Visual detection of Cu$^{2+}$ based on fluorescence quenching of green-synthesized gold nanoclusters using soy protein as template

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ABSTRACT
The fast emergence of various nanomaterials with characteristic properties has provided excellent tools in analytical sciences. Herein, environment-friendly luminescent gold nanoclusters (AuNCs) were synthesized by using soy protein as template. We first investigated the optimum reaction condition for the brightest emission, and then employed the luminescent AuNCs to develop a visual sensor for the detection of Cu$^{2+}$. This sensor showed good sensitivity and selectivity with a linear response of 40–400 μM and a detection limit of 10 μM. The assay was facile to operate and the optical results could be read by naked eyes under a portable UV light, showing great application potential in environmental, biochemical and food analysis.

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Introduction

Environmental and food safety issues caused by heavy metals, such as Pb$^{2+}$, Cd$^{2+}$ and Cu$^{2+}$, have received more and more attention. It is known that Cu$^{2+}$ is toxic to cells due to cell apoptosis caused by Cu$^{2+}$-induced hydroxyl radicals (Shao et al., 2017). It was also reported that a relatively higher level of Cu$^{2+}$ was deleterious to different organs including liver, kidney and brain (Kumar, Kalita, Bora, & Misra, 2016), leading to organ dysfunction and neurodegenerative diseases. River water as well as drinking water contaminated with waste from mines or cable factories increased the possibility of excess intake of Cu$^{2+}$.

Conventional methods for the detection of Cu$^{2+}$ including inductively coupled plasma optical emission spectrometry (Xiu and Zhang, 2009), inductively coupled plasma atomic emission spectrometry (Lee, Choi, Suh, & Lee, 2016) and inductively coupled plasma-mass spectrometry (Hare et al., 2017) suffered professional operation, time-consuming procedures and high-cost expense. Since the employment of gold nanoparticles in the biosays for colorimetric and visual detections (Guo, et al., 2015; Yuan, Hu, Chang, & Lu, 2016), scientists have been devoting increasing efforts to develop optical sensors for easy-to-read output in point-of-care or on-site assays. The synthesis and application of
noble metal nanoclusters have attracted enormous contributions from researchers in the recent years due to their unique optical, physical, chemical, electrical and catalytic properties (Tao, Li, Ren, & Qu, 2015; Wang & Li, 2011). The molecular-scale noble metal nanoclusters consist of two to several hundred metal atoms, and can exhibit very strong absorption and emission property. For instance, gold nanoclusters (AuNCs) emit strong luminescence under the UV light due to the intrinsic quantum effect (Qian, Zhu, Wu, & Jin, 2012; Vosch et al., 2007), providing robust tools for optical detections. For example, Pei’s group developed optical sensors for the visual detection of Ca$^{2+}$ and Pb$^{2+}$ using glutathione-capped AuNCs (Ji et al., 2015). Xie, Zheng, and Ying (2010) proposed visual assays for sensing Hg$^{2+}$ by employing bull serum albumin-capped AuNCs.

Among the materials that were employed to synthesize AuNCs, including polyelectrolytes, thiolate compounds, oligonucleotides, peptides and proteins (Yau, Varnavski, & Goodson, 2013), proteins have the most advantages due to the presence of multiple functional groups (–NH, –COOH, –OH and –SH) and their environmentally friendly property as natural compounds. For instance, bull serum albumin (Xie, Zheng, & Ying, 2009), lysozyme (Chen & Tseng, 2012), papain (Chen et al., 2013), egg white protein (Joseph & Geckeler, 2014) and soybean protein (Ling et al., 2016) all showed the capacity to cap luminescent AuNCs. Herein, we utilized soybean protein, a plant protein that is commercially available, naturally safe and environmentally degradable, to synthesize AuNCs for developing a visual assay for Cu$^{2+}$ sensing in a water-source lake. This work focused on the optimum conditions of AuNC synthesis using soybean protein and the development of the sensing method of Cu$^{2+}$.

