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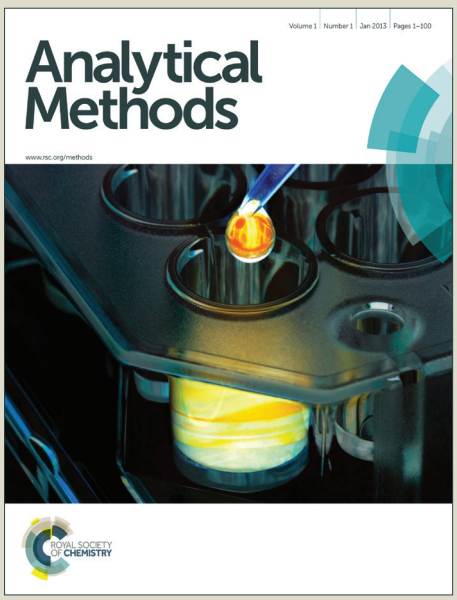

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ARTICLE TYPE

Detection of Fe(III) and bio-copper in human serum based on fluorescent AuAg nanoclusters

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In this study, a fluorescence assay for successive determination of Fe^{3+} and Cu^{2+} ions based on quenching fluorescence of composite AuAg nanoclusters (AuAg NCs) was developed. By using this binary fluorescence sensor, the Fe(III) level in human serum sample can be directly detected without pretreatment. After the nitrification of human serum, the bio-copper level in human serum may be measured with quick response. Human serum samples were analyzed and the average concentration of Fe(III) and bio-copper are 2.33×10^{-5} and 2.91×10^{-5} M, respectively. This assay is not only sensitively responsive to blood iron(III) but also serum copper, suggesting great potential applications for successively monitoring Fe(III) and bio-copper levels and their changes during the progression of biological process.

1. Introduction

Noble metal clusters are an emerging class of fluorescent nanomaterials, such as Au nanoclusters (NCs) and Ag NCs,[1,2] circumventing most of the drawbacks of common fluorescent compounds, which have drawn wide attention in single-molecule optoelectronic nanodevices, biological labeling, optical sensing, novel catalysis, and surface-enhanced Raman spectroscopy (SERS).[3-8] Recognition and quantification metal ions are considerable significant due to these ions play important roles in various biological and environmental processes.[9,10] In the past few years, there are numerous reports on the design of fluorescent sensors for detection of various metal ions, such as Hg^{2+},[11] Fe^{3+},[12] Cu^{2+} and Cd^{2+},[13, 14] Pb^{2+} [15] because of their high sensitivity, specificity, and real-time monitoring with fast response time.

Previous studies have demonstrated that copper and iron deficiency may lead to a wide variety of neurological problems, [16,17] cardiovascular disease and kidney damage.[18] Development of specific sensors determining these metal ions in aqueous media constantly grow attention. Current approaches of detecting Fe^{3+} and Cu^{2+} ions include inductively coupled plasma mass spectrometry (ICP-MS),[19,20] atomic absorption spectrometry [21,22] and electrochemical methods.[23,24] Although these methods offer excellent sensitivity, they are rather costly, time-consuming and complex. Most of the developed approaches to detect copper and iron ions are stressed on the design of selective sensors only for one kind of metal ion. In contrast, the investigation of sensors on successive or simultaneously detecting two kinds of these ions is rare. Thus it is important to develop a binary sensor to determine the iron and copper in the practical sample, especially in the biological samples.

In this study, a type of composite metal nanoclusters AuAg NCs with strong fluorescence was prepared. A fluorescent probe for successive determination of Fe^{3+} and Cu^{2+} ions based on the quenching fluorescence of AuAg NCs was developed. By using this binary fluorescence sensor, a practical application of human serum sample was performed to determine Fe(III) and bio-copper levels. This assay is quick response not only to blood iron level but also serum copper, suggesting great potential applications for successively monitoring Fe(III) and bio-copper concentrations and their changes during the progression of biological process.

