

ORIGINAL ARTICLE

Ultrasensitive detection of Cu²⁺ with the naked eye and application in immunoassays

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In this study, a novel method for the fast, sensitive and selective detection of Cu²⁺ using gold nanoparticles (AuNPs) was developed and used in immunoassays. In the presence of L-cysteine, L-cysteine can bind to the surface of citrate-stabilized AuNPs through Au-S bonds. As a result, aggregation of AuNPs occurs through electrostatic interactions between the cysteine-bound AuNPs. In contrast, in the presence of Cu²⁺, Cu²⁺ can catalyze O₂ oxidation of cysteine, leading to the quick formation of disulfide cystine. An increase in the concentration of Cu²⁺ decreased L-cysteine-induced AuNPs aggregation by decreasing the number of free cysteine thiol groups, and the solution color changed from purple to red. Therefore, the concentration of Cu²⁺ can be detected with the naked eye or with ultraviolet-visible spectroscopy, and the detection limits of Cu²⁺ were 20 nM and 10 nM, respectively. This sensitivity was approximately three orders of magnitude higher than that of traditional AuNPs-based colorimetric Cu²⁺ detection methods. Because of the high sensitivity of the proposed method, we further used it with a labeled antibody in colorimetric immunoassays. The detection limit of the cancer biomarker α -fetoprotein was 2 ng ml⁻¹, which is comparable to the detection limit of the enzyme-linked immunosorbent assay method.

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INTRODUCTION

Copper is an essential metal ion for biological functions. It is a cofactor and/or a structural component of numerous enzymes and other proteins needed in metabolic processes.¹ However, the accumulation of Cu²⁺ in humans leads to many serious conditions, including neurodegenerative diseases and prion diseases.^{2,3} As such, the detection and measurement of Cu²⁺ has become increasingly important. To date, there are many technologies that have been developed to detect Cu²⁺, including inductively coupled plasma⁴ detectors, electrochemical sensors,⁵ surface-plasmon resonance⁶ detectors, fluorescence-anisotropy assays,⁷ quantum-dot-based assays⁸ and Cu²⁺-specific-DNAzyme-based fluorescence sensors.⁹ Although these technologies can detect Cu²⁺ sensitively and selectively, the need for sophisticated instrumentation and highly trained operators limited their application in routine detection.

Recently, gold-nanoparticles (AuNPs)-based colorimetric methods, which without the aid of advanced instrumentation, have attracted attention for their use in many applications because they can be easily monitored with the naked eye or with low-cost portable instruments. AuNPs possess intrinsically strong surface-plasmon resonance absorptions and high extinction coefficients (approximately four orders of magnitude greater than typical organic dyes).¹⁰ The color of the AuNPs solution can change from red to purple, in response to the surface-plasmon resonance absorption of dispersed and aggregated

nanoparticles.¹¹ Systems based on analyte-induced aggregation of AuNPs have been used in a variety of detection formats for DNA,^{12,13} proteins,^{14,15} small molecules^{16–19} and cells.²⁰ AuNPs-based colorimetric methods can also be used in the detection of metal ions.^{21–27} For example, Mirkin *et al.*²⁴ reported a highly selective and sensitive colorimetric detection method for mercuric ions. Mercuric ions in aqueous media are detected by the formation of thymidine–Hg²⁺–thymidine coordination complexes, which raise the melting temperature of the DNA-hybridized AuNP probes, and thus the temperature at which the probes disperse, and cause a purple-to-red color change. Lu and Liu^{21–23} reported a highly sensitive and selective colorimetric lead biosensor based on DNAzyme-directed assembly of AuNPs. In this biosensor, Pb²⁺-specific DNAzyme was used as the target recognition element, and DNA-functionalized AuNPs were used as the signaling element.

Cu²⁺ detection can also use AuNPs-based colorimetric methods. Lu and Liu²⁸ used a Cu²⁺-dependent DNA ligation DNAzyme to develop a colorimetric sensor for Cu²⁺. The detection limit of this sensor was 5 μ M. Jiang *et al.*²⁹ reported a method for the detection of Cu²⁺ ions by azide- and terminal alkyne-functionalized AuNPs in aqueous solutions using click chemistry and recently extended this method to colorimetric immunoassays.³⁰ However, this method required overnight incubation to facilitate aggregation and was not quantitative. Mirkin *et al.*¹⁰ improved the method by using densely

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functionalized DNA AuNPs conjugates and click chemistry, and achieved a detection limit of Cu^{2+} of $20 \mu\text{M}$. However, the need for specifically modified ligands as well as the relatively complex detection steps and low sensitivity limit the application of the above methods.