Materials and methods

Instrumentation

Absorption spectra were obtained using a UV–Vis spectrophotometer (UV-1800, Shimadzu Company, Kyoto, Japan). The excitation and emission spectra were recorded on a fluorescence spectrophotometer (F-7000, Hitachi, Japan). The morphology of gold nanoclusters was characterized by transmission electron microscope (TEM) (JEM-2100, Electronics Co. Ltd., Japan). The hydrodynamic diameters of AuNCs were quantified by dynamic light scattering (DLS) conducted using a Malvern Zetasizer (Nano-ZS, Malvern Instrument, UK).

Reagents

Soybean seeds were provided by Shandong Yuwang Industrial Co. Ltd. (Shandong, China). Defatted soybean protein was laboratory-made from soybean seed according to a well-established process (Mateo & Veum, 1980), and its purity was measured by the Kjeldahl method ($N \times 6.25$). Chloroauric acid (HAuCl$_4$) was purchased from Shaen Chem. Tech. Co. Ltd. (Shanghai, China). CuSO$_4$·5H$_2$O was purchased from Sinopharm Chem. Reagent Co. Ltd. (Shanghai, China). All other reagents were of analytical grade and used as received. Ultrapure water obtained from a Millipore water purification system (18.2 MΩ cm$^{-1}$, Milli-Q, Millipore) was used in all experiments.
Preparation of soybean protein-templated AuNCs (SP-AuNCs)

Soybean protein-templated AuNCs (SP-AuNCs) were synthesized using a modified procedure reported by Xie et al. (2009). In the procedure, soybean protein functioned as both the stabilizer and the reducing agent. In a typical synthesis, under vigorous stirring, an aqueous solution of HAuCl₄ (5 mL, 10 mM) was added into the soybean protein solution (5 mL, 70 mg/mL), followed by 2-min stirring. Then NaOH (1 M) was added to adjust the pH to alkalinity, and the effect of different pH values ranging from 8 to 13 on the reaction was investigated. Finally, the volume of the mixture solution was adjusted to 11 mL by adding ultrapure water. The mixture was incubated in a water bath chader (200 rpm) at 60°C until the colour turned from light yellow to pale brown (6 h), indicating the formation of SP-AuNCs. Before measuring, the as-prepared SP-AuNCs were purified by using a dialysis bag with a molecular weight cut-off of 3.5 kDa. The purified products were pink-emitting SP-AuNCs and stored in a refrigerator at 4°C.

Assay protocol

Typically, Cu²⁺ detection was carried out as follows. Hundred microlitre of Cu²⁺ solutions of different concentrations (0–1600 μM) obtained by serial dilution of the stock solution (10 mM) was mixed with 100 μL phosphate-buffered solution (15 mM, pH8.0), followed by the addition of 50 μL pink-emitting SP-AuNCs. After 30 min of reaction, 200 μL of the reactant solution was transferred into a quartz cuvette for fluorescence spectra recording at ambient temperature.

To evaluate the selectivity towards Cu²⁺, all metal ions (800 μM) including K⁺, Ca²⁺, Na⁺, Mg²⁺, Al³⁺, Zn²⁺, Pb²⁺, Mn²⁺, Ba²⁺, Co²⁺, Cd²⁺ and Fe³⁺ were employed to investigate the sensor responses.

Results and discussion

Optimum conditions for the synthesis of SP-AuNCs

Soybean protein has similar physicochemical properties to BSA, which has been proven to work for the preparation of luminescent AuNCs. Soybean protein mainly consists of β-conglycinin (35%) and glycinin (52%) (Qi, Venkateshan, Mo, Zhang, & Sun, 2011), in which cysteine moieties could provide functional –SH as chelating groups for capturing Au ions (Riblett, Herald, Schmidt, & Tilley, 2001) and tyrosine moieties could act as reducing agents under alkaline conditions (Xie, Lee, Wang, & Ting, 2007). The pH value of the reaction condition undoubtedly played an essential role in the AuNC synthesis where proteins or peptides or enzymes were used as stabilizers. Soybean proteins tend to form aggregations at the isoelectric point (∼4.8) (Yuan et al., 2002); on the other hand, under acidic conditions, the protonation of various functional groups including –SH, –NH, –OH and –COOH would weaken the interactions, so the reaction pH was chosen far from this. In this study, no characteristic emission was observed at ∼600 nm even under alkaline conditions at pH values of 8, 9, 10 and 11 (data not shown). The pink-emitting SP-AuNCs could only be successfully synthesized at pH 12 and 13. As shown in Figure 1, after adjusting the pH to 13 from 12, a sharp decrease
in the emission intensity of AuNCs emerged accompanied with a slight red shift of 25 nm, indicating the decreased emission energy and low quantum yields. Therefore, pH 12 was selected as the optimum alkalinity.

