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A ratiometric fluorescent probe for sensitive, selective and reversible detection of copper (II) based on riboflavin-stabilized gold nanoclusters

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ABSTRACT

Most of the copper (II) fluorescent probes are based on the measurement of fluorescence at a single wavelength, which may be influenced by variations in the sample environment. To the end, the ratiometric fluorescent measurement, which involves the simultaneous measurement of two fluorescence signals at different wavelengths followed by calculation of their intensity ratio, can effectively eliminate the adverse effects on fluorescence signals and give greater precision to the data analysis relative to single-channel detection. In this work, we prepared novel luminescent gold nanoclusters (AuNCs) utilizing vitamin B_2 (riboflavin) as stabilizer by a simple, rapid and one-pot green (low-toxicity materials use) procedure. The as-prepared riboflavin-AuNCs (**Ri**-AuNCs) solution can be luminescent exhibiting two fluorescence emission peaks at 530 nm and around 840 nm with excitation at 375 nm, however, in the presence of Cu^{2+}, the fluorescence of the **Ri**-AuNCs was found to be quenched at around 840 nm and enhanced at 530 nm by Cu^{2+}. The resultant ratiometric fluorescent response can provide a novel sensory probe for the determination of Cu^{2+}. The present probe had excellent selectivity in the presence of several cations. The probe revealed a detection limit of 0.9 μM of Cu^{2+}. Moreover, our proposed probe can reversibly switch between the "on" and "off" states through the addition of Cu^{2+} and EDTA, which is reusable in practical application. Results and method reported here provide a unique strategy for performance of ratiometric assays demonstrated with a AuNCs-based fluorescent probe, which expands the application of AuNCs.

1. Introduction

Copper (II) plays significant roles in a wealth of regulations of biological process. However, Cu^{2+} can also give rise to hazardous environmental issues due to its wide-ranging utilization. Typically, Cu^{2+} in low-dose (<0.9 mg/day) is an indispensable trace nutrient, but short-time challenged with high-dose of Cu^{2+} can pose harm to the kidneys and liver [1]. The allowed concentration of Cu^{2+} in drinking water is less than 2 mg/L (32 μM), according to guidelines for drinking water quality of the World Health Organization (WHO) [2]. Many methods have been reported for detection of Cu^{2+} in water, such as by atomic absorption/emission spectroscopy [3], inductively coupled plasma mass spectrometry (ICP-MS) [4], and capillary electrophoresis [5]. However, most of them are intricate, time-consuming and impractical for a real-time or high-throughput format [6]. Therefore, there is an increasing demand for a simple, reliable, easily accessible and high-throughput routine assay for Cu^{2+} with high sensitivity, selectivity and flexibility. Among the approaches or techniques for Cu^{2+} sensing, the fluorescence detection is more favorable in terms of sensitivity and simplicity of process. Most of the Cu^{2+} fluorescent probes work by monitoring of fluorescence at a single wavelength, but this model may be sometimes problematic for precise fluorescent analysis in practical application, because it may be susceptible to diversity in the sample environment and instrumental fluctuations and thereafter may give rise to false signal readout due to the lack of self-calibrating ability [7]. To the end, a new mode of fluorescent detection (i.e., ratiometric measurement) has been applied to the simultaneous measurement of two fluorescence signals at different wavelengths followed by calculation of their intensity ratio, which can minimize the ambiguities on fluorescence signals, thus giving greater analytic accuracy relative to single-channel detection [8,9]. Therefore, the ratiometric fluorescence probes have widely been used in biochemical fields [10,11]. However, most of ratiometric fluorescent approaches used complex methods to synthesize their fluorescent sensor, using organic solvent and even toxic chemicals [12–14]. The main aim of this work is to

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exploit the green (low-toxicity reagent use, low reagent consumption) and environmental-friendly sensory probe with the advantages of ratiometric fluorescence for the identification of Cu^{2+}.

