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ORIGINAL PAPER

Simple method for visual detection of glutathione *S*-transferase activity and inhibition using cysteamine-capped gold nanoparticles as colorimetric probes

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Abstract An operationally simple colorimetric method for measuring glutathione *S*-transferase (GST) activity was developed using cysteamine-capped gold nanoparticles (AuNPs) in this work. This method was based that GST can catalyze the reaction of 1-chloro-2,4-dinitrobenzene (CDNB) and glutathione (GSH) to produce a conjugate (CDNB-SG). GSH could induce the aggregation of AuNPs, whereas the conjugate could not induce the aggregation of AuNPs. Thus, GST activity can be facilely assayed with the naked eye or a simple colorimetric reader. The convenient and simple colorimetric response of the assay makes them an attractive approach for drug-screening application.

Keywords Glutathione *S*-transferase · Colorimetric method · Gold nanoparticles · Inhibition

Introduction

Glutathione *S*-transferases (GSTs) constitute a family of detoxification enzymes that catalyze the conjugation of glutathione (GSH) with a variety of hydrophobic compounds, including drugs and their metabolites, to yield water-soluble derivatives that are excreted in urine or bile. GSTs are involved in multiple biological functions, including xenobiotic detoxification, clearance of oxidative stress products, protein transport, modulation of cell proliferation, and induction of the apoptosis signaling pathway [1]. Moreover, GST isozymes (Alpha, Mu, and Pi) are frequently over-expressed in neoplastic tissues and have been considered as an important marker for a number of tumors such as lung, ovarian, breast, and gastric carcinoma [2, 3]. The assay of GST activity is of high importance for the development of efficient therapeutics and the screening of new anticancer drugs. To date, 1-chloro-2,4-dinitrobenzene (CDNB), a ultraviolet chromogenic substrate, is the most prevalent probe for the assay of GST activity [4]. However, the chromogenic probe faces some obvious flaws like short absorption wavelength (340 nm), high background, and low sensitivity. While several elegant methods have so far been developed for GST activity assay [5, 6], these methods are limited by the expensive materials, sophisticated instruments, or their time-consuming experimental procedures. As a consequence, a facile, cheap, and sensitive method for GST activity assay is still highly desired.

Because of their easy preparation, excellent biocompatibility, and unique optoelectronic properties, gold nanoparticles (AuNPs) have attracted increasing attention in many fields. The well-dispersed AuNP solution is red, whereas the aggregated AuNP solution appears a blue color. The color change induced by aggregation of AuNPs provides an ideal platform for colorimetric analysis [7]. The major advantage of AuNP-based colorimetric assay is that the molecular recognition event can be transformed into color change, which can be easily observed by the naked eye, and therefore, no sophisticated instruments are required. AuNP-based colorimetric assay has been used for the detection of various substances including small molecules, DNA, and proteins [7–9]. The relatively simple measurement, inexpensive instrumentation, and robustness and potential for high-throughput assay make AuNP-based colorimetric assays excellent candidates for in vitro measurements of enzyme activity. There are already a number of AuNP-based colorimetric assays for transferases

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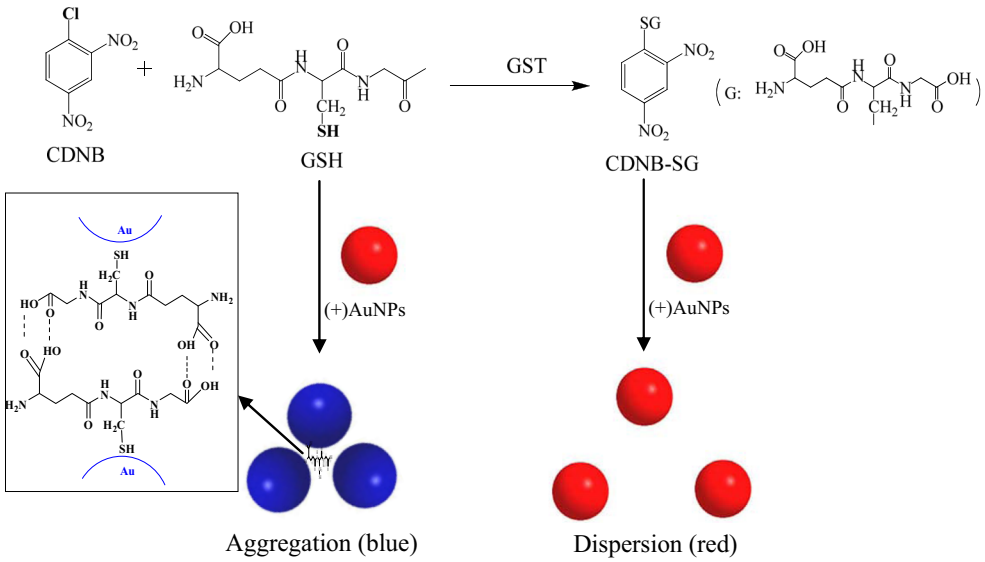