The incubation time in the process of AuNC synthesis was also investigated. When excited by 400 nm, a characteristic peak at about 600 nm was gradually generated with increasing intensities. As shown in Figure 2, the emission intensity of the SP-AuNCs reached a plateau after 6 hours of incubation. Therefore, the incubation time was adjusted to 6 h.

![Figure 1.](image1.png)  
**Figure 1.** Fluorescence emission spectra of SP-AuNCs prepared at different pH values: pH 13 (black) and pH 12 (red). The excitation wavelength was 400 nm.

![Figure 2.](image2.png)  
**Figure 2.** The fluorescence emission intensity of SP-AuNCs recorded at different incubation times.
Characterization of SP-AuNCs

As shown in Figure 3, the synthesized SP-AuNCs were pale brown in colour under visible light (a) and emitted a pink fluorescence at ~600 nm (red dotted line) under UV light (b). Photographs of the solutions of HAuCl₄ and pure soybean protein were also taken under UV light exposure. In distinct contrast, t, no fluorescence emission was found for HAuCl₄ and soybean protein. It is worth mentioning that the protein emitted a light blue fluorescence at ~485 nm (data not shown), but with no emission at 580–620 nm. It is known that many proteins bear blue emissions under UV irradiation, which is attributed to the aromatic amino acids (Try, Tyr, Thy and Phe) (Shore & Pardee, 1956). The difference between the emission spectra of soybean protein and SP-AuNCs indicated the formation of metal nanoclusters. The absorption spectrum of SP-AuNCs showed a broad band from 300 to 500 nm and no strong absorbance peak at ~500 nm of large nanoparticles. The enhanced absorbance at ~320 nm was due to the absorption of Au³⁺ (Eustis, Hsu, & El-Sayed, 2005), and the broad band of 300–500 nm could be attributed to the electronic transition between the protein ligand and the gold core (Le Guevel et al., 2011).

To further confirm the formation of SP-AuNCs, TEM analysis was conducted, and two TEM images of different magnifications were obtained. As shown in Figure 4, the as-prepared SP-AuNCs were spherical in shape without the formation of large-sized nanoparticles. The diameters of the SP-AuNCs ranged from 2.6 to 12.5 nm with an average of 6.5 nm, which is a little larger than that found in the previous literature. This could be attributed to the big capping ligand, soybean protein of molecular weight ranging from 8 to 600 kDa. It was generally considered that only noble metal nanoclusters with size smaller than 2 nm could emit under UV light excitation due to the quantum effect.

![Figure 3](image-url) **Figure 3.** UV–VIS absorption and fluorescence emission ($\lambda_{\text{ex}} = 400$ nm) spectra of the as-prepared SP-AuNCs (solid line in absorption spectrum, and dotted line in emission spectrum) and pure soybean protein (dash line in absorption spectrum, and blue dash-dotted line in emission spectrum), respectively. Inset: digital images of the solutions of HAuCl₄ (item 1), SP-AuNCs (item 2) and pure soybean protein (item 3) under (a) visible light and (b) 325-nm UV light.
TEM results in our study indicated that the essential mechanism of photoluminescence for nanoclusters could not be simply explained by the quantum effect. In addition, the photo-emission of nanoclusters was also strongly dependent on the type of surface ligands together with the ligand-to-metal–metal charge transfer (LMMCT) mechanism in the complex ligand–core–shell structure (Chen et al., 2014).

**Fluorescence measurement and visual assay of Cu²⁺**

**Sensitivity of fluorescence assay**
Due to the excellent optical properties of luminescent nanoclusters, many works have been reported for developing visual assays of heavy metal ions (Wei et al., 2010; Zang et al., 2016). For instance, Xie et al. developed a highly selective and ultrasensitive fluorescence sensor for Hg²⁺ detection, with a detection limit of 0.5 nM. In the present work, SP-AuNCs were employed for Cu²⁺ sensing since Cu²⁺ can quench the fluorescence emission of SP-AuNCs through promoting the intersystem crossing of SP-AuNCs in the excited state (Durgadas, Sharma, & Sreenivasan, 2011). The promotion might be due to the chelation between Cu²⁺ and the active moieties of soybean protein, such as –NH₂ and –COOH.

