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A turn-on fluorescent probe for phytic acid based on ferric ions-modulated glutathione-capped silver nanoclusters

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In this work, we describe a method for fluorometric determination of phytic acid (PA) by using glutathione-capped silver nanoclusters (GSH@AgNCs). This method is based on the finding that ferric ions (Fe³⁺) can quench the fluorescence of GSH@AgNCs. PA, a kind of multiphosphate with a high density of negatively-charged phosphate groups, shows a strong affinity for Fe³⁺ ions by the multiple ligands. As a result, the presence of PA can prevent the quenching of Fe³⁺ ions towards GSH@AgNCs thus resulting in the turn-on fluorescence of GSH@AgNCs. The developed fluorimetric analysis method can allow for the detection of PA with a detection limit of about 1.0 µM. Furthermore, this method was also successfully applied to the determination of PA in spiked corn grain samples with satisfactory recoveries in the range of 91.4 to 107%.

Introduction

Phytic acid (PA) is a natural plant constituent widely found in legumes, cereals, oil seeds, pollens and nuts. Historically, PA is considered solely as an antinutrient mainly due to its ability to bind essentail dietary minerals including calcium, iron, and zinc, as well as proteins and starch, and to consequently reduce their bioavailability in humans. In recent years, it has been reported that PA has also a variety of benefits on human health, since it can trigger the reduction of the risk of cancers, heart diseases, diabetes, and renal calculi. It can also bind potentially toxic mineral elements such as Cd²⁺ and Pb²⁺ in human body, and thus influences their toxicity and facilitates their excretion. Considering both potentially detrimental and beneficial effects of PA, it is highly necessary to develop a reliable method for the determination of PA in order to make a valuable evaluation on its metabolism in human body.

The known analysis methods for the PA determination are basically limited to the chromatography ones including ion pair chromatography, high-performance liquid chromatography, liquid chromatography, and gas chromatography. However, the procedures are time-consuming and needs some large-scale instruments. Therefore, it is necessary to develop a simple and economical in operation method for the determination of PA. Fluorimetric methods have received widespread attention because of their high sensitivity, simplicity, and rapid implementation. However, there are rarely fluorescent probes available for the determination of PA except organic fluorescent dyes and carbon nanodots. Therefore, it still remains highly desirable to develop a new and efficient fluorescent probe for the determination of PA.

Fluorescent noble metal nanoclusters (NCs), i.e., AuNCs, AgNCs, and PtNCs, which consist of several to tens of metal atoms have increasingly applied for fluorimetric analysis owing to their unique physical, electrical, catalytic and optical properties. These NCs are of significant interest because they provide the bridge between atomic and nanoparticle behavior in noble metals. In comparison with organic dye molecules, NCs have better luminescence properties such as outstanding photostability, large Stokes shift, and good biocompatibility. Among these metal nanoclusters, AgNCs with particularly fluorescence have been proved to be excellent fluorophores for bioimaging, biological probes, and chemical sensing.

As a result, a lot of efforts have been made to direct the synthesis of fluorescent and water-soluble AgNCs with the help of various stabilizers, including polyelectrolytes, thiolated DNA, thiolated and biomolecules. Especially, peptides have attracted increasing attentions in the synthesis of fluorescent AgNCs due to their excellent biocompatibility and abundant functional groups. Glutathione (GSH) is a natural tripeptide consisting of glutamic acid, cysteine, and glycine, which is widely used as a stabilizing agent in the synthesis of AgNCs. For example, Xie et al developed a facile boiling water synthesis protocol to prepare the GSH-protected AgNCs with strong luminescence via the aggregation-induced emission. Zhou et al prepared GSH stabilized AgNCs though a facile sonocatalytic approach for the selective detection of S²⁻ ions. Xie et al designed a cysteine sensor based on the GSH-protected AgNCs. Chen et al developed a Fe³⁺ sensor based on the Fe³⁺ ions-induced quenching of the fluorescence of GSH-capped AgNCs.