In this paper, we describe a rapid and sensitive colorimetric method for the detection of Cu^{2+} using citrate-stabilized AuNPs. According to a previous report,³¹ thiol compounds (such as homocysteine and L-cysteine) can bind to the surface of AuNPs through the formation of Au-S bonds and cause the aggregation of AuNPs through electrostatic interaction. In addition, Pecci *et al.*³² and Su *et al.*³³ have demonstrated that Cu^{2+} can catalyze O_2 oxidation of thiol compounds to form disulfide compounds. Therefore, we hypothesized that the thiol compound (L-cysteine) can bind to the surface of AuNPs (Figure 1). As a result, because the carboxyl group of cysteine is deprotonated in buffer solution while the amine group is still protonated, AuNPs aggregation occurs through electrostatic interaction between cysteine-bound AuNPs. In contrast, in the presence of Cu^{2+} , Cu^{2+} can catalyze O_2 oxidation of cysteine to quickly form disulfide cystine. With an increase in the concentration of Cu^{2+} , the L-cysteine-induced aggregation of AuNPs decreased because of the decrease in the free cysteine thiol groups, and the solution color changed from purple to red. Therefore, the concentration of Cu^{2+} can be detected with the naked eye or with ultraviolet-visible (UV-Vis) spectroscopy.

EXPERIMENTAL PROCEDURES

Synthesis of AuNPs

AuNPs with an average diameter of 13 nm were prepared by the citrate-mediated reduction of HAuCl_4 . Forty-nine milliliter of deionized water and 1.03 ml of HAuCl_4 (20 mg ml^{-1}) were introduced into a two-necked flask. This mixture was heated to reflux, and then 5 ml of trisodium citrate solution (38.8 mM) was quickly added, resulting in a change in solution color from pale yellow to deep red. The solution was heated under reflux for 10 min and then allowed to cool to room temperature. Finally, 50 ml of AuNPs was mixed with 2 ml of 1% surfactant Tween-80 to yield well-dispersed AuNPs.

Cu^{2+} detection

Ten micromolar of L-cysteine and different concentrations of Cu^{2+} in Tris-hydrochloric acid (Tris-HCl; pH 7.4, 100 mM) buffer were added to 96-well plates. The total incubation volume was $100 \mu\text{l}$, and the catalytic reaction was performed at 37°C . After a 10-min reaction time, $100 \mu\text{l}$ of the AuNPs ($\sim 7.48 \text{ nm}$) was added and the reaction continued for a further 10 min. Finally, the solution color was observed with the naked eye or with UV-Vis spectroscopy.

Kinetic behavior

Ten micromolar of L-cysteine was incubated with four concentrations of Cu^{2+} in Tris-HCl (pH 7.4, 100 mM) buffer. At different reaction times, an equal volume of AuNPs was added to the mixture solution and incubated for a further 10 min. Finally, the kinetic behavior of Cu^{2+} to catalyze L-cysteine was studied by measuring the absorption spectra of the reaction solutions.

Colorimetric immunoassays

One milliliter of CuO nanoparticles (NPs) (1 mg ml^{-1} ; Sigma-Aldrich, Inc. St Louis, MO, USA; diameter $< 50 \text{ nm}$) was sonicated for 30 min to disperse the NPs. Then, 1 ml of α -fetoprotein (AFP) polyclonal antibody (1 mg ml^{-1}) was added to the CuO NPs solution. The mixture was vortexed and then incubated at 37°C for 2 h. The mixture solution was subsequently centrifuged at 12000 r.p.m. for 10 min, and the suspension, which contained unlabeled AFP polyclonal antibody, was discarded. Finally, $200 \mu\text{l}$ of 10% BSA was added to stabilize the AFP polyclonal antibody-CuO solution.