The effect of the concentration of Cu²⁺ on the fluorescence quenching of SP-AuNCs was investigated. As shown in Figure 5, the fluorescence emission intensity of as-prepared SP-AuNCs gradually decreased with an increase in the Cu²⁺ concentration from 0 to 1600 μM, indicating that the sensor had a wide response range and was of great sensitivity. A good linear relation was obtained with an $R^2$ of 0.9951 between the relative fluorescence intensity ($I/I_0$) and Cu²⁺ concentration ranging from 40 to 400 μM (inset). The limit of detection (LOD) of the sensing method using the as-synthesized SP-AuNCs as the probe was estimated to be 10 μM based on a signal-to-noise ratio of 3, which was lower than...
the contaminant level (20.5 μM) in drinking water regulated by the United States Environmental Protection Agency. It is noteworthy that the linear range and LOD would change along with the concentration of SP-AuNCs. Lower concentrations in range and relatively lower LOD could be achieved by employing low concentrations of SP-AuNCs in the assays.

**Visual assay and selectivity of the assay**

The selectivity of the sensor constructed for Cu$^{2+}$ detection using the as-prepared SP-AuNCs was evaluated. Several metal ions including K$^+$, Ca$^{2+}$, Na$^+$, Mg$^{2+}$, Al$^{3+}$, Zn$^{2+}$, Pb$^{2+}$, Mn$^{2+}$, Ba$^{2+}$, Co$^{2+}$, Cd$^{2+}$ and Fe$^{3+}$ (800 μM) were tested as a contrast. As indicated in Figure 6(a,b), only Cu$^{2+}$ could cause a significant quenching of the pink-emitting SP-AuNCs, exhibiting apparent contrast compared with other ions. The results could be read by a portable UV light and its ability demonstrated for on-site visual and fast assays. As shown in Figure 6(c), the emission intensity showed a sharp decrease when challenged with only Cu$^{2+}$ (800 μM), indicating that the proposed method had an excellent selectivity towards Cu$^{2+}$. In addition, this quenching selectivity of our assay could be visualized with naked eyes (b), demonstrating it could be a rapid online visual evaluating method for Cu$^{2+}$ samples, especially at high Cu$^{2+}$ concentrations.

**Recovery performance**

A suitable volume of tap water was appropriately spiked with the standard solution of Cu$^{2+}$ and three samples with the same concentration of Cu$^{2+}$ (180 μM) were obtained for the analysis. The analysis results showed that quantitative recoveries of all cases between 113% and 125% were achieved. These results showed that this method has potential for the quantitative determination of free Cu$^{2+}$ in tap water samples.

![Graph showing relative fluorescence intensities of SP-AuNCs probes challenged with increasing concentrations of Cu$^{2+}$.](image)
Conclusions

To summarize, biodegradable and environmentally friendly AuNCs were synthesized by using natural soybean protein, and were employed as optical probes for the facile assay of Cu$^{2+}$. To get the brightest emission, the optimum conditions for AuNC synthesis were investigated. The pH and time-dependent effects on the fluorescence emission of SP-AuNCs were studied. It was found that the pink-emitting AuNCs could only be synthesized at pH $\geq 12$, and their emission intensity would reach the plateau level after 6 h. More interestingly, we found that AuNCs using soybean protein as template were luminescent but had sizes larger than 2 nm, which was considered un-luminescent according to the classical quantum effect theory. This will pave a way for further research on the clarification of mechanism. The AuNCs emitted a characteristic fluorescence at 600 nm by 400 nm excitation. The sensor constructed based on the AuNCs showed high selectivity and sensitivity for sensing Cu$^{2+}$ with an LOD of 10 μM and a linear detection concentration ranging from 40 to 400 μM. We successfully employed the AuNCs for the detection of Cu$^{2+}$ in real samples of tap water.

Disclosure statement

No potential conflict of interest was reported by the authors.

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