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and their coworkers developed a fluorimetric method for the rapid determination of trace-level Hg\(^{2+}\) by using GSH-capped AgNCs.\(^{52}\) Recently, our group also used GSH-passivated AgNCs as the fluorescent probes for the analysis of Cu\(^{2+}\) ions.\(^{53}\)

In the present work, aiming to seek for the further application of GSH@AgNCs, a sensitive fluorimetric assay by the turn-on fluorescence has been developed for probing PA based on the Fe\(^{3+}\)-modulated GSH@AgNCs. Herein, Fe\(^{3+}\) ions can quench the fluorescence intensity of GSH@AgNCs efficiently through the weak interaction from collision.\(^{51}\) PA has a strong binding affinity for Fe\(^{3+}\) ions, thus they could effectively restrain the quenching effect of Fe\(^{3+}\) ions to GSH@AgNCs. Compared with the traditional analysis methods using organic dyes, the developed fluorimetric assay with AgNCs is less toxic, easier to synthesis, and more sensitive. This is the first time that AgNCs are applied in the detection of PA in real samples. The detection performances of the developed fluorimetry, including sensitivity, selectivity, repeatability, stability, and recovery, were evaluated for sensing PA both in the standard and real samples.

**Experimental**

**Reagents and apparatus**

Phytic acid (PA), silver nitrate (AgNO\(_3\)), sodium borohydride (NaBH\(_4\)), GSH (>98%), ferric chloride hexahydrate (FeCl\(_3\)·6H\(_2\)O), lysine, and threonine were bought from Aladdin Chemical Reagent Co. Ltd. All reagents were of analytical grade and used as received without further purification. Ultrapure water prepared from a Millipore water purification system was used throughout the experiments.

The sonochemical synthesis was performed on Ultrasonic Cleaner KQ-100B (Kun Shan Ultrasonic Instruments Co., Ltd., Jiangsu, China). The fluorescence measurements were performed on fluorescence spectrophotometer (Max-4, HORIBA, USA). The UV-vis absorption spectra were recorded on a UV-vis spectrophotometer (Shimadzu, UV-3600, Japan). Transmission electron microscopy (TEM) images were obtained using a FEI Tecnai G2 F20 microscopy.

**Synthesis of GSH@AgNCs**

GSH@AgNCs were prepared as previously reported.\(^{49}\) Briefly, AgNO\(_3\) (25 mM, 5.0 mL) and GSH (25 mM, 20 mL) were mixed together with vigorous stirring. The solution became deep turbid and then 1.0 M NaOH solution was added drop-wising to adjust the pH to about 5.0 (the solution became clear, kept stirring). After 15 min, the mixture was irradiated with ultrasound in the dark for 8 hours. The obtained GSH@AgNCs solution was preserved in 4 ºC in the dark without further purification.

**Fluorescence measurements**

Aqueous Fe\(^{3+}\) solutions (5.0 mM) were freshly prepared before use. An aliquot of 10 µL PA of different concentrations was separately introduced into the acetate-sodium acetate (HAc-NaAc) buffer (50 mM, pH 4.5, 480 µL) containing Fe\(^{3+}\) (0.050 mM) to be incubated for 5 min at room temperature. Then, 25 µL AgNCs (>100) were added into the above solution. After incubation for 15 min, the fluorescence intensities of the solutions were measured with an excitation at 350 nm. The selectivity of PA detection was investigated by the addition of other interferences following the same procedure. All experiments were performed at room temperature.

**Preparation of real samples**

To prepare the samples, the corn grains were collected from local supermarket and were treated following the previously reported procedure.\(^{18}\) Briefly, a 0.50 g amount of fine-ground corn grains was extracted with 10 mL of 0.50 M HCl for 2 h at room temperature. The suspension was centrifuged at 5000 rpm for 30 min and then the supernatant was purified by anion-exchange treatment with 0.70 M NaCl solution. Furthermore, the collected samples containing PA were diluted with HAc-NaAc buffer to a 50 mL volumetric flask to be detected with three replicates for each sample.

**Results and discussion**

**Characterization of the GSH@AgNCs**

The as-prepared GSH@AgNCs were characterized by TEM, UV-vis absorption, and fluorescence spectroscopy as indicated in Fig. 1 and Fig. 2.

From the TEM image (Fig. 1), it can be seen that the GSH@AgNCs were spherical, well dispersed and uniform with an average size of about 2.0 nm in diameter. Fig. 2 showed the UV-vis absorption spectra and fluorescence spectra of GSH@AgNCs. From the absorption spectra, it was observed that the GSH@AgNCs exhibited a sharp absorption peak at 350 nm, which is according with the previous report.\(^{51}\) GSH was not fluorescent. In contrast, when excited at 350 nm, the GSH@AgNCs exhibited a strong emission at 430 nm, which indicated the formation of the nanoclusters. In addition, the GSH@AgNCs synthesized in this work were very stable in the dark at 4 ºC. As shown in Fig. S1, no obvious change was seen (<10% decrease in fluorescence intensity) for the as-synthesized GSH@AgNCs even after four weeks storage, indicating the excellent stability of the AgNCs.

