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Visual and fast detection of trace copper ions using biosensor based on FRET

Yaqin Zhao *, Dong Chen [1], Jing Yang [1], Binsheng Yang *

Key Laboratory of Chemical Biology and Molecular Engineering of Ministry of Education, Institute of Molecular Science, Shanxi University, Taiyuan 030006, China

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ABSTRACT

The development of a simple, rapid and sensitive sensor to detect copper ion is very important for environmental detection. Here, we constructed a fluorescence biosensor based on fluorescence resonance energy transfer (FRET) between copper resistance operon coded protein C (CopC), a copper binding protein, and dansyl chloride (DNS-Cl) to selectively detect copper ion. At alkaline conditions, DNS-Cl was contently attached to CopC forming biosensor of DNS-Cl/CopC, in which fluorescence emission of CopC at 320 nm was absorbed by DNS-Cl. After binding with copper ion, the fluorescence of DNS-Cl was quenched significantly to 47%. Within the range of 0.04–11 μM ($R = 0.989$), the good linearity was obtained and the detection limit reached to 7 nM. More importantly, the biosensor of DNS-Cl/CopC has been successfully used to detecting of copper ion level in purified water, tap water and waste water drained from steel mill. And the results were consistent well with those obtained from the inductively coupled plasma mass spectrometry. Therefore, the established biosensor DNS-Cl/CopC is a creditable method to detect copper ion with high sensitivity and selectivity, which can be utilized as a powerful tool to monitor copper pollution in the environments.

1. Introduction

Copper is one of the most bioessential elements and serves as an useful cofactor for various metalloenzymes of cytochrome coxidase, tyrosinase, and super-oxide dismutase, etc. [1–5]. However, redundant copper ion can lead to a series of diseases including liver or kidney damage, Menkes disease, gastrointestinal distress, and serious Alzheimer's disease, [6,7]. Hence, appropriate nutritional levels of copper should be strictly regulated in organism by copper homeostatic system. The allowed maximum level of copper in water is at 1.3 ppm (20 μM) by the United States Environmental Protection Agency (EPA) [8]. The fast and simple detection method of trace copper ion from environmental becomes very necessary. So far, the most widely used methods for metal ions determination focused on the atomic absorption spectrometry (AAS) inductively coupled plasma-mass spectrometry (ICP-MS), and voltammetry methods [9,10]. Recent years, various methods on the basis of fluorimetric detection, chemi-luminescence immunoassay, colorimetric assay, electrochemical investigation, and so on [11–17] have been developed for the detection of copper ions. In spite of good detection limits and working ranges, these methods are not suitable for convenient and fast detection requirements due to disadvantages of requiring expensive instrument, tedious operation procedures and complicated sample preparations and so on. Pursuing for a simple, selective, sensitive, and inexpensive method for detection of copper ions is very urgent for both environment and human health. In comparison, biological macromolecules can be directly obtained from organisms. For their special bio-functions as well as good biocompatibility, biosensor of macromolecules was preferred by scientific researchers.

Copper resistance operon coded protein C (CopC) is a member of the operon CopABCD that is responsible for copper resistance. In the oxidizing environment of the periplasmic space of *Pseudomonas syringae* pathovar *tomato*, it acts as a copper chaperone [18]. It takes on topology barrel structure by nine β-sheets. The protein of CopC is a small soluble molecule protein, which contains nine positive residues (Arg, Lys) and eight negative residues (Glu, Asp). So far, no papers reported that other metal ions apart from cooper take part in the function of CopC. It is an important copper binding protein. The NMR results (PDB: 1ot4) of CopC indicated that residues of His1, Glu27, Asp89 and His 91 in the N-terminal domain involved in the coordination of Cu^{2+} with protein [19,20]. Its stability and good biocompatibility makes it a promising probe for sensing applications. Considering the specificity of CopC for copper and high fluorescence quantum yield of dansyl chloride (DNS-Cl), we prepared a new biosensor by covalent bonding DNS-Cl with amine groups from CopC at alkaline conditions (at pH 9.3). Based on the fluorescence resonance energy transfer (FRET), fluorescence of DNS-Cl can be excited by emission of Trp83 on CopC (Scheme 1). The

* Corresponding authors.
E-mail addresses: zhaoyaqin@sxu.edu.cn (Y. Zhao), yangbs@sxu.edu.cn (B. Yang).
[1] These authors contributed equally.