Rapid growth in nanotechnology provides marvelous opportunities for the application of nanomaterials in biochemical fields, which utilize the unique structural and photophysical features of nanomaterials, such as quantum dot [15], carbon-based nanomaterials [16–18], metal nanoparticles [19,20]. Recently, extensive efforts have been devoted to the facile preparation of fluorescent, water-soluble metal nanoclusters (MNCs) utilizing biocompatible scaffolds [21–24]. These MNCs, e.g., silver nanoclusters (AgNCs) and gold nanoclusters (AuNCs), can present dramatical fluorescence and thus have been used as probes for the detection of various analytes [2,25–30]. Most fluorescence MNCs-based probes are single-channel output, which is usually made on the basis of fluorescence quenching [24,25,27] or fluorescence enhancement [23,28–30]. However, few reports have revealed their applications for ratiometric measurements. Wang et al. developed a ratiometric fluorescent probe array of bovine serum albumin (BSA)-AuNCs for the detection and identification of amino acids [31]. MacLean et al. demonstrated ratiometric detection of Hg^{2+} using DNA-stabilized AgNC [32]. To the best of our knowledge, there are still no reports about ratiometric fluorescence measurement of Cu^{2+} using MNCs-based probes. In the past two years, our group has reported some MNCs-based probes for sensing targets with single signal readout [27–30], herein, we describe our ongoing effort to exploit the facile preparation of novel MNCs for the design of Cu^{2+} probe with the advantages of ratiometric fluorescence.

Riboflavin, also known as vitamin B_2, is a micronutrient with a key role in maintaining health in humans and animals. Riboflavin (**Ri**) is a nucleoside analog compound with yellow–green fluorescence, which has been showed to be a stronger complexing agent absorbing on metal surfaces (gold, silver etc.) through the carbonyl and amine group [33,34]. The binding riboflavin with metal surfaces indicates that there is a potential for riboflavin to act as a ligand in the preparation of some novel metal nanoparticles or nanoclusters [35]. In this respect, we exploit the feasibility of the synthesis of novel gold nanoclusters (AuNCs) using riboflavin as a stabilizer in this work. Moreover, the as-prepared riboflavin-stabilized AuNCs (**Ri**-AuNCs) have been characterized, and luminescence studies indicated that these **Ri**-AuNCs exhibit dual emission peaks at 530 nm and around 840 nm with excitation at 375 nm, however, in the presence of Cu^{2+}, the fluorescence of the **Ri**-AuNCs was found to be quenched at around 840 nm and enhanced at 530 nm by Cu^{2+}, which can act as a fluorescent probe for ratiometric measurement of Cu^{2+} (Scheme 1). Moreover, the ratiometric fluorescent response of **Ri**-AuNCs can reversibly switch between the "on" and "off" states through the addition of Cu^{2+} and EDTA. These **Ri**-AuNCs may help to develop an easier method to detect Cu^{2+} based on the marriage of AuNC's surface properties and riboflavin's physicochemical properties.

