed by microscope in bright-field image and fluorescence image, respectively.

2.4 Optimization of the detection conditions

1.0 μM Hg^{2+} ions was added to R-PE solution at different reaction time (1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 min), pH (5.0, 6.0, 7.0, 8.0, 9.0), and temperatures (20, 25, 30, 35, 40, 45, 50 °C), respectively, to explore the optimum conditions. In addition, the interference of other negative ions (I^-, PO_4^{3-}, SO_4^{2-} and CO_3^{2-}) at 1.0 μM and compounds (0.101 g L^{-1} KBr, 0.695 g L^{-1} KCl, 24.530 g L^{-1} NaCl, 1.537 g L^{-1} $CaCl_2$, 4.090 g L^{-1} Na_2SO_4 and 0.201 g L^{-1} $NaHCO_3$), which possibly co-exist in sea water samples were evaluated under the optimal conditions.

2.5 Standard curve for detecting Hg^{2+} ions

Hg^{2+} ions at different concentrations (0, 0.0010, 0.005, 0.010, 0.10, 0.50, 1.0, 5.0, 25.0 μM) were analyzed under the optimum condition of Tris–HCl (50 mM, pH 8.0) at 40 °C and 10 min reaction time. Then the fluorescence spectra of the reaction mixtures were recorded at the excitation of 495 nm. A calibration curve was thereby prepared to show the relationship between the logarithmic concentrations of Hg^{2+} ions and fluorescence quenching efficiencies of R-PE. The quenching efficiencies of R-PE caused by Hg^{2+} ions were calculated in accordance with the following equation reported previously: quenching efficiencies $= (F_0 - F)/F_0$, where F_0 and F refer to the fluorescence intensities of R-PE ($\lambda_{em} = 570$ nm) in the absence and presence of Hg^{2+} ions, respectively.

2.6 Detection of Hg^{2+} ions in diverse water samples

Tap water and sea water were collected from Weihai, Shandong province, China. The samples were centrifuged at 10 000*g* for 10 min and then filtered through a 0.22 μm filtered membrane. To further investigate the feasibility and possible application of the developed R-PE fluorescent probe for analysis of Hg^{2+} ions in the complex water sample, Hg^{2+} ions standards including 0, 0.050, 0.100, 0.500 μM were initially added into blank tap-water solution and sea water solution. R-PE with Tris–HCl (50 mM, pH 8.0) solution was mixed with diverse water samples containing various concentrations of Hg^{2+} at 40 °C for 10 min. Then, the fluorescence quenching intensity at λ_{em} of 570 nm was measured by a fluorescence spectrometer. The recovery (%) = 100 × (found concentration)/(spiked concentration). Data from the experiment was analyzed by SPSS16.0 statistic software.

3 Results and discussion

3.1 Characterization of purified R-PE

The purified R-PE was determined by SDS-PAGE (Fig. 1A). It was revealed that purified R-PE from the HA column contained three main bands. On the basis of the positions of the protein markers, the molecular weights of three bands could be estimated to be approximately 18.0 kDa, 21.0 kDa and 30.0 kDa. It was reported that SDS-PAGE analysis previously confirmed the presence of two major subunits with 18.0 kDa and 20.0 kDa,

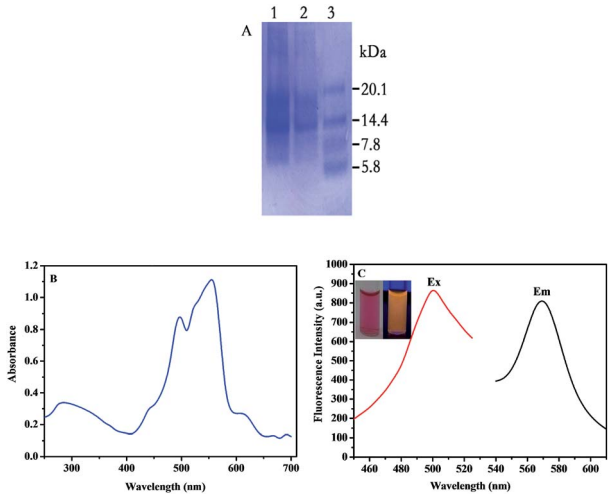

Fig. 1 Characterization of purified R-PE. (A) SDS-PAGE of purified R-PE. Lane 1 and lane 2, purified R-PE; lane 3, molecular mass markers. (B) Absorption spectra of purified R-PE. (C) Fluorescence excitation (red line) and emission (black line) spectra of R-PE after purification with the described procedure. Inset shows R-PE under visible light (left) and UV light (right). Then the relevant fluorescence changes were recorded by the fluorescence spectrometer.