2. Experimental

2.1 Chemicals

$HAuCl_4\cdot3H_2O$, silver nitrate ($AgNO_3$), glutathione (GSH), disodium ethylenediamine tetraacetate (EDTA), NH_4F, ferric chloride ($FeCl_3$), cupric chloride ($CuCl_2$) were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). A series of different concentration of solutions were obtained by dilution. The aqueous solutions were prepared with doubly distilled water. The K^+, Ca^{2+}, Na^+, Mg^{2+}, Zn^{2+}, Mn^{2+}, Cd^{2+}, Ni^{2+}, Br^-, I^-, F^-, bovin serum albumin (BSA), cysteine, pepsin, Co^{2+}, Fe^{3+}, Cu^{2+}, Pb^{2+} and Al^{3+} metal ion solutions and molecules were prepared to examine the metal ion induced emission enhancement or quenching. The concentration of all prepared metal ion solutions is 1×10^{-2} M. All reagents were of analytical reagent grade, and used as received.

2.2 Prepartion of AuAg NCs.

In a typical synthesis, under vigorous stirring (1000 rpm), the Au/Ag NCs were synthesized using GSH as template by chemical reduction of $HAuCl_4$, followed the literature reported earlier.[25] Freshly prepared aqueous solutions of $HAuCl_4$ (20 mM, 0.25 mL), GSH (100 mM, 0.15 mL) and $AgNO_3$ (20 mM, 0.25 mL) were mixed with 4.35 mL of distilled water at 25 °C in a round

bottomed flask. The reaction mixture was heated to 110 °C under gentle stirring for 12 h. An aqueous solution of strongly orange-emitting Au/Ag NCs was formed. The orange-emitting Au/AgNC solution could be stored at 4 °C for 6 months with negligible changes in the optical properties.

2.3 AuAg NCs-based sensor for Fe(III) and Cu^{2+} ions.

A series of 20 μL of Fe^{3+} standard solutions with different concentrations were added to 1 mL of Au/AgNCs, respectively. The reactions were allowed to proceed for 10 min at room temperature, the fluorescence quenching spectra was then recorded (excitation 450 nm; maximum emission 570 nm). For the determination of Cu^{2+}, there is a certain concentration of Fe^{3+} (4.9×10^{-7} M) in the target, thus a series of 20 μL of Cu^{2+} standard solutions with different concentrations were added to 1 mL of Au/AgNCs, respectively. The EDTA was added after the sufficient NH_4F (0.1 mol/L) was full reaction with the Fe^{3+}, the fluorescence quenching spectra was then recorded (excitation 450 nm; maximum emission 570 nm).

2.4 Human serum sample processing

The serum samples were supplied by healthy volunteers that were stored at 4 °C until use. All experiments procedures were performed in compliance with the relevant laws and institutional guidelines. The untreated serum was used for the detection of Fe^{3+}.

50 μL of blood sample was diluted with 50 μL of double distilled water and then 50 μL of concentrated HNO_3 was added for reaction 2 h in the disposable centrifuge tube at the room temperature. The sample was briefly centrifuged at 5000 rpm for 7 min when it was be completely nitrification. The supernatant was adjusted to neutral use the NaOH solution. This treated serum was used for the detection of Cu^{2+}.

2.5 AuAg NCs-based sensor for Fe (III) and copper in human serum.

Take 20 μL of the untreated human serum to the 1 mL Au/AgNCs to proceed for 20 min at room temperature, the decrease of the fluorescence intensity was induced by the Fe^{3+} and the concentration of Fe^{3+} in the sample was quantified according to the calibration curve.

Take 50 μL of the treated serum to the 1 mL Au/AgNCs, then added 50 μL 0.1 M NH_4F and 50 μL 0.1 M EDTA sequentially. The fluorescence enhancement after the addition of EDTA was induced by Cu^{2+}. The concentration of Cu^{2+} in the sample was quantified according to the calibration curve.

2.6 Characterization.

The photoluminescence spectra were recorded on an F-4500 (Hitach, Japan) fluorescence spectrometer. UV/Vis absorption spectra were recorded by a Lambda 35 spectrophotometer (PerkinElmer, USA). Transmission electron microscopy (TEM) was performed on a JEM-2010 transmission electron microscope at 80 kV. The copper level of human serum was also determined by atomic absorption spectrometry using a Perkin Elmer atomic absorbance spectrophotometer (ASS) (PEAAnalyst 300).