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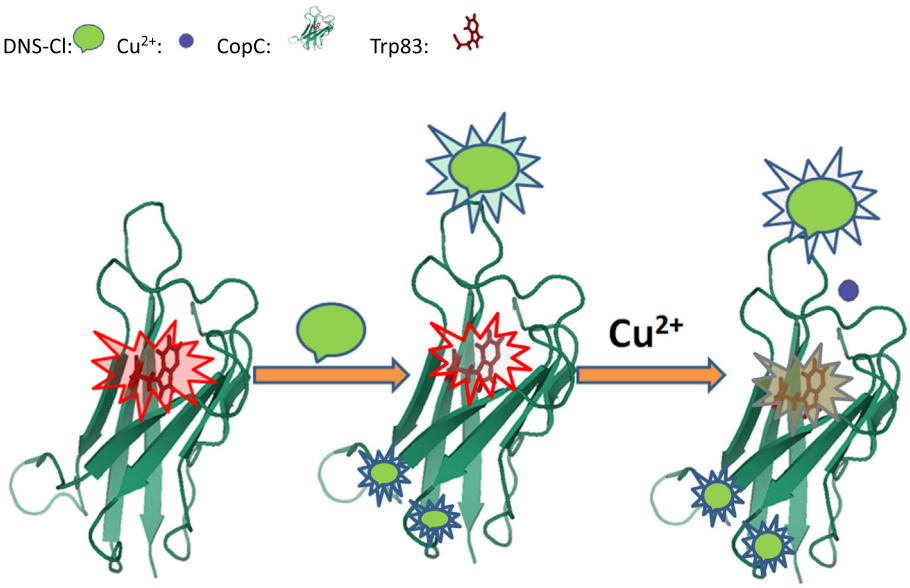


Scheme 1. Proposed mechanism for the detection of copper ion by DNS-Cl/CopC.

results indicated that biosensor DNS-Cl/CopC possessed excellent selectivity, good sensitivity and strong anti-interference ability for the detection of Cu^{2+}. The limit of detection was calculated to be 7 nM, which was much lower than the maximum level of Cu^{2+} (20 nM) in drinking water by EPA [8]. Furthermore, the biosensor DNS-Cl/CopC was successfully used to detecting copper levels in tap water and waste water drained from steel mill with satisfactory recovery results.

2. Experimental

2.1. Reagents and apparatus

$CuCl_2$, KCl, $CaCl_2$, $MgCl_2$, $ZnCl_2$, $MnCl_2$, $CdCl_2$, $FeCl_2$, $CoCl_2$, $NiCl_2$, $AlCl_3$, $FeCl_3$ and N-(2- Hydroxyethyl)ethylenedinitrio-N,N′,N′-triacetic acid (HEDTA) were purchased from Sangon Chemical Reagent Co., Ltd. (Shanghai, China). Dansyl chloride (DNS-Cl) was purchased from Sigma. All chemicals and metal salts used in this research were of analytic grade. And other chemicals utilized in protein purification are of analytical grade. Deionized water was purified by Milli-Q system (Millipore, Bedford, MA, USA).

Tryptone, yeast extract, ampicillin (Amp^r) and isopropyl-β-D-thiogalactopyranoside (IPTG) were purchased from Amresco Ltd. Other biochemical reagents in construction, expression and purification of proteins were purchased from TaKaRa.

PD-10 column was purchased from GE Healthcare.

DNS-Cl concentration was determined from its molar extinction in acetone at 369 nm.

2.2. Expression and purification of CopC

After verification by DNA sequence analysis, the recombinant plasmid pET-28a-CopC was transferred into *E. col.* (DE3), which was incubated 3 h at 37 °C. Protein synthesis was induced using IPTG (0.2 mM) for 5 h, at optical density of 0.6–0.8 (at 600 nm). As previously reported [21], proteins of CopC were purified by CM-25 cation exchange and DEAE anion exchange chromatography. The purity of the intermediate and final samples was assessed by SDS-PAGE. After purification, the proteins were concentrated and kept at −80 °C. Protein concentration was measured by the absorption at 280 nm with an extinction coefficient of $\varepsilon_{280} = 6970\ M^{-1}$.

2.3. Preparation of biosensor DNS-Cl/CopC

Dansyl chloride solution of 0.2 M was made fresh in acetone. Appropriate portions were added into the CopC solution ([DNS-Cl]/[CopC] = 50:1) in 30 mM Na_2CO_3/$NaHCO_3$ buffer at pH 9.3. The mixed solution was incubated at 25 °C for 12 h in the darkness. The un-reacted DNS-Cl was removed through PD-10 column, which was monitored by measuring absorbance of fraction at 280 nm and 330 nm. Labeled protein was stored in darkness at −20 °C until use.