Based on the results and referring to the previous report,\(^{49}\) a possible mechanism has been proposed for the fluorescent property of AgNCs. Firstly, Ag\(^+\) reacted with GSH to form a kind of silver thiolate complex, then ultrasonic irradiation destroyed the C-S bond of the silver thiolate complex and released a radical R\(^-\). After the radical R\(^-\) reduced Ag\(^+\) to Ag\(^0\), AgNCs were thus generated to be kept stable in the presence of excess GSH, which was taken as a stably capping agent.
The mechanism of the proposed fluorimetry method

Fig. 3 shows the fluorescence emission spectra of GSH@AgNCs under the different analysis conditions. The GSH@AgNCs shows a strong fluorescence at 430 nm. As expected, once Fe$^{3+}$ ions were introduced, the fluorescence of GSH@AgNCs can significantly decrease by about 82% of the fluorescence intensity due to the quenching by Fe$^{3+}$ ions through the weak interaction from collision. PA, a kind of multiphosphate with a high density of negatively charged phosphate groups, shows a high affinity for Fe$^{3+}$ ions by the multiple ligands. Thus, it is expected that the presence of PA can inhibit the quenching of Fe$^{3+}$ ions towards the fluorescence of GSH@AgNCs. The above results confirmed well this assumption. As shown in Fig. 3, the fluorescence intensity of GSH@AgNCs remained about 92% in the presence of 50 µM PA. The photographs of the three samples under UV light (inset of Fig. 3) coincided with the variation of fluorescence. To get rid of the possible adverse impact on GSH@AgNCs caused by PA, PA was also employed to the GSH@AgNCs solution (without Fe$^{3+}$ ions) as a contrast. The intense fluorescence intensity indicated that PA caused negligible effect on the fluorescence intensity of GSH@AgNCs (Fig. S2). Therefore, we speculate that a turn-on fluorescence method can be proposed based on the anti-quenching ability of Fe$^{3+}$ ions to GSH@AgNCs caused by the coordination of PA and Fe$^{3+}$ ions.

Optimization of the sensing system

Prior to the application of our fluorescent method for the detection of PA, some key factors including the concentrations of Fe$^{3+}$, the pH values, and the reaction time need to be further optimized.

We firstly investigated the concentrations of Fe$^{3+}$ on the fluorescence intensity of GSH@AgNCs. As shown in Fig. S3, with the increase of Fe$^{3+}$ levels, the fluorescence intensity decreased quickly at the beginning and then gradually tended to be stable. When the concentrations of Fe$^{3+}$ reached 50 µM, nearly 82% fluorescence intensity of GSH@AgNCs was quenched. When the concentrations of Fe$^{3+}$ increased continually up to 100 µM the quenching degree only increased from 82% to 87%. It is conceivable that the limited amounts of PA might be unlikely to restrain excess Fe$^{3+}$ effectively. Thus, 50 µM Fe$^{3+}$ was selected in the whole detection process. Such a concentration of Fe$^{3+}$ was not only large enough to achieve effective fluorescence quenching but also within the ability for PA to restrain them.

Fig. S4 showed the effect of pH values on the fluorescence intensities of GSH@AgNCs in the absence and presence of Fe$^{3+}$ ions, and Fe$^{3+}$ ions with PA, respectively. Obviously, the fluorescence intensity of GSH@AgNCs decreased with increasing pH values. Upon addition of Fe$^{3+}$ ions, the fluorescence intensities of the AgNCs with Fe$^{3+}$ ions system decreased firstly and then increased slightly with the increasing pH values.

Fig. 4 shows the effect of pH values on the fluorescence enhanced factor ($F_{enh}$) with $F_0$ where $F$ and $F_0$ are the fluorescence intensities of GSH@AgNCs in the presence of both PA and Fe$^{3+}$ and only Fe$^{3+}$ ions. The results indicated that the fluorescence enhanced factor ($F_{enh}$) is the largest when the pH value of
the media is around 4.5. Therefore, a comparatively weak acidic environment with the pH value of 4.5 is the optimal condition for the sensing system.

According to the previous report, the reactions between Fe$^{3+}$ ions and the GSH@AgNCs reached a state of equilibrium within 15 min. Thus, the optimum incubation time of the system in our experiments was also chosen as 15 min. Furthermore, the reaction process between PA and Fe$^{3+}$ ions was also investigated. The time-dependent fluorescence changes upon addition of 20 µM PA were monitored, as shown in Fig. S5. As time went by, the fluorescence changed slightly, indicating a quick reaction process. Taking the complete reactions with the lower concentrations of PA into account, the reaction time of 5 min should be chosen for the subsequent experiments.