3. Results and discussion

3.1 Preparation and characterization of AuAg NCs

The preparation of AuAg NCs was performed according to the previous method with slight modification.[26] Briefly, $HAuCl_4$, $AgNO_3$ and GSH aqueous solutions were allowed to react under gentle stirring at 110 °C for 12 h. A gradual color change appeared within minutes from yellow to colorless and then to light yellow. The resultant solution exhibited light yellow under visible light and there was intense orange luminescence from the solution when irradiated by the UV light. Fig. 1 shows the TEM image of the synthesized AuAg NCs, indicating the size of the nanoparticles less than 2 nm with quite narrow distribution. The UV–vis absorption and fluorescence spectra acquired from AuAg NCs are displayed in Fig. 2. When the as-prepared AuAg NCs are excited by 450 nm light, an emission band at 570 nm is observed, implying a successful preparation of AuAg NCs. The AuAg NCs show excellent stability in water, maintaining excellent photoluminescence. With rhodamine B as the reference, the quantum yield of the as-prepared Au NCs was calculated to be 15.0%.

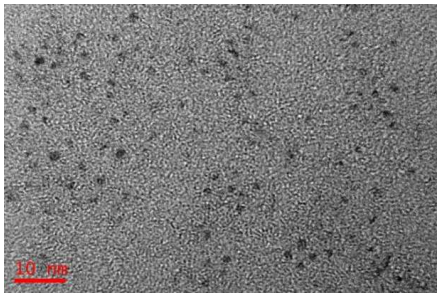


Fig. 1 TEM image of the AuAg NCs.

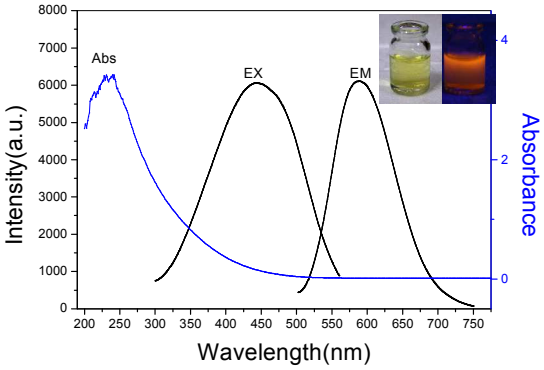


Fig. 2 UV-vis absorption (blue line) and excitation and emission spectra (black line, λex=450 nm，λem=570 nm) of the orange-emitting AuAg NCs. Inset: Photographs of an aqueous solution of AuAg NCs under visible light (left) and UV irradiation (λ = 365 nm) (right).

3.2 Response of fluorescent AuAg NCs toward different metal ions

The study of sensing metal ions was carried out based on the fluorescent response of AuAg NCs. With the addition of Fe^{3+} or Cu^{2+} ion into the AuAg NCs solution, a rapidly quenched fluorescence occurred. As a comparison, other common metal ions, protein and small thiol-containing molecules, such as K^+, Ca^{2+}, Na^+, Mg^{2+}, Zn^{2+}, Mn^{2+}, Cd^{2+}, Ni^{2+}, Co^{2+}, Fe^{3+}, Cu^{2+}, Pb^{2+}, Al^{3+}, Br^-, I^-, F^- as well as BSA, cysteine, pepsin were investigated under the same conditions. A clear quenching effect like that of the Fe^{3+} and Cu^{2+} ions was not observed when the concentration of these metal ions and molecules increased up to 10^{-4} M, as

shown in Fig. 3. Therefore, the quenching fluorescence provides effective assay for the respective determination of Fe^{3+} and Cu^{2+} ions.

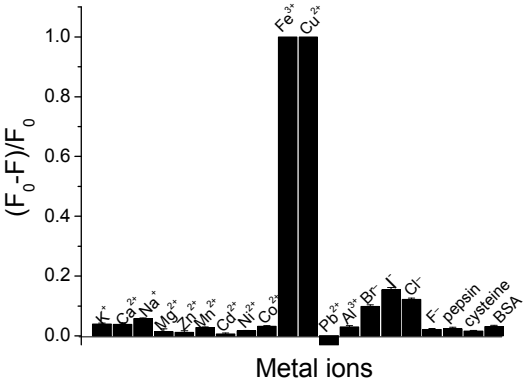


Fig 3. The fluorescence intensity ratio of changes of the AuAg NCs induced by various ions and biological molecules (1×10^{-4}M), F_0 is the initial intensity of AuAg NCs and F is the fluorescence after the addition of metal ions, the error bars represent the standard deviation of three measurements.