2.4. Fluorescence detection of Cu^{2+} with the DNS-Cl/CopC

Fluorescence change of biosensor DNS-Cl/CopC in the absence or presence of metal ions was observed by naked eyes with triple 365 nm UV lamp (Jing ke Industrial Co., Ltd., Shanghai, China). And fluorescence intensity changes of DNS-Cl/CopC in the absence or presence of metal ions was quantitatively investigated by FluoroMax-4 spectrofluorometer equipped with a xenon lamp (HORIBA Scientific, Japan). The fluorescence spectra were recorded ranging from 290 to 550 nm with excitation at 280 nm. The solution of 1500 μL DNS-Cl/CopC in Na_2CO_3/$NaHCO_3$ buffer at pH 9.3 was added to a 1 cm sample cuvette, and then the metal ions solution was added to the above solution gradually. During the titration, the sample was maintained at 25 °C by using a jacketed cell holder connected to an external circulating water bath (Huber). To correct for dilution during each titration and to normalize the results from different titrations, the fluorescence intensity were converted to molar fluorescence intensity by dividing the fluorescence intensity by the analytical concentration of DNS-Cl/CopC.

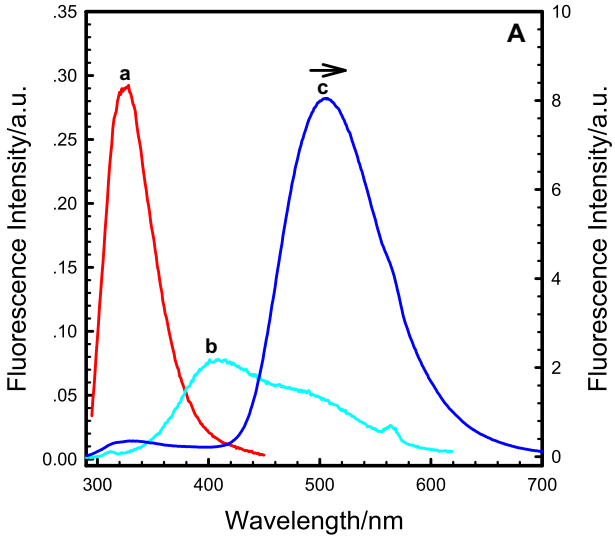
3. Results and discussion

3.1. Establishment of Cu^{2+} biosensor

Dansyl chloride (DNS-Cl) is the most widely used fluorogenic derivatizing reagent for amino acid determination in proteins and peptides [22,23]. It displayed different fluorescence emission in solvents with different polarity. As reported methods [24,25], we labeled protein of CopC with DNS-Cl. In Na_2CO_3/$NaHCO_3$ buffer at pH 9.3, DNS-Cl was firstly added into the solution of protein CopC with the ratio to be 50:1 and then incubated overnight at 25 °C. For removing the excess DNS-Cl molecules, the mixed solutions were passed through PD-10

column. With excitation of 280 nm, fluorescence emission of DNS-Cl in Na_2CO_3/$NaHCO_3$ buffer at pH 9.3 appeared at 405 nm (curve b, Fig. 1A). The protein of CopC contains only one Trp residue as well as one Tyr residue. Its fluorescence emission appeared at 320 nm (curve a, Fig. 1A). And the reaction production of mixed solution of DNS-Cl with CopC displayed different fluorescence emission at 505 nm (curve c, Fig. 1A). Hence, we inferred that new complex of DNS-Cl with CopC formed. For acryl chloride complex (for example DNS-Cl), it can bind to functional amine groups on proteins and form a fluorescent sulphonamide or to phenol group of tyrosine [22]. As indicated from PDB (1ot4), CopC contains five Lys residues (Lys4, Lys23, Lys29, Lys38 and Lys74) and one Tyr residue (Tyr79) (Fig. 1B), which may be the potential binding sites of DNS-Cl. After forming covalent bond with CopC, fluorescence emission of DNS-Cl was shifted to 505 nm. By measuring CopC and DNS-Cl concentration, it can be obtained that per molar CopC may bind three DNS-Cl molecules (data not shown). Then, we obtained biosensor DNS-Cl/CopC.