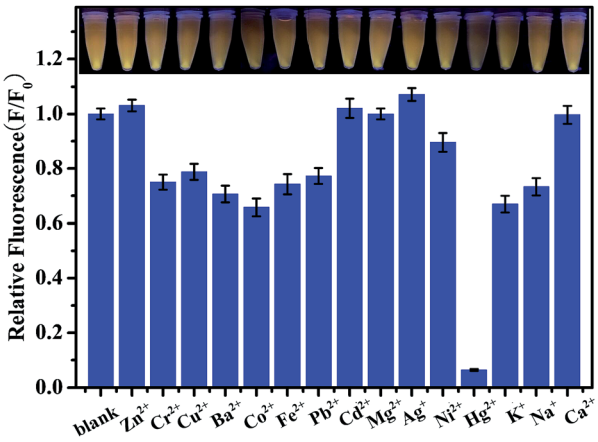

Fig. 2 Relative fluorescence (F/F_0) of R-PE solution in the presence of various metal ions at 1.0 μM. F_0 and F correspond to the fluorescence intensity of R-PE in the absence and presence of 1.0 μM of various metal ions.

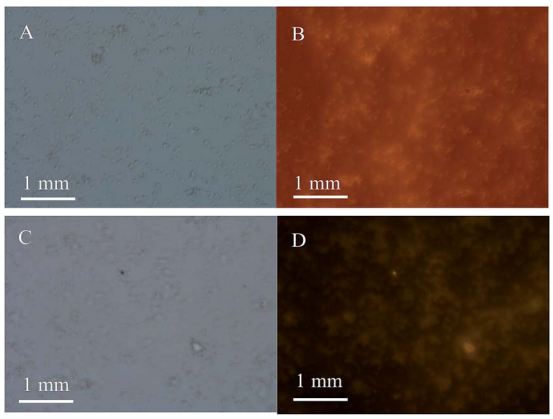

Fig. 3 (A) Bright-field image of R-PE (0.080 mg mL^{-1}). (B) Fluorescence image of (A). (C) Bright-field image of R-PE (0.080 mg mL^{-1}) treated with Hg^{2+} (5 μM) for 10 min at 30 °C. (D) Fluorescence image of (C).

respectively, and a minor subunit of 30.0 kDa, which were much smaller and slighter.[17,18] The optical properties of R-PE were characterized by UV-vis absorbance and fluorescence spectroscopy. Fig. 1B indicated that there were two adsorption peaks at 495 nm and 560 nm with a shoulder at 540 nm, corresponding to the previous reports (495 nm, 566 nm and 540 nm shoulder; 499 nm, 565 nm, and 545 nm shoulder).[19,20] As shown in Fig. 1C, the R-PE showed red color under visible light, while exhibited powerful orange color when irradiated with UV light. When excited at 495 nm, R-PE displayed a maximum emission peak at 570 nm. As being mainly in accordance with a former literature report,[21] the features could demonstrate that the substance extracted from *Porphyra yezoensis* was a kind of R-PE.

3.2 Sensing response of R-PE to metal ions

To explore the sensing selectivity, R-PE was used to investigate the influence of various biologically and environmentally relevant metal ions under the same conditions. Fig. 2 presented the difference in relative fluorescence against the common metal ions including Zn^{2+}, Cr^{3+}, Cu^{2+}, Ba^{2+}, Co^{2+}, Fe^{2+}, Pb^{2+}, Cd^{2+}, Mg^{2+}, Ag^{+}, Ni^{2+}, Hg^{2+}, K^{+}, Na^{+} and Ca^{2+} ions at 1.0 μM.