Hundred microliter of AFP monoclonal antibody ($50 \mu\text{g ml}^{-1}$) was incubated overnight in a 96-well plate at 4°C , and the wells were blocked with 5% fetal bovine serum for 1 h. After washing with buffer, different concentrations of AFP were added to the wells and allowed to incubate at 37°C for 1 h. The wells were then washed again and the AFP polyclonal antibody-CuO solution was added and allowed to incubate at 37°C for a further 1 h.

After immobilization of the AFP polyclonal antibody-CuO, $20 \mu\text{l}$ of HCl (1 mM) was added to release the Cu^{2+} from the AFP polyclonal antibody-CuO. L-cysteine in Tris-HCl (pH 7.4, 125 mM) buffer was introduced into the wells to react with the Cu^{2+} for 10 min. The volume of each well was $100 \mu\text{l}$, and the concentration of L-cysteine was $10 \mu\text{M}$. Then, an equal volume of AuNPs was added, and the reaction was continued for a further 10 min. Finally, the solution color was recorded with a digital camera or with UV-Vis spectroscopy.

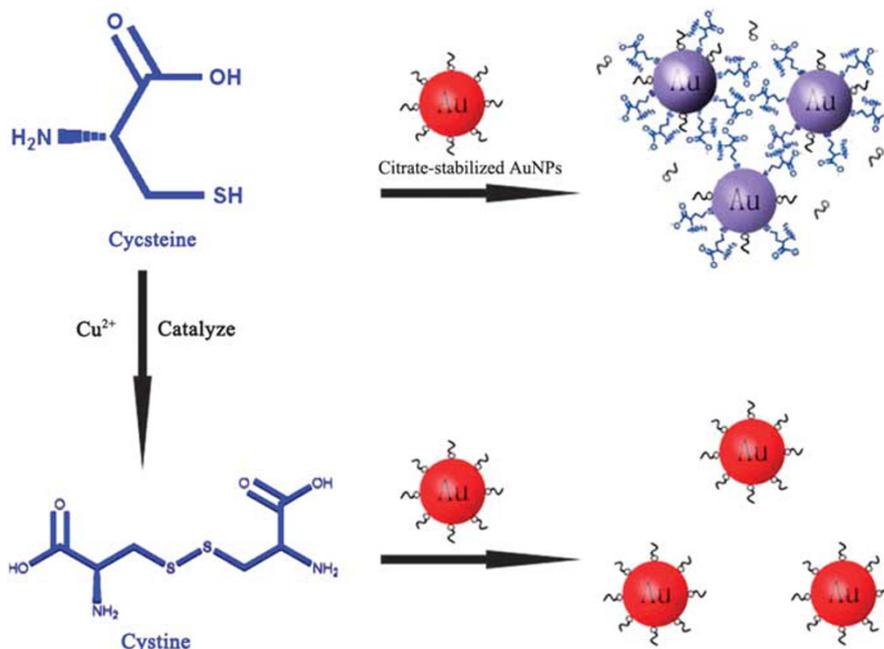


Figure 1 Schematic for the Cu^{2+} colorimetric detection method.

RESULTS AND DISCUSSION

Characterization of AuNPs

Figure 2 shows transmission electron microscope images of dispersed and aggregated AuNPs. Note that to improve the stability of AuNPs in a high ionic strength solution, we used the surfactant Tween-80 to modify the surface of the AuNPs. As shown in Figure 2a, the diameter of the as-prepared AuNPs is ~ 13 nm. When the appropriate amount of L-cysteine was added to the AuNPs solution, the solution color changed from red to purple, and aggregated AuNPs could be observed by transmission electron microscope (Figure 2b). This result proved that L-cysteine could bind to the surface of AuNPs through formation of Au-S bonds, and AuNPs aggregation occurred through electrostatic interaction between cysteine-bound AuNPs. The extinction coefficient of 13 nm AuNPs was $2.7 \times 10^8 \text{ M cm}^{-1}$,³⁴ and the concentration of dispersed as-prepared AuNPs used in the experiment was ~ 7.48 nM.

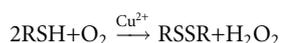
Optimum reaction conditions

To improve the sensitivity and efficiency of the detection of Cu²⁺, we first optimized the buffer concentration. Surfactant-capped AuNPs have relatively high stability in high ionic strength solutions. When Tris-HCl buffer was used as the reaction buffer, the AuNPs were well dispersed at buffer concentrations < 100 mM. However, when the buffer concentrations were > 100 mM, the AuNPs began to aggregate, and the solution color changed from red to purple. Therefore, 100 mM Tris-HCl (pH 7.4) buffer was chosen as the optimum buffer condition.