The sensors of DNS-Cl/CopC reported here are based on fluorescence resonance energy transfer (FRET) between Trp from CopC and DNS-Cl (Scheme 1). With excitation at 280 nm, fluorescence emission of Trp83 on CopC appeared at 320 nm, which was absorbed by DNS-Cl. At the same time, this emission was used to excite fluorescence of DNS-Cl bound on protein. As shown in Fig. S1, fluorescence emission



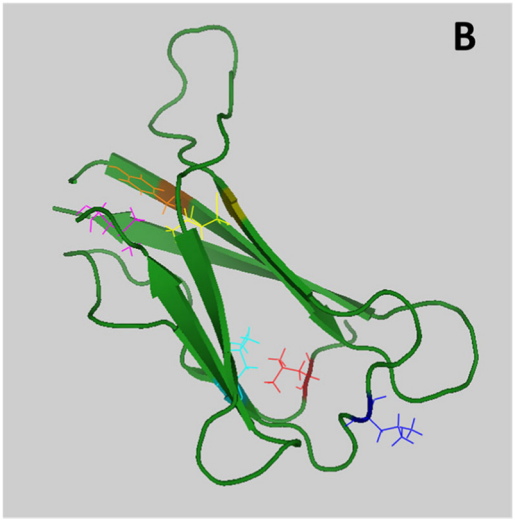


Fig. 1. A: Fluorescence emission of CopC (a), DNS-Cl (b) and DNS-Cl/CopC complex (c). B: X-ray structure of CopC (PDB: 1ot4). red: Lys4, cyan: Lys23, blue: Lys29, yellow: Lys38, magenta: Lys74, and orange: Tyr79.

of DNS-Cl may be various with different excited wavelength. Its emission located at 505 nm with optimal excitation of 320 nm. With excitation of 280 nm, only one peak at 405 nm can be observed. And, fluorescence emission of DNS-Cl appeared at 405 nm as well as 505 nm with excitation of 290 nm. Thus, we can infer that for biosensor DNS-Cl/CopC, emission at 505 came from FRET from Trp on CopC to DNS-Cl.

3.2. Selectivity and mechanism of biosensor DNS-Cl/CopC for Cu^{2+}

For obtaining optimal conditions, fluorescence emission of DNS-Cl/CopC was carried out at different pH and different temperatures. It can be seen from Fig. S2A that DNS-Cl/CopC displayed significant fluorescence emission at pH 10.3 and 9.3. However, copper ions maybe can't bind with CopC at pH 10.3 (Fig. S2B). Hence, the buffer of 30 mM Na_2CO_3/$NaHCO_3$ (pH 9.3) was used in the manuscript. Comparing curve a, b and c in Fig. S2C, it can be indicated that only collisional quench can be observed at different temperatures. We chose room temperature of 25 °C as experimental conditions. To verify the selectivity, biosensor of CopC labeled with DNS-Cl (DNS-Cl/CopC) with 20 μM Cu^{2+} or 1 mM Na^+ or K^+ or Ca^{2+} or Zn^{2+} or Mg^{2+} or Mn^{2+} or Cd^{2+} or Fe^{2+} or Co^{2+} or Ni^{2+} or Pb^{2+} or Fe^{3+} or Al^{3+} was incubated in 30 mM Na_2CO_3/$NaHCO_3$ buffer solution (pH 9.3) at 25 °C for 10 min, respectively. As shown in Fig. 2A, the fluorescence emission of DNS-Cl/CopC was quenched only by copper ion, which can be recognized by naked eyes (Fig. 2A insert). It indicated that DNS-Cl/CopC could be selectively quenched by Cu^{2+}. Moreover, there was no obvious interference on the detection of Cu^{2+} in the presence of other interfering metal ions for DNS-Cl/CopC (Fig. 2B). In addition, the presence of other amino acids for example His, Asp, Glu and so on has not effect on the Cu^{2+} quenching emission from DNS-Cl (Fig. 2C). Therefore, DNS-Cl/CopC could be used as a selectively fluorescence sensors for Cu^{2+} in aqueous medium.