3.3 Evaluation of fluorescence response to Fe^{3+} ion and Cu^{2+} ion.

The fluorescence intensity of the AuAg NC decreased greatly after the addition of Fe^{3+}. Apparently, the fluorescence intensity of AuAg NC clearly decreases with the increase of the concentration of Fe^{3+}. Finally, the fluorescence might quench completely along with the increase of concentration of Fe^{3+}. Fig. 4 shows that the fluorescence intensity of AuAg NCs decreases with the increasing concentration of Fe^{3+}. Due to the high probability of the coexistence of Fe^{3+} and Cu^{2+} ions, it is difficult to tell the source of the quenching fluorescence under the coexistence of these metal ions. The popular chelants, NH_4F and EDTA, might be used to differentiate the quenching fluorescence arisen from Fe^{3+} or Cu^{2+}, as illustrated in scheme 1.

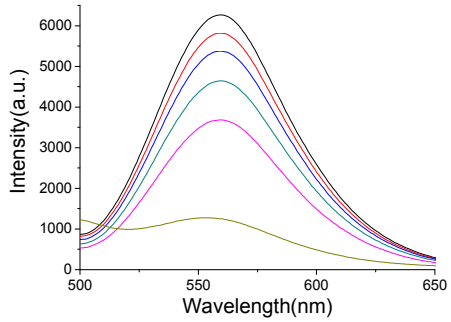
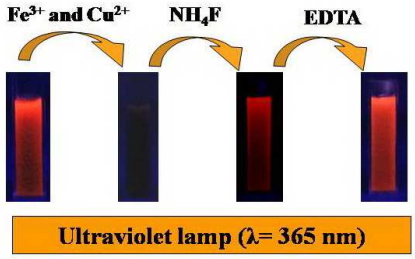


Fig 4. Fluorescence quenching of AuAg NCs with increasing Fe(III) concentration (from top to bottom: 0M , 2×10^{-8} M, 1.9×10^{-7} M, 7.2×10^{-6} M, 3.5×10^{-5} M, 1.7×10^{-4} M).

The Fe^{3+} and Cu^{2+} ions may combine with the COOH and NH_2 groups of GSH stabilizing AuAg NC, the combination of metal ions to the surface of the AuAg NCs would induce the fluorescence quenching. However, the response of AuAg NCs toward Cu^{2+} and Fe^{3+} were found to be reversible, namely, the quenched florescence was almost recovered to 100% after the addition of NH_4F into the mixture system of Fe^{3+} and AuAg NCs. EDTA was also used to further test the Cu^{2+} ion. With the addition of EDTA to mixture of Cu^{2+} and AuAg NCs, an enhancement of fluorescence up to the initial intensity of fluorescent AuAg NCs was found owing to the formation of complex between EDTA and Cu^{2+}. These results indicate that the formation of metal complexes makes the metal cations departed from the surface of AuAg NCs. Therefore, the introduction of NH_4F or EDTA to AuAg NCs provides a possibility to determine Fe^{3+} and Cu^{2+} ions successively.



Scheme 1. Schematic diagram of successive detection of Fe^{3+} and Cu^{2+} based on the binary fluorescence sensor of AuAg NCs.

3.4. Application to human serum samples analysis

As nutrient elements, iron and copper are of wide existence in biological tissues. Most of the iron existed in the form of protein complexes in the biological body fluids and organs. Similarly, the main existed form of copper-containing compounds is present as undissociated copper such as protein in the biological body fluids and organs. Human serum samples were analyzed to demonstrate the practical application to determination of the Fe(III) and copper level. When the human serum sample was added to AuAg NCs, a significant decrease of fluorescence intensity was found, which indicated that the iron and/or copper in the human serum might be directly determined without pretreatment. With the addition of NH_4F to the mixture system, a quickly remarkably enhancement of fluorescence occurred and recovered to the initial intensity of the AuAg NCs. As a control experiment, the chelant NH_4F was first added to the human serum, then the biological sample mixed with the AuAg NCs and no detectable variation of fluorescence occurred, as shown in Fig. S1 (supplementary material). These results indicate that quenched fluorescence resulted from Fe(III) rather than bio-copper in the human serum, which demonstrate that the proposed fluorescent probe can only respond to Fe(III) in the serum, implying the fluorescence of AuAg NCs would not be interfered by other components in human serum, and this fluorescence assay may selectively determine the Fe(III) in the human serum.