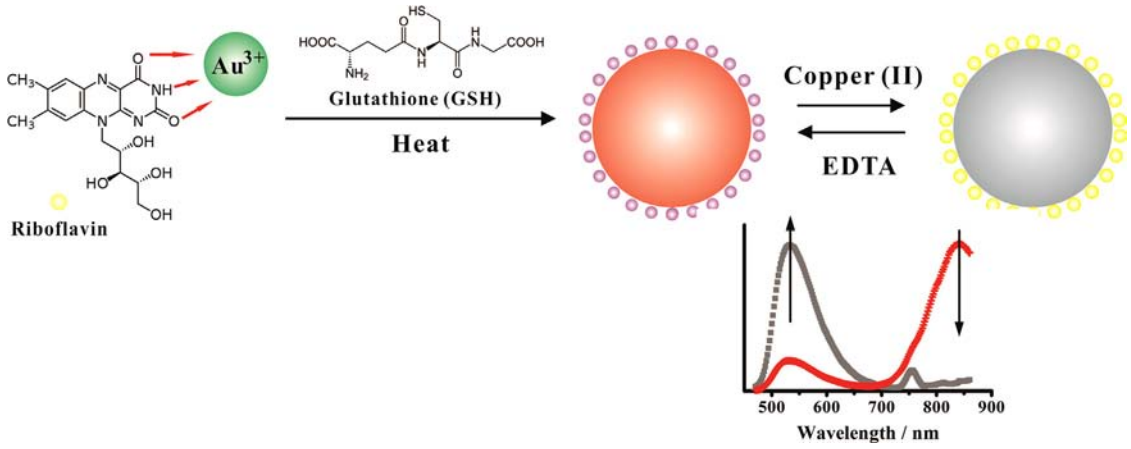
2. Materials and methods

2.1. Materials and instruments

Hydrogen tetrachloroaurate (III) dehydrate ($HAuCl_4$) was purchased from Sinopharm Chemical Reagent Company (Shanghai, China). $NaCl_2$, KCl, $CuCl_2$, $Hg(NO_3)_2$, $PbCl_2$, $ZnCl_2$, $MgCl_2$, $BaCl_2$, $AlCl_3$, $CoCl_2$, $MnCl_2$, $FeCl_3$, $NiCl_2$ and $CaCl_2$ were purchased from Lingfeng Fine Chemical Co., Ltd. (Shanghai, China). Riboflavin was purchased from Generay Biotech Co., Ltd. (Shanghai, China). L-Glutathione (Reduced, GSH) was purchased from Solarbio Co., Ltd. (Beijing, China). EDTA disodium salt was purchased from Pharmacia Biotech Co., Ltd. (Uppsala, Sweden). All chemicals used in this work were of analytical reagent and obtained from commercial sources and directly used without additional purification. $5\times$ PBS buffer ($5\times$ 2.7 mM KCl, $5\times$ 2 mM KH_2PO_4, $5\times$ 136 mM NaCl, $5\times$ 10 mM Na_2HPO_4, pH=7.4) was prepared using metal-free reagents in distilled water purified by a Milli-Q water purification system (Millipore Corp., Bedford, MA) with an electrical resistance of 18.2 MΩ. All glassware was thoroughly cleaned overnight with freshly prepared 3:1 HCl/HNO_3 (*aqua regia*) and rinsed thoroughly with Mill-Q water prior to use. Fluorescence was measured in a fluorescence microplate reader (Bio-Tek Instrument, Winooski, USA) using a black 384 well microplate (Fluotrac 200, Greiner, Germany). Transmission electron microscope (TEM) measurements were performed on Jeol JEM-2100 instrument. Samples for TEM studies were prepared by placing a drop of riboflavin-stabilized gold nanoclusters (**Ri**-AuNCs) solution on a copper grid. The films on the TEM grids were allowed to dry for 2 min following that the extra solution was removed using a blotting paper. Inductively coupled plasma mass spectrometry (ICP-MS) was carried out by a NexION 300 ICP-MS Spectrometer (PerkinElmer Corporation, USA).

2.2. Preparation of riboflavin-stabilized gold nanoclusters (Ri-AuNCs)

Briefly, 2.5 μM riboflavin (**Ri**) and 3 mM $HAuCl_4$ were sequentially added and mixed in an aqueous solution, and the reaction mixture was incubated at room temperature, in the dark, for 30 min. 2.4 mM GSH was added and the reaction mixture was vigorous stirred for 0.5 min. Then the mixture was heated at 90 °C



Scheme 1. Schematic illustration of the ratiometric fluorescent probe for detection of copper (II) utilizing riboflavin-stabilized gold nanoclusters (**Ri**-AuNCs) .

for 35 min. The resulting yellow solutions were centrifuged at 3750 rpm for 20 min, and then the supernatant was further purified and collected by a 10 kDa MWCO centrifuge filter (Millipore) after several runs of the dilution and concentration cycles. The resultant **Ri**-AuNCs were produced with fluorescence emission at 530 nm and around 840 nm (excitation at 375 nm). This as-prepared **Ri**-AuNCs were stored at 4 °C for further use.

2.3. Fluorescence assay for Cu^{2+}

An aliquot of 90 μL **Ri**-AuNCs in aqueous PBS buffer (2.7 mM KCl, 2 mM KH_2PO_4, 136 mM NaCl, 10 mM Na_2HPO_4, pH=7.4) was placed in the wells of a transparent 384-well microtiter plate. Then, 10 μL sample or Milli-Q water was added to the corresponding wells and the resulting solutions were incubated for 10 min before measuring their emission spectra excited at 375 nm. The **Ri**-AuNCs solution can be luminescent exhibiting two fluorescence emission peaks at 530 nm and around 840 nm with excitation at 375 nm, however, in the presence of Cu^{2+}, the fluorescence of the **Ri**-AuNCs was found to be quenched at around 840 nm and enhanced at 530 nm by Cu^{2+}, and the resultant ratios of the fluorescence at 530 and 840 nm ($FI_{530/840}$) are related to the concentrations of Cu^{2+} added, which can be used as a ratiometric indicator for quantitatively detecting Cu^{2+}. The GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA) was employed to perform the data processing.