The results showed that only Hg^{2+} ions could trigger the quenching of the fluorescence obviously, while the other ions displayed weak or even negligible effects on the fluorescence intensities, as shown in their corresponding photographs (Fig. 2, insert). These observations reflected that the prominent sensing response to Hg^{2+} ions endowed R-PE with high selectivity. In order to assess the quenching reactions between Hg^{2+} ions and R-PE, the morphological characteristics of R-PE was observed by microscope in Fig. 3. As shown in Fig. 3D, the introduction of Hg^{2+} ions caused the fluorescence quenching of R-PE compared with Fig. 3B. The fluorescence quenching of R-PE might be partly attributed to the energy transfer between R-PE and Hg^{2+} ions.[15,22]

3.3 Optimization of the detection conditions

In order to obtain a highly-sensitive response to Hg^{2+} ions, the effects of the experimental conditions, such as the reaction time, pH values and temperature, were investigated respectively. The relative fluorescence of R-PE and Hg^{2+} ions in various times was measured in Fig. 4A. The figure showed that the fluorescence intensity reduced at first ten minutes and became constant later. So the reaction time at ten minutes was chosen for the following experiments. Compared with some other fluorescent probes such as Pvi,[23] long lifetime fluorescence quantum dots and gold nanoparticles,[24] colorimetric method[25] and flame atomic absorption spectrometry,[26] R-PE as a fluorescent probe to Hg^{2+} ions was more rapid in this work. Fig. 4B illustrated the relative fluorescence of reactions conducted at

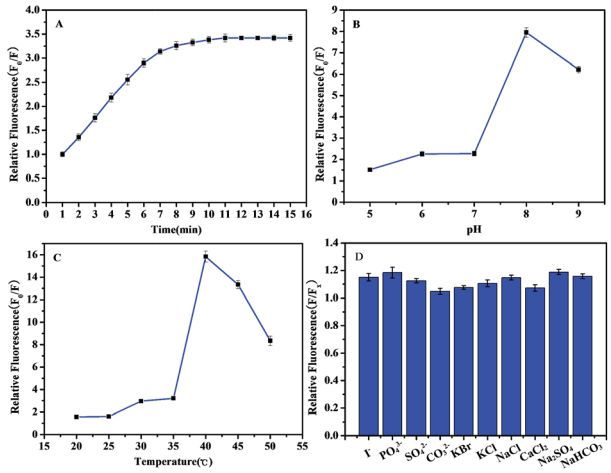

Fig. 4 Effects of time (A), pH (B) and temperature (C) on the relative fluorescence (F_0/F) of R-PE (0.080 mg mL^{-1}) for Hg^{2+} detection. (D) Effects of potential interferents on relative fluorescence (F/F_x) of R-PE (0.080 mg mL^{-1}) for Hg^{2+} detection. F and F_x correspond to the fluorescence intensity of R-PE with Hg^{2+} ions in absence and presence of 1.0 μM potential interferents.

different pH values. It was obviously found that the highest relative fluorescence was obtained at pH 8.0. As shown in Fig. 4C, the relative fluorescence reached the maximum when temperature reached up to 40 °C. Thus, 40 °C was selected for the optimal detection conditions. Fig. 4D showed that relative fluorescence values of potential interferents (I^-, PO_4^{3-}, SO_4^{2-}, CO_3^{2-}, KBr, KCl, NaCl, $CaCl_2$, Na_2SO_4 and $NaHCO_3$) were around 1.0, indicating that no significant interference occurred when co-existing with Hg^{2+} ions.

3.4 The response of R-PE fluorescent probes to Hg^{2+} ions

Besides the selectivity, the sensitivity was another important parameter to assess the performance of the sensing system. Therefore, the capability of R-PE fluorescent probe for quantitative detection of Hg^{2+} ions was evaluated under the optimal conditions. As shown in Fig. 5A, the R-PE was sensitive to Hg^{2+} ions and the fluorescence intensity decreased gradually with increasing concentrations of Hg^{2+} ions. When the concentration of Hg^{2+} ions was increased to 25.0 μM, no further restoring fluorescence could be obtained, meaning that the sensing response had reached to the maximum. Fig. 5B exhibited the relationship between the logarithmic concentrations of Hg^{2+} ions and fluorescence quenching efficiencies of R-PE (0.080 mg mL^{-1}), corresponding to photographs under UV light (Fig. 5B). The calibration curve was obtained with the concentrations of Hg^{2+} ions ranging from 0.0010 μM to 25.0 μM. A linear regression equation for Hg^{2+} ions was thereby acquired. The limit of detection (LOD) was estimated to be 0.0130 μM based on the standard deviation rule (LOD = 3Sd/s), which was much lower than the toxicity level of Hg^{2+} ions in drinking water (30 nM) defined by the World Health Organization (WHO).[27] Besides, the detection limit of Hg^{2+} ions was much lower than that of other fluorescent sensors such as nitrogen-doped carbon quantum dots (N-CQDs) (230 nM),[28] fluorescent carbon dots (FCDs) (20 nM)[29] and label-free colorimetric sensor (50 nM),[30] which were not natural and required high cost. It was widely recognized that the traditional analytical methods such as colorimetric biosensors,[25] flame atomic absorption spectrometry[26] and electrochemistry,[31] can detect Hg^{2+} ions with high selectivity and sensitivity, but most of them might suffer from expensive specialized instruments, time-consuming and complex operations. Moreover, some organic probes and quantum dots are generally applied but involve either of complicated synthesis and toxic agent, or cost ineffective and time-consuming analysis.[28,29] R-PE, as a kind of natural fluorescent protein, is widely existed and easy to purify from *Porphyra yezoensis*. By contrast, R-PE used in the current work that was obtained from *Porphyra yezoensis* as fluorescent probe is natural and nontoxic probe with strong fluorescence. The developed fluorimetric analysis with R-PE is rapid (ten minutes), simple, cost-effective, and highly sensitive for detecting Hg^{2+} ions.