Scheme 1 Visual detection of GST activity based on the color change of (+)AuNPs

and hydrolases [10–16]. However, to the best our knowledge, AuNP-based colorimetric assay is not used to detect GST activity.

In this study, we propose one AuNP-based colorimetric assay for GST activity. The overall strategy is illustrated in Scheme 1. The cysteamine-capped AuNPs were synthesized and used as probes. GSH is the GST substrate, and GST can catalyze the reaction of CDNB and GSH to produce a conjugate (CDNB-SG). Thiol group of GSH tends to readily adsorb onto the surface colloidal particles via Au–S bond, and then, GSH can induce aggregation of the AuNPs. As a result, the color of AuNP solution changes from red to blue. In the presence of GST, GSH is converted into CDNB-SG, and the AuNP solution remains red. Thus, GST activity can be facilely assayed with the naked eye or a simple colorimetric reader. Moreover, the colorimetric method developed here is also employed for GST inhibitor evaluation.

Experimental

Reagents and chemical

Chloroauric acid ($HAuCl_4$), GST, GSH, CDNB, dihydroquercetin, and sodium borohydride were purchased from Shanghai Chemical Reagent Company (Shanghai, China). Cysteamine was purchased from Sinopharm Chemical Reagent Company (Beijing, China). All other solvents and reagents in this investigation were of analytical grade and used without further purification. Millipore water (18 MΩ cm) was used in all experiments. The experiments were conducted at room temperature (ca. 20 °C).

Apparatus

UV-visible adsorption spectra were recorded on a U-3900H UV–Vis Spectrophotometer (Hitachi, Japan) at room temperature using a 500-μL black-body quartz curette with 1-cm path length. The photographs were taken with a Cannon 500 digital camera. The pH measurements were carried out on model PB-10 digital ion analyzer (Sartorius Scientific Instruments Co., Ltd., China, Beijing). Transmission electron microscopy (TEM) measurements were made on a JEM-2100 transmission electron microscope (Jeol Co. Ltd., Japan). The samples for TEM characterization were prepared by placing a drop of colloidal solution on carbon-coated copper grid and dried at room temperature.

Preparation of cysteamine-capped AuNPs

All glassware used in the following procedure was cleaned in a bath of freshly prepared 3:1 HNO_3/HCl, rinsed thoroughly in water and dried in air prior to use. The positively charged AuNPs were prepared according to the published protocol [17]. Briefly, a cysteamine solution (400 μL, 213 mM) was added to 40 mL of 1.42 mM $HAuCl_4$ solution. After stirring for 20 min at room temperature, 10 μL of 10 mM $NaBH_4$ solution was added, and the mixture was vigorously stirred for 10 min at room temperature in the dark. Then, the mixture was further stirred 15 min, and the resulting win-red solution was stored in the refrigerator (4 °C) and ready for use. The as-prepared AuNPs were charactered with UV-visible absorption spectra and TEM. The results of TEM showed that the average size of the AuNPs was about 34 nm. The concentration of the AuNP solution was 10.5 nM, which was estimated by the original concentration of the gold solution [18].

Colorimetric detection of GST activity

A typical colorimetric assay of GST was realized by following the procedure given in Scheme 1. First, to a 1.5-mL Eppendorf tube were added 25 μL of GSH (12 mM), 45 μL of CDNB (3.0 mM), 50 μL of GST (appropriate concentration), and 200 μL of triethanolamine–HCl buffer (20 mM, pH 6.5), and then, the mixed solution was incubated for 60 min at 25 °C. Second, 50 μL of the reacted solution, 180 μL of AuNPs (10.5 nM), 180 μL of Britton–Robinson (BR) buffer (0.04 M H_3PO_4, 0.04 M HAc, 0.04 M H_3BO_3, pH 3.6), and 90 μL of H_2O were orderly added into a 1.5-mL Eppendorf tube, and the solution was allowed to react for 15 min at room temperature (ca. 20 °C). Finally, the picture was taken, and the UV/Vis spectra were recorded. The control assays contained no enzyme and were performed under the above conditions.