To quantitative detect the Fe(III) in the human serum, calibrate curve was first established and a linear relationship between the degree of quenching fluorescence and Fe^{3+} concentration within the range of 9×10^{-8} ~ 1.1×10^{-6} M was found (Fig. S2), which the fluorescence intensity was inversely proportional to concentration of Fe(III). The equation is displayed as following: F_0-F/F_0 =0.03173+0.1531c, where F_0 is the initial intensity of fluorescent

AuAg NCs, and F is the fluorescence intensity after the addition of Fe(III). The linear regression coefficient is 0.9965 and the limit of detection of Fe(III) is 10^{-8} M. By using this fluorescence sensor, the human serum samples were analyzed to demonstrate the practical application from four volunteers and the blood Fe(III) concentration were illustrated in Table 1.

As discussed aforementioned, this fluorescent probe can only respond to the Cu^{2+} rather than undissociated copper. Thus, the bio-copper in the human serum cannot be directly measured utilized this fluorescent probe due to few copper ions present in the human body fluids. The same four human serum samples were analyzed to determine bio-copper levels following the general procedure. After human serum nitrification, the iron and copper were existed as ionic state in the aqueous solution. Before the quantitative analysis of bio-copper, the calibration curve of copper ions was established, as shown in Fig. S3. Clearly, the addition of various concentrations of Cu^{2+} to the fluorescent AuAg NCs led to varying degrees of quenching fluorescence shown in Fig. S4. The corresponding ΔF ($\Delta F=F_0-F$) of AuAg NCs increased with the concentration of the metal ion in the solution and shows a linear relationship with the Cu^{2+} concentration. Therefore, the degree of quenching fluorescence of fluorescent AuAg NCs may be directly reflected by the concentration change of the copper ion. To eliminate the interference of Fe^{3+} toward the detection of copper, NH_4F was added to the nitrification solution of human serum, as shown in Fig. S5. Then, with the addition of nitrification solution to the AuAg NCs solution, the fluorescence intensity was decreased. The copper concentration can be calculated from the calibration curve. As a result, the bio-copper levels of human serum detected by this assay were also displayed in Table 1. The samples were also determined by AAS, and the obtained results were closely comparable with the result given by the proposed method, suggesting this assay is of high accuracy. The experimental results indicate that the recovery of this assay 101.8% corresponding to Cu^{2+} and 99.6% corresponding to Fe(III), as shown in Table S1.

Table1. The result obtained from the AuAg NCs binary sensor for Fe(III) and copper levels in human serum samples.

Sample	Fe(III) (M)	Cu^{2+} (M)
1	2.11×10^{-5}	2.75×10^{-5}
2	2.34×10^{-5}	1.61×10^{-5}
3	1.44×10^{-5}	4.62×10^{-5}
4	3.44×10^{-5}	2.65×10^{-5}
5	2.62×10^{-5}	4.33×10^{-5}

4. Conclusions

In summary, a type of composite metal nanoclusters AuAg NCs were prepared and a binary fluorescence sensor for successively determining Fe(III) and copper level in human serum was developed. The Fe(III) level of human serum can be directly determined without pretreatment with high sensitivity. Human serum samples were analyzed and the average concentration of Fe(III) is 2.33×10^{-5} M. On the other hand, the bio-copper was detected after the nitrification of human serum and the result is corroborated by AAS, suggesting this assay is of high accuracy. This proposed assay for successive detection of Fe(III) and bio-copper level has high selectivity, low cost, and high sensitivity, which is of a potential application in clinical diagnosis.

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Notes and references

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† Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/b000000x/

‡ Footnotes should appear here. These might include comments relevant to but not central to the matter under discussion, limited experimental and spectral data, and crystallographic data.

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