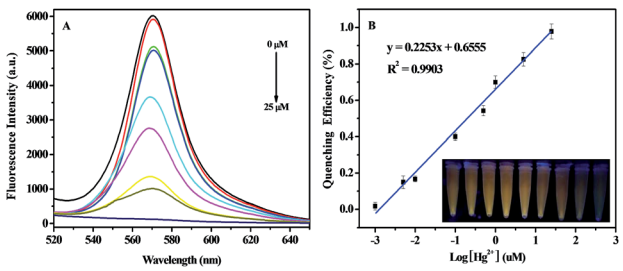

Fig. 5 (A) Fluorescence spectra of R-PE (0.080 mg mL^{-1}) in the presence of various concentrations of Hg^{2+} ions (from bottom to top: 0, 0.0010, 0.005, 0.010, 0.10, 0.50, 1.0, 5.0, 25.0 μM) in the test solutions. (B) Fluorescence quenching efficiencies of R-PE (0.08 mg mL^{-1}) *versus* the logarithmic concentrations of Hg^{2+} ions (0.0010, 0.0050, 0.010, 0.10, 0.50, 1.0, 5.0, 25.0 μM). All measurements were taken in Tris–HCl (50 mM, pH 8.0) at 40 °C. The error bar represents the standard deviation of five measurements. Insert: photographic images of the corresponding solutions under UV light.

3.5 Detecting Hg^{2+} ions in diverse water samples

In order to evaluate the feasibility in practical applications, the fluorimetric assay mentioned above was employed to detect Hg^{2+} ions in tap water and sea water (Weihai) samples. The accuracy of the sample analysis was examined through standard

Table 1 Detection of Hg^{2+} ions in two kinds of water samples

Sample	Added (μM)	Found (μM)	RSD (%, n = 3)	Recovery (%)
Tap water	0	Not detected		
	0.050	0.046	1.2	92.0
	0.100	0.096	0.5	96.0
	0.500	0.477	0.8	95.4
Sea water (Weihai)	0	Not detected		
	0.050	0.054	1.6	108.0
	0.100	0.106	1.3	106.0
	0.500	0.523	0.4	104.6

addition method. And the detection results were revealed in Table 1. Recoveries of different added Hg^{2+} concentrations were acquired between 92.0% and 108.0% with the relative standard deviations (RSDs) lower than 1.60%. The results demonstrated that the fluorescent probe of R-PE possessed high sensitivity and selectivity, which made R-PE become an excellent tool to detect Hg^{2+} ions in water samples.

4 Conclusions

In summary, a rapid, sensitive and selective fluorescence detection method has been successfully established for detecting Hg^{2+} ions utilizing R-PE from *Porphyra yezoensis* as fluorescent probe. Under the optimal conditions, there is a good linear relationship for Hg^{2+} ions in the range of 0.0010–25.0 μM, with the LOD was 0.0130 μM. The developed R-PE-based fluorescence method has presented some outstanding advantages over the traditional detection methodologies, such as natural nontoxic fluorescent probe, short reaction time, mild reactive conditions, simple operation, high selectivity and sensitivity. All these remarkable advantages of this method suggest that the proposal has promising application prospects in the field of environment monitoring.

Acknowledgements

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