It is well know that dansyl chloride DNS-Cl reacts with the free amino groups on CopC resulting in proteins that fluoresce at blue. There is significant spectra overlay between absorptivity of DNS-Cl and fluorescence spectra of CopC (Fig. 2D). Thus, FRET between CopC and DNS-Cl happened. Fluorescence emission of CopC at 320 nm, with excitation at 280 nm, may excite fluorescence of DNS-Cl, whose emission appeared at 505 nm. And, fluorescence emission of DNS-Cl can't be excited by 280 nm (Fig. S1). After binding with CopC, copper ion coordinated with His1, His91, Glu27 and Asp89 on protein (PDB: 1ot4). Due to heavy metal ions effects, the emission of fluorophore can be turned off by their partially filled orbital, heavy-atom effects or unpaired electrons. It may quench the fluorescence via electron-transfer pathways [26]. The electron transfer takes place between coordination amino acids from CopC and copper ion, which further resulted in the fluorescence quenching of Trp at 320 nm on CopC. By virtue of decrease of emission of CopC, significant fluorescence quenching of DNS-Cl bound on CopC can also be observed (Fig. 2A). The fluorescence quenching of DNS-Cl/CopC in the presence of copper ion can be quantitatively detected and the fluorescence quenching efficiency is calculated to be 56.3% by $(F_0 - F_1)/F_0 \times 100\%$, where F_0 and F_1 mean the fluorescence intensity of DNS-Cl/CopC in the absence and presence of copper ion, respectively [27].

3.3. Sensitivity and fluorescence restore of DNS-Cl/CopC

The detection of various metal ions with selective and sensitive probes is of considerable interest due to their vital role in the human body even in trace amounts [28,29]. In order to investigate the sensitivity to Cu^{2+}, fluorescence spectra of DNS-Cl/CopC upon addition of different amount of Cu^{2+} ions in 30 mM Na_2CO_3/$NaHCO_3$ buffer solution at pH 9.3 at 25 °C were obtained. As can be seen from Fig. 3A, upon gradual addition of Cu^{2+} to DNS-Cl/CopC, fluorescence intensity at 505 nm decreased gradually. Upon addition of 1.0 equiv of Cu^{2+}, the