3. Result and discussion

3.1. Design of the sensing strategy

As a starting point of our study, **Ri**-AuNCs were synthesized by thermal reduction with the addition of reduced glutathione (GSH) (see Experimental section). To synthesize **Ri**-AuNCs, $HAuCl_4$ and **Ri** were mixed in aqueous solution. Following reduction with GSH under high temperature, the transmission electron microscopy (TEM), absorption and fluorescence emission were investigated to confirm the formation of AuNCs (Fig. 1). As shown in Fig. 1C, the as-prepared **Ri**-AuNCs exhibit two fluorescence emission peaks at 530 nm and around 840 nm with excitation at 375 nm. The maximum excitation spectra of the **Ri**-AuNCs were also investigated upon the emission peaks at 530 nm and 840 nm, respectively (Fig. S1), the results indicate that the maximum excitation peaks are both around 375 nm, unless noted otherwise, the following fluorescent measurement was all excited at 375 nm. The **Ri**-AuNCs are highly dispersed in aqueous solution, and a typical TEM image in Fig. 1B shows that the as-prepared **Ri**-AuNCs are monodispersed and uniform. The **Ri**-AuNCs solution can be luminescent exhibiting two fluorescence emission peak at 530 nm and around 840 nm with excitation at 375 nm, however, in the presence of Cu^{2+}, the fluorescence of the **Ri**-AuNCs was found to be quenched at around 840 nm and enhanced at 530 nm by Cu^{2+}, which can be used as a ratiometric indicator for Cu^{2+} (Fig. 1C).

In order to decipher the sensing mechanism, we investigated the fluorescent response of **Ri**, **Ri**+$HAuCl_4$, **Ri**+GSH, and the as-prepared **Ri**-AuNCs upon addition of Cu^{2+} and sequential presence of ethylenediaminetetraacetate (EDTA), a strong Cu^{2+} chelator, respectively (Fig. S2). As depicted in Fig. S2, the fluorescence of **Ri** at 530 nm can be effectively quenched by $HAuCl_4$ due to the **Ri**–Au^{3+} interaction, while GSH shows no influence on it. The as-prepared **Ri**-AuNCs by the thermal reduction of **Ri**-Au^{3+}/GSH can exhibit another fluorescence emission peak at 840 nm, which attributes to the characteristic production of **Ri**-AuNCs. Cu^{2+} is a well-known highly efficient fluorescent quencher toward metal nanoclusters due to its paramagnetic properties via electron or energy transfer [27,36]. Upon challenged with Cu^{2+}, **Ri**-AuNCs was found to be quenched at around 840 nm and enhanced at 530 nm