**Sensitivity of the sensing system**

Under the optimum experimental conditions, we evaluated the capability of this analytical system for the quantitative detection of PA. Fig. 5 displays the fluorescence spectra of the Fe$^{3+}$-GSH@AgNCs system in the presence of various concentrations of PA. As shown in Fig. 5A, the fluorescence intensity of GSH@AgNCs at 430 nm increases gradually with the increasing concentrations of PA, indicating that the addition of PA can efficiently coordinate with Fe$^{3+}$ ions, preventing the fluorescence quenching process induced by Fe$^{3+}$. From Fig. 5B, the developed method exhibited a good behavior to the detections of PA in the linear range of 5.0 - 20 µM (R = 0.996) with a detection limit of 1.0 µM at a signal-to-noise ratio of 3. The reproducibility of the sensing system was also investigated by operating six repeated measurements of 10 µM PA, and the relative standard deviation (RSD) was found to be 4.2 %, which suggested the reliability of the proposed method.

**Selectivity of the fluorescent method**

The selectivity of this fluorescent probe was investigated by examining the fluorescence responses of the Fe$^{3+}$-GSH@AgNCs complex toward various co-existing substances, including different metal ions, anions, ethylenediaminetetraacetic acid (EDTA), amino acids. As shown in Fig. 6, no obvious signal change was observed for these substances, whereas the addition of PA resulted in significant fluorescence enhancements, as described previously. The results indicated that these substances had little influence on the fluorescence intensity of the Fe$^{3+}$-GSH@AgNCs system. In addition, an experiment was carried out in which both 20 µM PA and competitive substances were added (Fig. S6). The results revealed that the competitive substances had minor or no influence on the fluorescence recovery response to PA, indicating the high selectivity for PA in the presence of these interfering substances. The good selectivity of this Fe$^{3+}$-GSH@AgNCs system might be ascribed to the larger stability constant between PA and Fe$^{3+}$.19
expensive instruments. To the best of our knowledge, this is the first time that GSH@AgNCs are employed for the detection of PA. It paves the potential ways to the extensive applications of AgNCs for different sensing detections.

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Notes and references


Fig. 6 The selectivity of Fe³⁺-GSH@AgNCs toward PA. The concentration of PA and other interferences were both 20 µM. From 1 to 13 is PA, Cl⁻, CO₃²⁻, SO₄²⁻, Mg²⁺, Cu²⁺, Ca²⁺, K⁺, Zn²⁺, Al³⁺, EDTA, lysine, and threonine, respectively.

Analysis application for PA in real samples

The highly selectivity of this fluorescent platform provides an analysis potential for the detection of PA in the complex samples. To evaluate the practicality of the present method, the proposed fluorimetry method was applied for determining the recovery ratios by adding different concentrations of PA into the real corn grain samples. As shown in Table 1, the recovery ratios of these measurements ranges from 91.4 % - 107 % with a RSD of less than 5.0 %, indicating that the proposed method is suitable for the quantitative determination of PA in real samples.

Table 1 Determination of PA in corn grain samples with different concentrations.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Determined (µM)</th>
<th>Added (µM)</th>
<th>Measured (µM)</th>
<th>Recovery (%)</th>
<th>RSD (n=3, %)</th>
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<tr>
<td>1</td>
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<td>5.0</td>
<td>12.58</td>
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<td>3.43</td>
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<td>99.6</td>
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<td>12.49</td>
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<td>16.79</td>
<td>91.35</td>
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</tbody>
</table>

Conclusions

In summary, a sensitive fluorimetry method has been developed by the turn-on fluorescence of GSH@AgNCs for the detection of PA. This strategy relies on anti-quenching ability of the Fe³⁺ ions to fluorescent GSH@AgNCs through the strong coordination of PA with Fe³⁺ ions. The proposed fluorimetry method displays the high selectivity, low detection limit, and well reproducible performances in the determination of PA in real samples, without the complicated modifications and expensive instruments. To the best of our knowledge, this is the first time that GSH@AgNCs are employed for the detection of PA. It paves the potential ways to the extensive applications of AgNCs for different sensing detections.
A sensitive fluorescent detection assay for phytic acid (PA) in real samples was developed based on the Fe$^{3+}$-modulated GSH@AgNCs, with the high detection selectivity, repeatability, and stability.