We also optimized the L-cysteine concentration. Hundred microliter of the AuNPs solution was added to an equal volume of different concentrations of L-cysteine, and the mixtures were incubated for 10 min at 37 °C. The concentrations of L-cysteine were 2, 5, 10, 15 and 20 μM . As shown in Figure 3, at concentrations of L-cysteine 10 μM or higher, a redshift of the surface-plasmon resonance absorption band occurred and the solution color changed from red to purple. Therefore, 10 μM of L-cysteine was chosen for the subsequent experiments.

Kinetic behavior

To evaluate the kinetic behavior of the Cu²⁺ catalysis of L-cysteine, we tested four concentrations of Cu²⁺ with 10 μM of L-cysteine. The Cu²⁺ concentrations before adding the AuNPs were 0, 50, 100, 200 and 400 nM. According to a previous report,³² Cu²⁺ can catalyze O₂ oxidation of L-cysteine to cystine. The catalytic equation is as follows:



As shown in Figure 4, the abscissa denotes the reaction times of L-cysteine and Cu²⁺, and the ordinate denotes the relative absorption

value when the AuNPs were added to the mixture of L-cysteine and Cu²⁺. The relative absorption value was defined as the value of A_{650}/A_{520} , which was the ratio of the extinction coefficients at these two wavelengths that expressed the molar ratio of the aggregated to the dispersed AuNPs.³⁵ With the increase in the Cu²⁺ concentration, more L-cysteine was catalyzed by Cu²⁺ to form cystine. Thus, the value of A_{650}/A_{520} decreased. Taking the time course of 400 nM of Cu²⁺ as one example, the value of A_{650}/A_{520} significantly decreased with time until it reached a plateau in ~ 10 min. As shown in Figure 4, different concentrations of Cu²⁺ can catalyze the O₂ oxidation of different amounts of L-cysteine within 10 min, thus, the degree of aggregation of the AuNPs is different. Therefore, to quantitatively analyze the Cu²⁺ concentration, 10 min was chosen as the reaction time for the L-cysteine and Cu²⁺ mixture.

Detection of Cu²⁺

Under the optimized conditions, L-cysteine was reacted with different concentrations of Cu²⁺ for 10 min and then incubated with AuNPs for a further 10 min. The color of the solutions changed from red to purple, and a digital camera was used to record the color. As shown in Figure 5, we can observe a purple-to-red color change when the concentration of Cu²⁺ increased from 20 to 200 nM. The detection limit of Cu²⁺ was 20 nM. This result demonstrates that the proposed method can be used for the direct detection of Cu²⁺ with the naked eye.

To evaluate the minimum concentration of Cu²⁺ in aqueous solution that can be detected by this colorimetric method, UV-Vis spectroscopy was also used to detect the Cu²⁺ concentration. As shown in Figure 6a, with a decrease in Cu²⁺ concentration, less L-cysteine was catalyzed by Cu²⁺. Therefore, the AuNPs tended

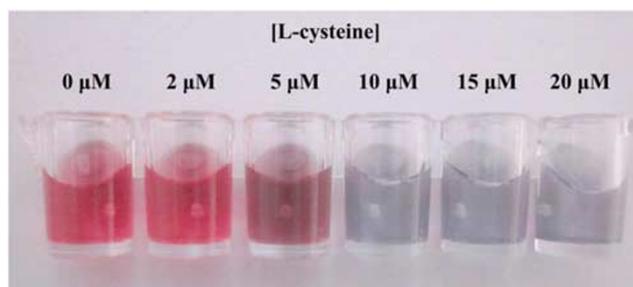


Figure 3 Different concentrations of L-cysteine induce the aggregation of AuNPs.