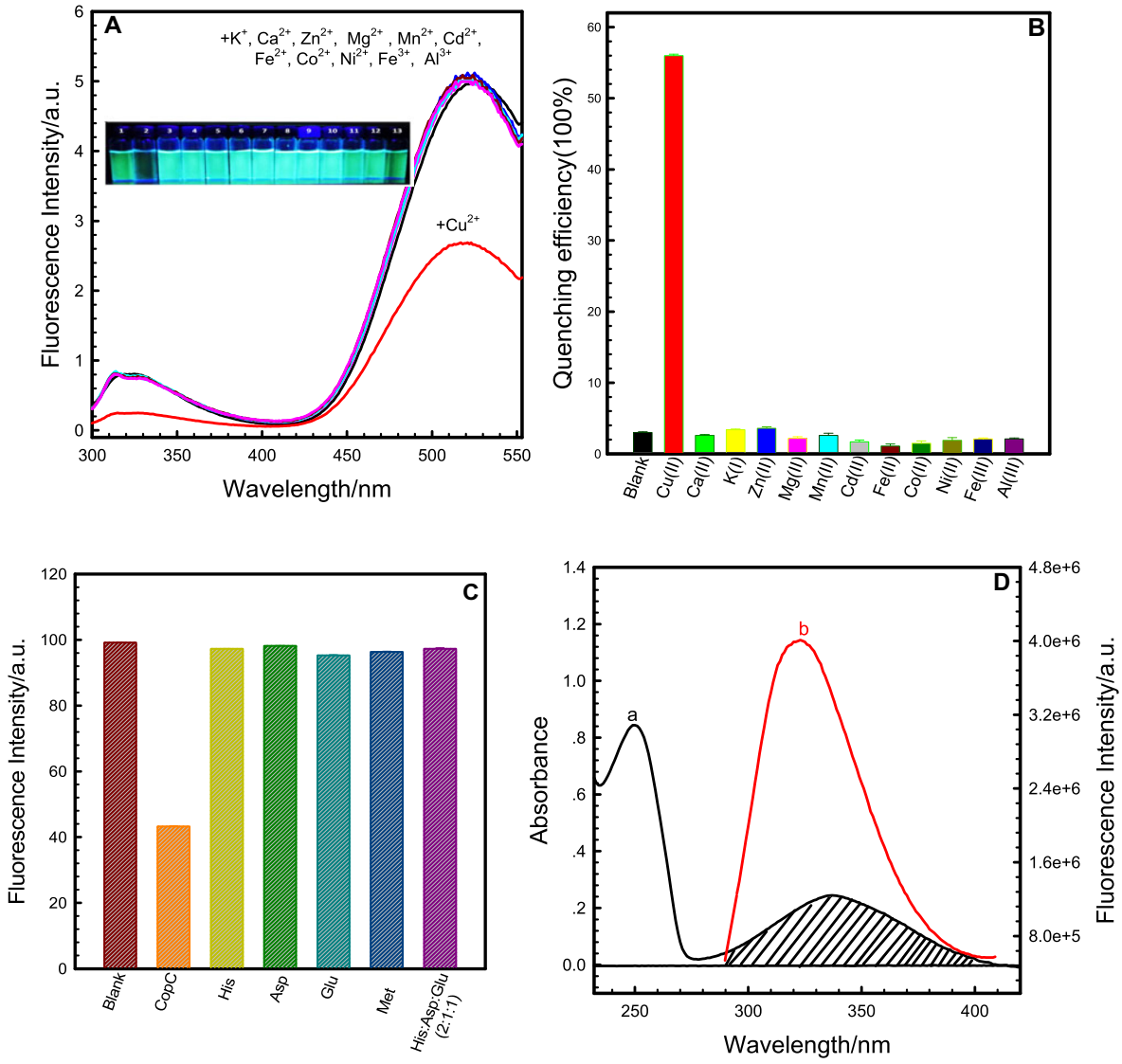


Fig. 2. A: Fluorescence spectra of 10 μM biosensor DNS-Cl/CopC upon addition of various metal ions (2.0 equiv.) in 30 mM Na_2CO_3/$NaHCO_3$ buffer solution (pH 9.3) at 25 °C. insert: Images of DNS-Cl/CopC with metal ions, from left to right: blank, Cu^{2+}, Na^{+}, Ca^{2+}, K^{+}, Zn^{2+}, Mg^{2+}, Mn^{2+}, Cd^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+} and Fe^{3+}, respectively. B: Fluorescence quenching efficiency of metal ions (M^{n+}) to DNS-Cl/CopC at 505 nm ([Cu^{2+}]/[DNS-Cl/CopC] = 2.0, [M^{n+}]/[DNS-Cl/CopC] = 10.0.) C: Fluorescence intensity of residues His or Asp or Glu or Met or mixed solutions ([His]:[Asp]:[Glu] = 2:1: 1) with the addition of 5 equiv Cu^{2+}. D: Spectral overlap between CopC (a) emission and DNS-Cl (b) absorption, in 30 mM Na_2CO_3/$NaHCO_3$ buffer (pH 9.3) at 25 °C.

fluorescence intensity quenched almost 53%. At the same time, the solution color changed from green to colorless with 365 nm UV lamp (Fig. 3A, insert). As shown in Fig. 3B, a good linear correlation was obtained between the quenching efficiency and the concentration of copper ion from 0.04 to 11 μM. The detection limit of DNS-Cl/CopC for Cu^{2+} was determined to be (6.81 ± 0.07) nM using the eq. DL = 3 s/k, where s is the standard deviation of the blank solution (11 times) and k is the slope of the calibration curve [30,31]. The probe in the present manuscript was more superior to the reported ion fluorescence probes by polyamine-functionalized carbon quantum dots and pyoverdine-similar chemosensors [27,30]. Titration of DNS-Cl/CopC by adding the solutions of copper was shown in Fig. 3C. It can be seen that a sharp inflexion appeared at about [Cu^{2+}]/[DNS-Cl/CopC] = 1.0, which confirmed 1:1 stoichiometric ratio of the copper to DNS-Cl/CopC. It further proved that labeling DNS-Cl did not change binding ratio of copper on CopC.

The time course for the fluorescence response of 10 μM DNS-Cl/CopC upon addition of Cu^{2+} in 30 mM Na_2CO_3/$NaHCO_3$ buffer solution (pH 9.3) at room temperature was shown in Fig. 4A. It was found that upon addition of Cu^{2+} to DNS-Cl/CopC, the obvious spectral decrease of DNS-Cl/CopC at 505 nm can be observed within 30 s. Therefore, this system can be used for real-time and fast tracking of Cu^{2+}. In addition, we also investigated the restore and re-usability of DNS-Cl/CopC biosensor. In 30 mM Na_2CO_3/$NaHCO_3$ buffer solution (pH 9.3), fluorescence intensity of DNS-Cl bound with CopC at 505 nm, which was quenched by cupric initially, can be restored after adding N-(2-Hydroxyethyl) ethylenedinitrio-N,N′,N′-triacetic acid (HEDTA) within 30 s (Fig. 4B). We can infer that cupric could be competed efficiently by HEDTA. This process is reversible. And then the DNS-Cl/CopC could be regained by PD-10 column, which still could be utilized again to detect copper ion.