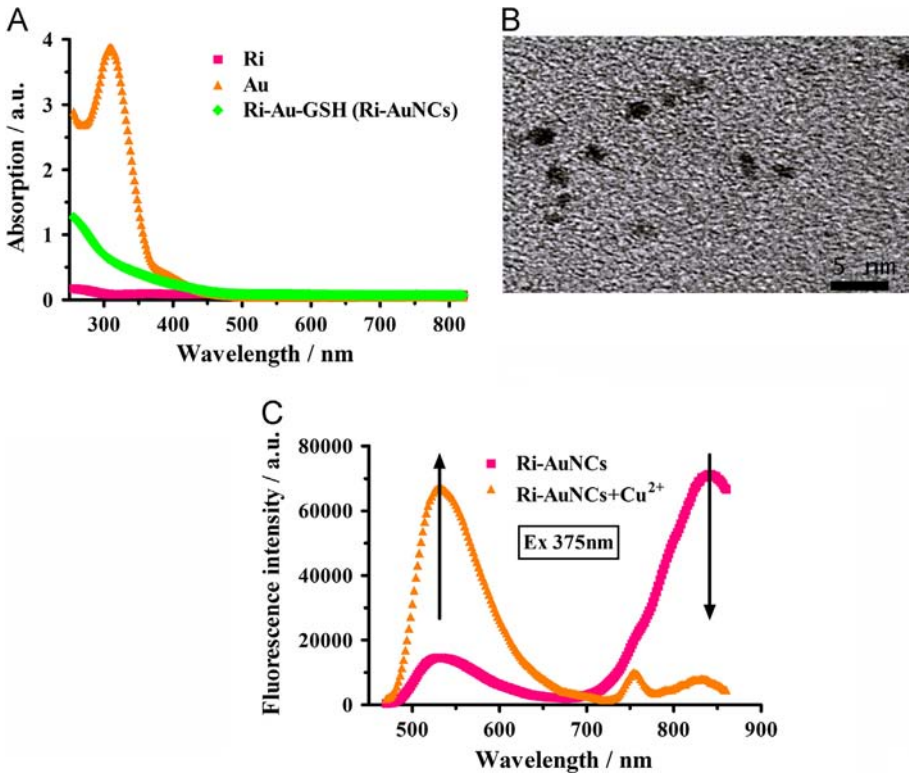


Fig. 1. (A) Typical absorption spectra of the riboflavin, $HAuCl_4$ (Au), and **Ri**-AuNCs, respectively; (B) TEM image of the **Ri**-AuNCs; and (C) typical emission spectra of the riboflavin-stabilized gold nanoclusters (**Ri**-AuNCs) excited at 375 nm.

(Fig. S2). In order to verify whether the quenching fluorescence of **Ri**-AuNCs was due to the fluorescence quenching by metal–metal interaction [27], EDTA was used in competition with **Ri**-AuNCs for Cu^{2+}. As a result, the above Cu^{2+}-triggered fluorescent response of **Ri**-AuNCs (i.e., quenching at around 840 nm and enhancing at 530 nm) can be switched to its default state (Fig. S2). Additionally, the solution of **Ri** was found to be luminescent exhibiting fluorescence emission peak at 530 nm, and upon challenged with various concentrations of Cu^{2+}, its fluorescence intensity at 530 nm shows negligible change (Fig. S3). Therefore, a cascade reaction occurs between **Ri**-AuNCs and Cu^{2+}, in which the as-prepared AuNCs suppress or quench the fluorescence of **Ri** binding on its surface due to the **Ri**–Au^{3+} interaction, and Cu^{2+} can quench the fluorescence of AuNCs to break the **Ri**–Au^{3+} interaction resulting in the fluorescent restoration of **Ri**.

3.2. Ratiometric measurement of copper (II)

Considering the ratiometric changes in fluorescent properties of **Ri**-AuNCs toward Cu^{2+}, the potential of developing a novel fluorescent probe for analytical determination of Cu^{2+} was assessed based on the concept demonstrated above. The different concentrations (0, 0.01, 0.1, 1, 2, 4, 6, 8, 10, 20 and 30 μM) of Cu^{2+} from one stock solution were added to the **Ri**-AuNCs probes. As presented in Fig. 2A, with the addition of an increasing concentration of Cu^{2+} to the suspension of **Ri**-AuNCs, an obvious decrease in the emission peak at around 840 nm and an increase in the emission peak from 530 nm were clearly detected. The sensitivity of the **Ri**-AuNCs probe for the Cu^{2+} was investigated. From Fig. 2B, it can be seen that the fluorescence ratio ($FI_{530}/_{840}$) is sensitive to the concentration of Cu^{2+}, the fitting range is from 0 to 30 μM with a Boltzmann sigmoidal equation $Y=0.1959+13.2041/[1+\exp(8.184-X)/1.805]$, where Y is the fluorescence ratio ($FI_{530}/_{840}$) and X is the concentration of Cu^{2+} (regression coefficient $R^2=0.9856$). A series of 8 repetitive measurements with 4 μM Cu^{2+} was used for investigating the precision of **Ri**-AuNCs probe response, and obtained a relative standard deviation (RSD) of 5.4%, demonstrating an excellent reproducibility of the assay. The limit of detection of Cu^{2+} using **Ri**-AuNCs probe based on 3σ was approximately 0.9 μM, which was better than the level (32 μM) of drinking water defined by WHO. Compared to the detection limit of the reported AuNCs-based method of 0.5 μM [2], this sensing platform is one of the most sensitive Cu^{2+} detection methods. This reported AuNCs-based method is a single-channel output on the basis of fluorescence quenching of the sensing unit by the Cu^{2+}, while our proposed method performed with the advantages of ratiometric fluorescence for the identification of Cu^{2+}. In order to test the feasibility of our proposed method in real applications, we evaluated its ratiometric fluorescent response of Cu^{2+} in tap water and river water (Table 1 and Table S1). Before spiking Cu^{2+} in real water samples, no ratiometric fluorescent response of the **Ri**-AuNCs probe was observed in the real water samples, indicating Cu^{2+} in these real water samples was not detected (below the limit of detection of concentration in the proposed method). However,

Table 1
Comparison of the concentrations of Cu^{2+} in tap water and spiked tap water by our proposed method and the ICP-MS method.