Results and discussion

The cysteamine-capped AuNPs were synthesized by sodium borohydride reduction of hydrogen tetrachloroaurate(III) in the presence of cysteamine [17]. Because of the $–NH_3^+$ group of cysteamine, the cysteamine-capped AuNPs are positively charged at low pH. The positive-charged AuNPs ((+)AuNPs) solution is stabilized against aggregation due to the positive capping agent's electrostatic repulsion between AuNPs [11]. The AuNPs were characterized by UV–Vis spectroscopy and TEM. The absorption spectrum of the AuNP solution showed a peak at 520 nm and appeared red (Fig. 1). The TEM images (Fig. 2) showed that the AuNPs were spherical in shape and nearly monodispersed with an average diameter of 34 nm.

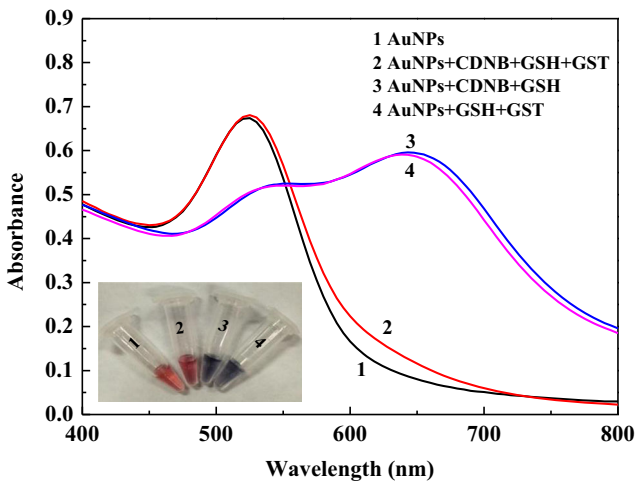

Fig. 1 Absorption spectra of the AuNPs (*1*), AuNPs–CDNB–GSH–GST (*2*), AuNPs–CDNB–GSH (*3*), and AuNPs–GSH–GST (*4*). The *inset* shows the corresponding photographs. Experimental conditions: *GST*, 0.3 U/mL; *CDNB*, 3.0 mM; *GSH*, 2.5 mM; *(+)AuNPs*, 180 μL; *BR buffer*, pH 3.6, 200 μL

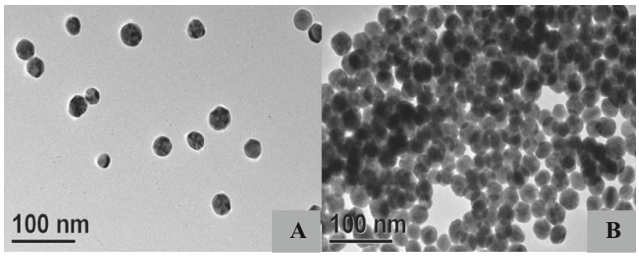

Fig. 2 TEM images of AuNPs–CDNB–GSH in the presence (**a**) and the absence (**b**) of GST. Experimental conditions: *GST*, 0.3 U/mL; *CDNB*, 3.0 mM; *GSH*, 2.5 mM; *(+)AuNPs*, 180 μL; *BR buffer*, pH 3.6, 200 μL

In order to verify the feasibility of the AuNP-based colorimetric assay for GST activity in the Scheme 1, the UV–Vis absorption spectra of the AuNP solution were measured in the presence and the absence of GST (Fig. 1). Curve 1 in Fig. 1 displays the absorption peak at 520 nm, clearly indicating that AuNPs are dispersed under the reaction media. When GSH and CDNB were added into the AuNPs, a noticeable decrease at 520 nm and a significant increase at 670 nm in AuNP absorption spectra were observed (curve 3). This typical change of A_{670}/A_{520} has been assigned to AuNP aggregation [11, 19]. In the absence of GST, GSH–CDNB could induce aggregation of AuNPs, and a significant color change from red to blue occurred. However, in the presence of GST, the aggregation of the AuNPs would not take place and the red-to-blue color change would not be observed. The changes in solution color and absorption spectra of the AuNPs thus allow one to probe GST activity.