3.4. Applications of the biosensor to drinking water and waste water

To investigate the applicability of our biosensor for practical samples, we analyzed the copper concentration in tap water and waste water drained from steel mill. Samples were also detected by our biosensor and ICP-MS, respectively. As shown Table 1, the concentrations of copper ion from purified water were very low, in which no detectable

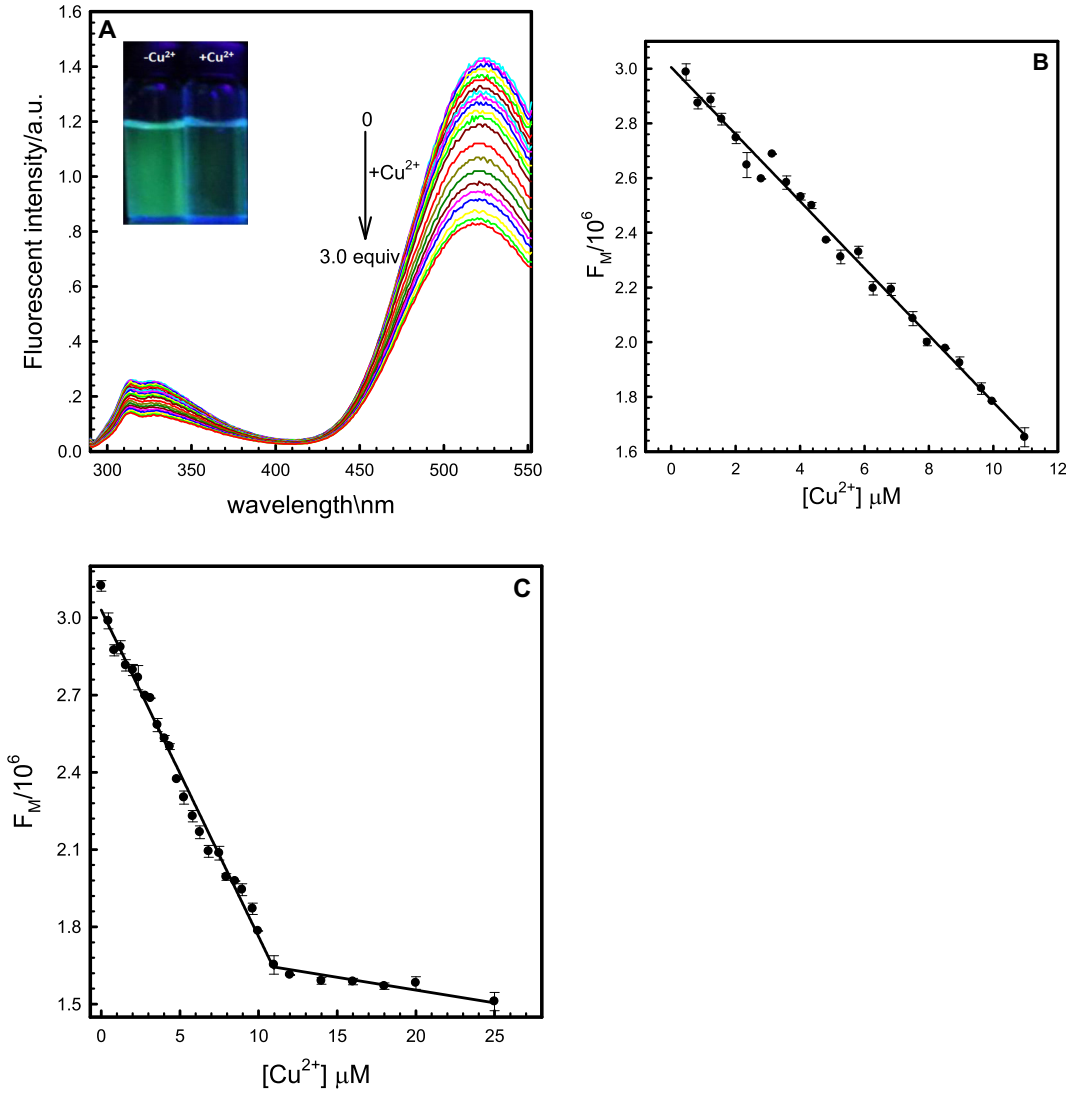


Fig. 3. A: Fluorescence spectra of 10 μM biosensor DNS-Cl/CopC with the addition of 0–3 equiv. Cu^{2+} in 30 mM Na_2CO_3/$NaHCO_3$ buffer (pH 9.3) at 25 °C. (Insert: Images of DNS-Cl/CopC in the absence or presence of Cu^{2+}). B: Changes of fluorescence intensity at 505 nm with Cu^{2+} concentration between 0.04 and 11 μM with liner regression equation of $y = 3.0–1.21\times$, $r = 0.9936$, where 'y' represents the fluorescence intensity of DNS-Cl/CopC and 'x' represents the concentration of copper ion. C: Titration curve for the addition of DNS-Cl/CopC with Cu^{2+} in 30 mM Na_2CO_3/$NaHCO_3$ (pH 9.3) at 25 °C.