Sample	Concentration (μM)		
	Added	The presented method	ICP-MS
Tap water	0	Not found	Not found
Spiked tap water	3	3.03	3.01
Spiked tap water	5	5.13	5.02

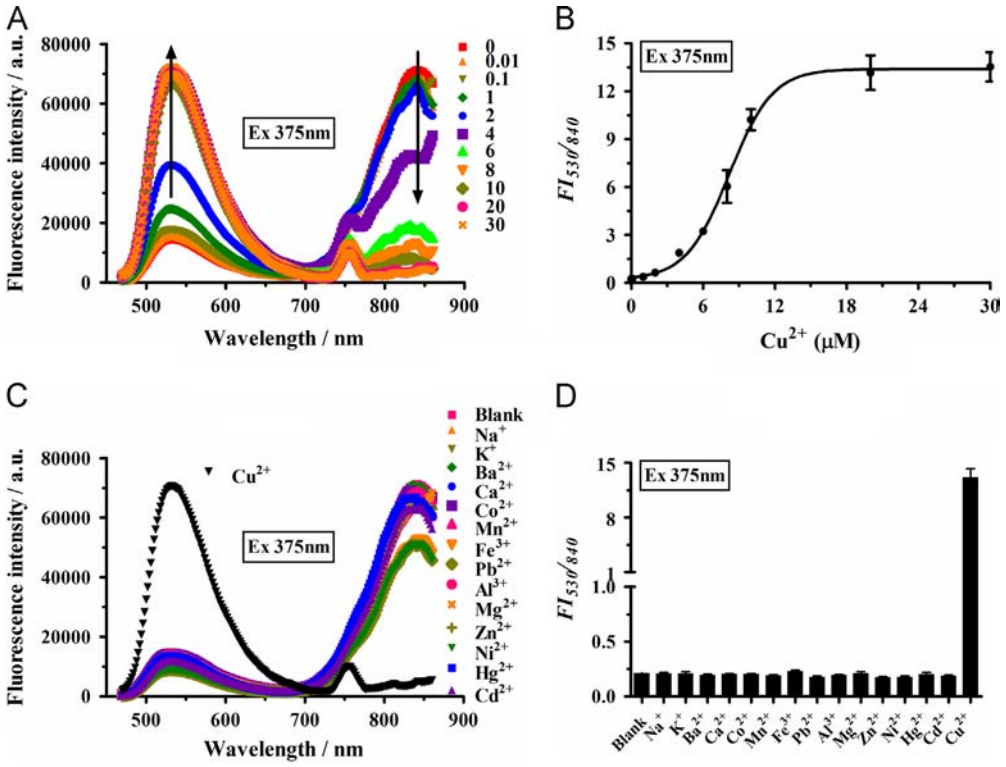


Fig. 2. (A) The fluorescence emission spectra are shown for various Cu^{2+} concentrations of 0, 0.01, 0.1, 1, 2, 4, 6, 8, 10, 20 and 30 μM and (B) plot of the fluorescence ratio ($FI_{530}/_{840}$) vs. the increasing concentrations of Cu^{2+} of the same data. (C) Fluorescence response of **Ri**-AuNCs solution upon addition of 100.0 μM other metal ions (Na^+, K^+, Ba^{2+}, Ca^{2+}, Co^{2+}, Mn^{2+}, Fe^{3+}, Pb^{2+}, Al^{3+}, Mg^{2+}, Zn^{2+}, Ni^{2+}, Hg^{2+} and Cd^{2+}) and 20.0 μM Cu^{2+} and (D) bars represent fluorescence ratio ($FI_{530}/_{840}$) of **Ri**-AuNCs incubated with various metal ions mentioned above.