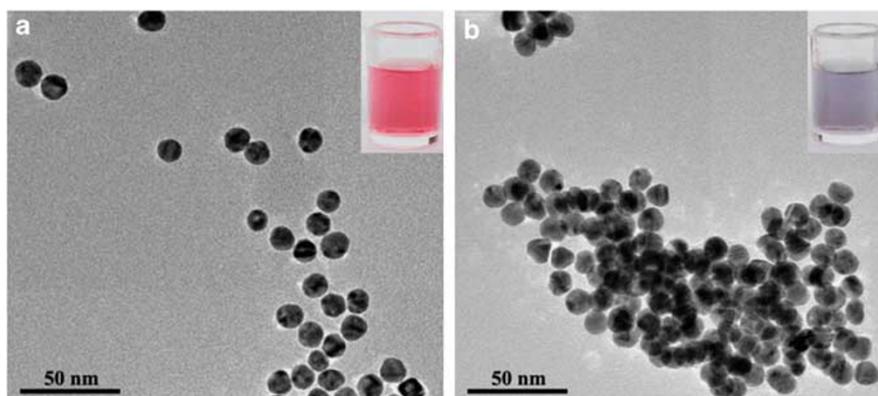


Figure 2 Transmission electron microscope (TEM) images of dispersed (a) and aggregated (b) AuNPs.

to aggregate, and the value of A_{650}/A_{520} increased. As shown in Figure 6b, the calibration curve for the value of A_{650}/A_{520} against Cu^{2+} concentration was linear in the range from 10 to 250 nM and fit the linear equation $A = -0.0041c + 1.2025$ ($R^2 = 0.9908$). The detection limit of Cu^{2+} was 10 nM, which is lower than the US Environmental Protection Agency-defined maximum contaminant level for copper in drinking water (20 μM). As shown in Table 1, the sensitivity of the proposed method was approximately three orders of magnitude

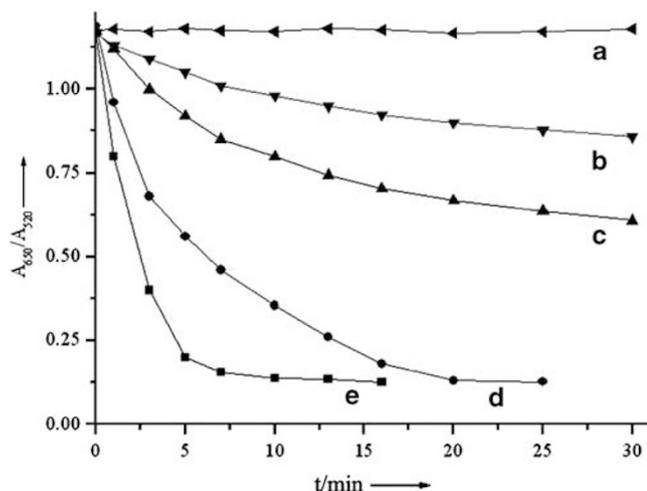


Figure 4 A_{650}/A_{520} values of the AuNPs solutions after the addition of L-cysteine and Cu^{2+} mixture solutions at different reaction times. From a to e, Cu^{2+} concentrations are 0, 50, 100, 200 and 400 nM, respectively.

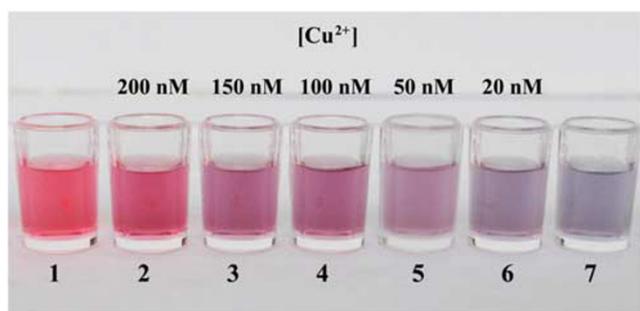


Figure 5 Naked-eye observation of different concentrations of Cu^{2+} . The concentrations of Cu^{2+} range from 20 to 200 nM. Sample 1 and 7 were the AuNPs solutions without and with the L-cysteine.