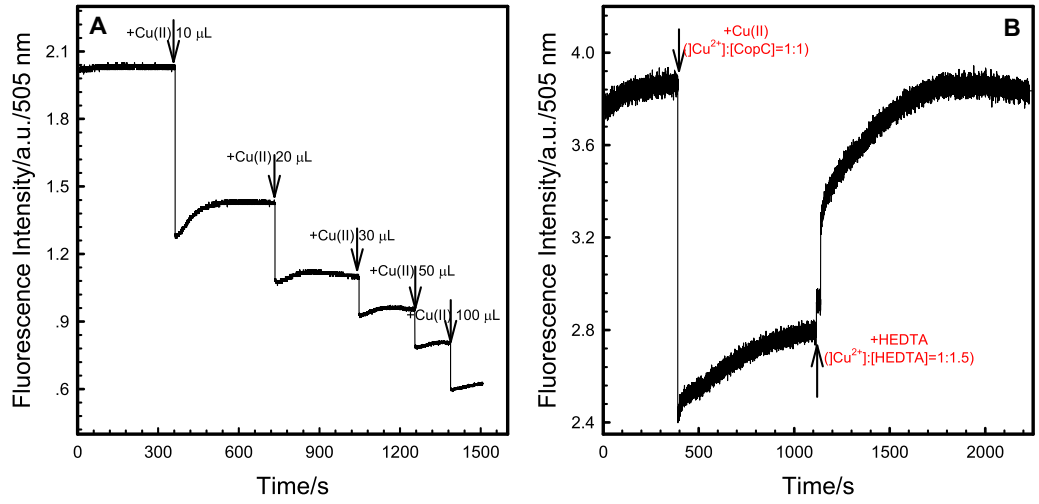


Fig. 4. The changes of fluorescence emission intensity of DNS-Cl/CopC in the presence of Cu^{2+} with different concentration ratios Cu^{2+} to DNS-Cl/CopC (A) or HEDTA ([Cu]/[HEDTA = 1:1.5) (B).

Table 1
Comparison of the results obtained by biosensor DNS-Cl/CopC and ICP-MS for the detection of copper ion in samples (n = 5).

Sample	Added Conc./μM	Detected/μM	Recovery (100%)	ICP-MS/μM
Purified water	0	ND	ND	ND
	0.8	0.78 ± 0.02	97.5 ± 2.5	0.82
	8.0	7.91 ± 0.05	98.9 ± 0.6	7.92
Drinking water	0	ND	ND	0.013
	0.8	0.81 ± 0.04	101.2 ± 5	0.79
	8.0	8.19 ± 0.1	102.3 ± 1.2	8.02
Waste water	0	ND	ND	9
	0.8	0.79 ± 0.03	98.7 ± 3.8	0.82
	8.0	7.95 ± 0.4	99.3 ± 5	8.04

ND means no detectable.

copper can be monitored by ICP-MS. In spite of using our biosensor DNS-Cl/CopC, no corresponding copper level was obtained. As for samples of the tap water and waste water drained from steel mill, their copper concentrations may be detected directly by our biosensor, which is consistent with the results from ICP-MS. Therefore, our biosensor could be used to detect whether sewage from steel mill can be discharged directly. All these results indicated that our biosensor were practically feasible for monitoring copper ion in real samples with good reliability and high accuracy.

4. Conclusion

We have designed an effective fluorescent biosensor for detecting copper ion in aqueous. The biosensor was prepared based on fluorescence resonance energy transfer (FRET) between luminescent compound DNS-Cl and copper binding protein of CopC. The obtained biosensor DNS-Cl/CopC displayed excellent selectivity and the detection limit reached to 7 nM. Additionally, the biosensor has been successfully applied to detect copper ion in drinking water, especially waste water drained from steel mill. It can be used to estimate whether the waste water can be discharged directly.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.saa.2019.03.082.

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