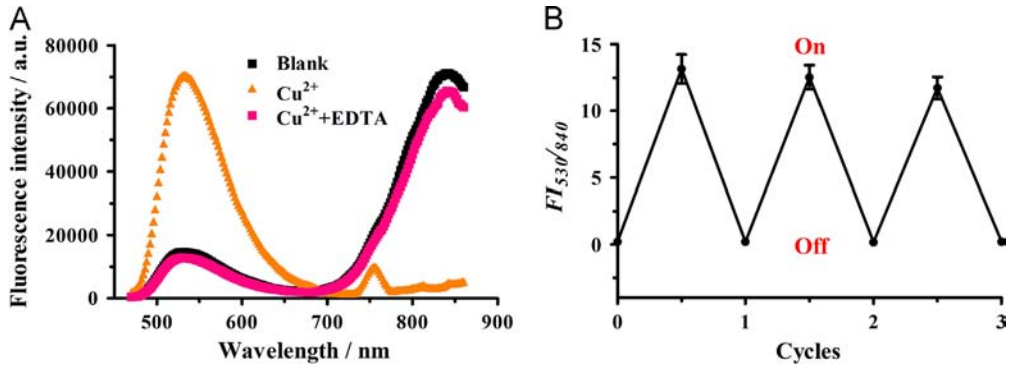


Fig. 3. Reversible switching of the described sensor between the on and off states through the alternating addition of Cu^{2+} and EDTA.

upon Cu^{2+} present in the real water samples, the **Ri**-AuNCs probe was induced with a ratiometric fluorescent response. The unknown concentrations of Cu^{2+} in different samples were measured by the standard addition method using both **Ri**-AuNCs probe and an ICP-MS method. Recovery of added known amount Cu^{2+} to the samples was in general larger than 95%, and the proposed method in this work has similar results for Cu^{2+} detection as that of the ICP-MS method, which indicated that the present method has a promise in practical application with great accuracy and reliability (Table 1). The ICP-MS method usually requires expensive and complicated instruments and professional procedures, while our proposed method can provide a simple, cost-effective sensory probe for rapid monitoring of Cu^{2+} within minutes.

To test selectivity, competing stimuli including 100 μM other metal ions (Na^+, K^+, Ba^{2+}, Ca^{2+}, Co^{2+}, Mn^{2+}, Fe^{3+}, Pb^{2+}, Al^{3+}, Mg^{2+}, Zn^{2+}, Ni^{2+}, Hg^{2+} and Cd^{2+}) were examined under the same conditions as in the case of 20 μM Cu^{2+}. It was found that Cu^{2+} results in an obvious change in the $FI_{530/840}$ (around 15), while there was nearly negligible $FI_{530/840}$ change (below 0.25) upon in the presence of other stimuli (Fig. 2D). The results demonstrated the excellent selectivity of this approach applied in Cu^{2+} detection over other metal ions (Fig. 2CD).

3.3. *The reversibility of Cu^{2+} sensing operation*

For a chemical sensor to be extensively employed in the detection of specific analytes, the reversibility is an important aspect. We also studied the reversibility of Cu^{2+} sensing operation based on **Ri**-AuNCs probe (Fig. 3). Fig. 3B shows the repeated switching behavior with alternating addition of Cu^{2+} and EDTA. This process could be repeated at least three times without loss of sensitivity, which clearly demonstrates the high degree of reversibility of the complexation/decomplexation process, which further confirmed the same conclusion suggested in the above discussion.

4. Conclusion

In this work, we have successfully synthesized a novel luminescent AuNCs (**Ri**-AuNCs) employing riboflavin as a stabilizer by a simple, rapid and one-pot green procedure. The **Ri**-AuNCs solution can be luminescent exhibiting two fluorescence emission peaks at 530 nm and around 840 nm with excitation at 375 nm, however, in the presence of Cu^{2+}, the fluorescence of the **Ri**-AuNCs was found to be quenched at around 840 nm and enhanced at 530 nm by Cu^{2+}, which can be used as a ratiometric indicator for quantitatively detecting Cu^{2+}. A cascade reaction occurs between **Ri**-AuNCs and Cu^{2+}, in which the as-prepared AuNCs suppress or quench the fluorescence of **Ri** binding on its surface due to the **Ri**–Au^{3+} interaction, and Cu^{2+} can quench the fluorescence of AuNCs to break the **Ri**–Au^{3+} interaction resulting in the fluorescent restoration of **Ri**. The resultant ratiometric fluorescent response was successfully applicable for the sensitive and selective detection of Cu^{2+} in water samples within 10 min. The limit of detection of Cu^{2+} using **Ri**-AuNCs probe based on 3σ was approximately 0.9 μM. Moreover, the reversibility of Cu^{2+} sensing operation based on **Ri**-AuNCs probe can perform with alternating addition of Cu^{2+} and EDTA. This is a new concept for performance of ratiometric assays demonstrated with a AuNCs-based fluorescent probe, which exhibits intrinsic attractive properties and expands the application of AuNCs.

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Appendix A. supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2013.09.034.

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