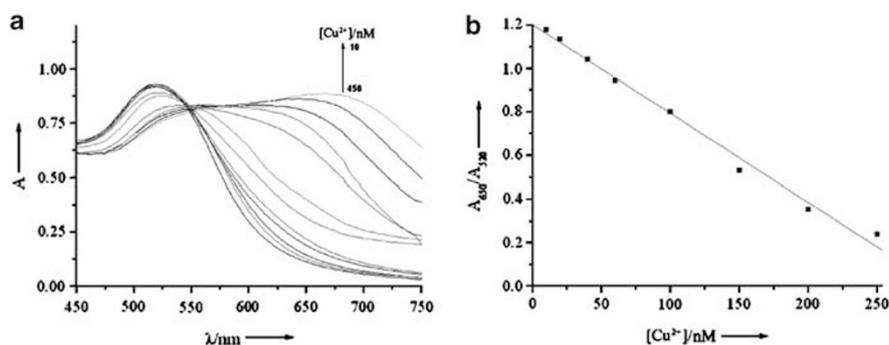


Figure 6 (a) Effect of Cu^{2+} concentrations on the extinction spectra of AuNPs. (b) Plot of the A_{650}/A_{520} value versus the concentrations of Cu^{2+} .

higher than that of traditional AuNPs-based colorimetric Cu^{2+} detection methods.^{10,11,28} This higher sensitivity of this method is due to the use of the Cu^{2+} analyte as the catalyst and the high catalytic efficiency of Cu^{2+} . Even a low concentration of Cu^{2+} could catalyze O_2 oxidation of a significant amount of L-cysteine to form disulfide cystine, thus affecting the aggregation of the AuNPs.

Metal selectivity

To evaluate the selectivity of the proposed method, the proposed method was challenged with other environmentally relevant metal ions, including Co^{2+} , K^+ , Na^+ , Mg^{2+} , Zn^{2+} , Ba^{2+} , Mn^{2+} , Cd^{2+} , Hg^{2+} , Al^{3+} , Fe^{3+} , Ni^{2+} , Ca^{2+} , Ag^+ and Pb^{2+} . In a typical experiment, one of these metal ions was added to the L-cysteine solution at a final concentration of 30 μM (except that the concentration of Cu^{2+} was 300 nM) for a 10 min reaction time, and then incubated for a further 10 min in the presence of AuNPs. As shown in Figure 7, high metal selectivity was observed from the AuNPs-based sensing. In the presence of a 100-fold concentration of interfering ions, only Co^{2+} showed slight interference. Although a decrease in the A_{650}/A_{520} ratio was detected upon addition of Co^{2+} , the degree of AuNPs aggregation produced by Cu^{2+} was still higher than that produced by Co^{2+} . Therefore, the proposed method was specific for Cu^{2+} ions.

Real water samples analysis

The proposed method was used to determine Cu^{2+} concentration in water samples, and the results are shown in Table 2. Meanwhile, inductively coupled plasma-mass spectrometry was also used to detect Cu^{2+} to verify the accuracy of the proposed method. The results obtained by the proposed method were in good agreement with those obtained by the inductively coupled plasma-mass spectrometry method. From the experimental results, it is obvious that the proposed method has great potential for practical sample analysis.

Table 1 The comparison of dynamic range, detection limit of several colorimetric methods

Detection principle	Dynamic range	Detection limit	Reference
Electrostatic interaction	10–500 μM	10 μM	11
Click chemistry	50–500 μM	50 μM	29
Click chemistry	20–100 μM	20 μM	10
Cu^{2+} -dependent DNA ligation DNAzyme	5 μM –1 mM	5 μM	28
Catalyst	10–450 nM	10 nM	This work

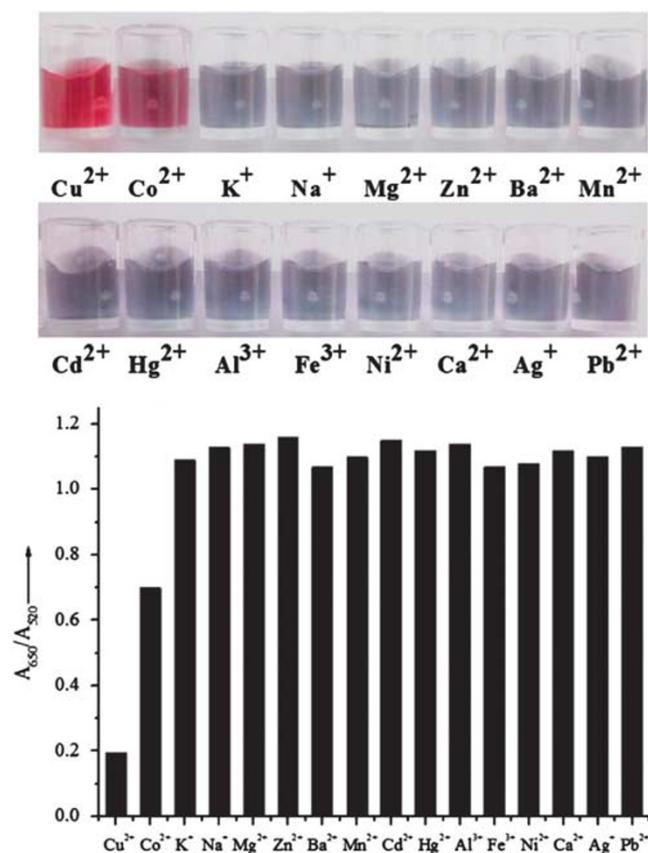


Figure 7 Selectivity of the AuNPs-based colorimetric Cu²⁺ detection method.

Table 2 The comparison of proposed method and ICP-MS detection of Cu²⁺ ($n=9$)

Sample	Detected by ICP-MS (μM)	Detected by this method (μM)	RSD (%)
Tap water	1.35	1.52	3.8
River water	3.41	3.73	5.4

Abbreviations: ICP-MS, inductively coupled plasma-mass spectrometry; RSD, relative standard deviation

Application in immunoassays

As the proposed method has high sensitivity for the detection of Cu²⁺, we considered applying this method to biological analysis. Therefore, in a further study, the proposed method was used with a labeled antibody to develop a colorimetric immunoassay. Conventional laboratory procedures for protein analysis, including western blot methods³⁶ and enzyme-linked immunosorbent assays (ELISAs),³⁷ enable sensitive detection and quantification of proteins. However, these methods need instruments for detection, and hence are costly and requires hours of work and skilled labor. Therefore, combined with the above Cu²⁺ detection method, we developed a rapid colorimetric immunoassay for protein detection with the naked eye or with UV-Vis spectroscopy.

The cancer biomarker AFP was assayed to illustrate the application of the proposed colorimetric immunoassay for cancer diagnosis. Figure 8 is a schematic representation of the colorimetric immunoassay. The experiment can be divided into five steps. First, AFP

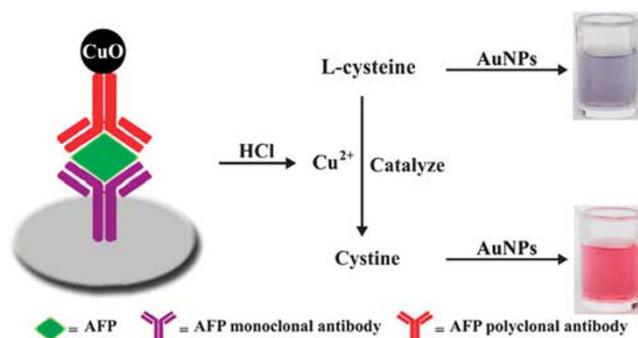


Figure 8 Schematic of the colorimetric immunoassay.

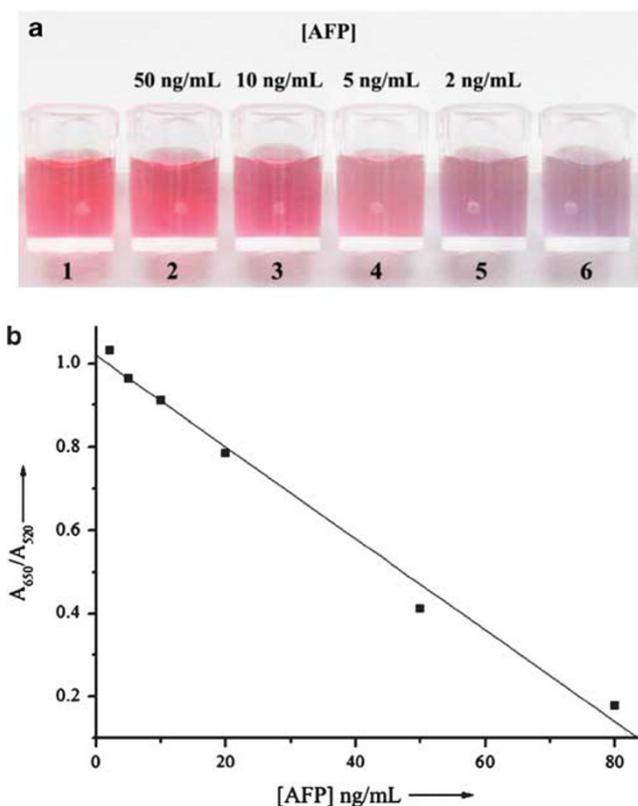


Figure 9 (a) Naked-eye observations of different concentrations of AFP. Sample 1 and 6 are the AuNPs solutions without and with the L-cysteine. (b) Plot of the A_{650}/A_{520} value versus the concentration of AFP.

monoconal antibody was incubated in a 96-well plate, and the well was blocked with 5% fetal bovine serum. Second, samples containing AFP with different concentrations were added into the wells. Third, AFP polyclonal antibody-CuO was specifically adsorbed onto AFP. Fourth, after the immobilization of the AFP polyclonal antibody-CuO, HCl was used to react with CuO to release Cu²⁺, and the Cu²⁺ subsequently catalyzed the O₂ oxidation of L-cysteine to form cystine. Finally, AuNPs were added for colorimetric detection.

As shown in Figure 9a, there was a visually observable color change for the different concentrations of AFP. Higher concentrations of AFP corresponded to higher concentrations of Cu²⁺, resulting in the catalysis of more L-cysteine and leading to the dispersion of the AuNPs. Different concentrations of AFP could be detected with the

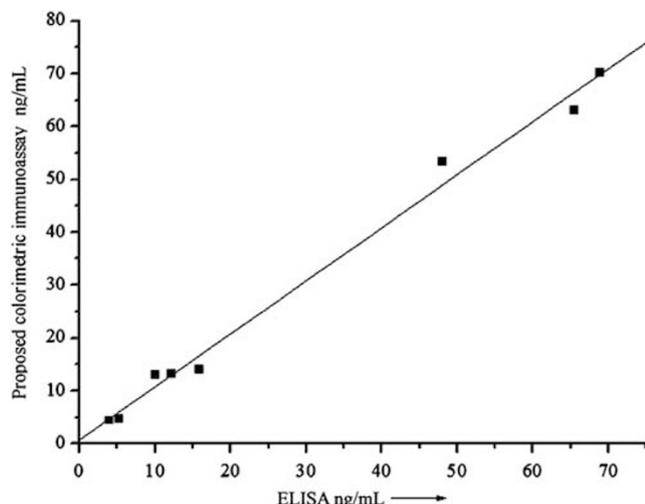


Figure 10 Comparison between the proposed colorimetric immunoassay and the standard ELISA for AFP.

naked eye. UV-Vis spectroscopy was also used to detect the Cu²⁺. As shown in Figure 9b, the calibration curve for the value of A_{650}/A_{520} against Cu²⁺ concentration was linear in the range from 2 to 80 ng ml⁻¹, and follows the linear equation $A = -0.011c + 1.0198$ ($R^2 = 0.9891$). The detection limit of Cu²⁺ was 2 ng ml⁻¹, which was comparable to the standard AFP ELISA. Therefore, the proposed colorimetric immunoassay can provide sensitive detection of AFP without using advanced equipment.

To further investigate the potential application of the proposed colorimetric immunoassay for clinical analysis, we examined eight serum specimens (five from normal sera and three from patient's sera) by using the proposed method and the standard AFP ELISA (Figure 10). The regression equation (linear) for these data is as follows (x axis, standard AFP ELISA; y axis, proposed colorimetric immunoassay): $y = 1.0043x - 0.6586$ ($R^2 = 0.9916$) for AFP. These data show no significant difference between the results of the two methods.

CONCLUSIONS

In conclusion, we developed a novel method using AuNPs for the rapid, sensitive and selective detection of Cu²⁺, and studied the application of this method in immunoassays. Compared with traditional AuNPs-based colorimetric Cu²⁺ detection methods, the proposed method has several excellent features, including ease of operation, low cost, rapid results (within 20 min) and a high sensitivity that was approximately three orders of magnitude higher than that of traditional AuNPs-based colorimetric methods. In a further study, the proposed method was used with a labeled antibody in a colorimetric immunoassay. The proposed colorimetric immunoassay can provide sensitive detection of AFP without using advanced equipment. The proposed method may have potential use in many application areas because immunoassays can be used widely in clinical diagnostics, drug discovery and nanoscience.

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