To explore the mechanism of AuNP aggregation in this system, GSH, GST, and CDNB were added into the AuNP solution, respectively. The absorbance spectra and the solution color showed that GSH could induce the AuNP aggregation, and GST or CDNS itself did not cause AuNP aggregation. Furthermore, in order to know the microstructure of the AuNPs–CDNB–GSH without and with GST, the TEM images (Fig. 2) were obtained. Note that in the presence of GST, the AuNPs were monodispersed, while in the absence of GST, the AuNPs aggregated together. The TEM results gave the direct evidence for GSH-induced aggregation. The TEM results were consistent with the changes in solution color and absorption spectra. The thiol group exhibits intriguing reactivity with AuNPs [20]. GSH binds onto the gold surface through thiol group. Two –COOH groups of GSH have a pK_a of 2.05 and 3.40, respectively [21]. Both the carboxylic acid groups of GSH can form intermolecular hydrogen bonding at pH <4.5 [22]. The media pH in this system was 3.6. Thus, as depicted in Scheme 1, hydrogen bonding interaction between neighboring –COOH groups overcome the interparticle repulsive force (electrostatic and/or steric repulsion) and draw AuNPs to aggregate. In the presence of GST, GSH would conjugate CDNB through thiol group. There is no thiol group (–SH) in the conjugate product (CDNB-SG) molecule, and the conjugate product cannot bind onto the surface of AuNPs

through Au–S bond. Thus, the conjugate product cannot induce the aggregation of AuNPs.

Furthermore, we prepared the citrate-capped AuNPs (ca.13 and 20 nm) for the comparison study, and the zeta potential of the citrate-capped AuNPs was negative at pH 3.6. Experimental results showed that the GSH could not lead to the aggregation of the negatively charged AuNPs. At pH 3.6, the main molecular form of GSH is the anion (pK_a of 2.05 and 3.40). The anionic GSH does not easily interact with anionic citrate-capped AuNPs because of electrostatic repulsion, and thus, GSH cannot induce the aggregation of citrate-capped AuNPs. However, the cysteamine-capped AuNPs are positively charged at pH 3.6, and the electrostatic attraction between GSH and cysteamine-capped AuNPs would help to bind GSH on the AuNPs.

To improve the performance of GST activity assay, we optimized the experimental conditions. The effect of the amount of AuNPs was examined in the range 90–240 μL. The experimental results (Fig. 3a) showed that the highest sensitivity was obtained when using 200 μL AuNPs (10.5 nM). When 200 μL of pH 3.6 BR buffer solution (0.04 M) was used as the reaction medium for GSH and AuNPs, the highest absorption ratio (A_{670}/A_{520}) was obtained (Fig. 3b, c). The interaction between AuNPs and GSH was rather rapid, and a binding time of 15 min was enough at room temperature (Fig. 3d). In this work, 25 μL GSH (2.5 mM) and 45 μL CDNB (3.0 mM) were used as the substrate of the enzyme reaction, and pH 6.5 Tris–HCl (20 mM) buffer was used as the enzyme reaction media.

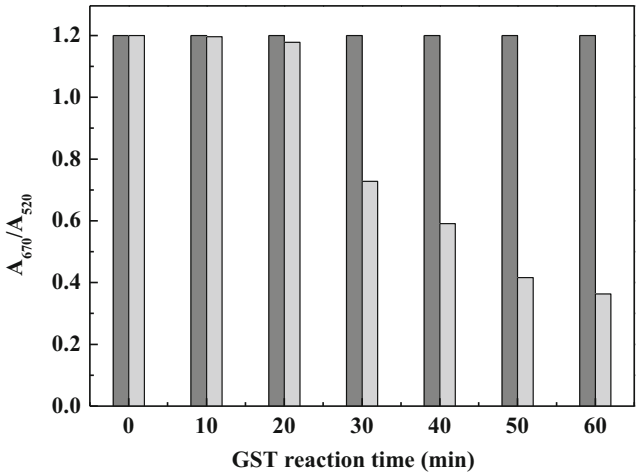

Fig. 4 Time-dependent A_{670}/A_{520} absorbance ratio as a function of enzyme reaction time: blank (*black*) and 0.4 U/mL GST (*gray*). Experimental conditions: *CDNB*, 3.0 mM; *GSH*, 2.5 mM; *(+)AuNPs*, 180 μL; *BR buffer*, pH 3.6, 200 μL

Under the optimized conditions, AuNPs was used as probe to monitor the enzyme reaction process. A reaction mixture containing GSH and GST in Tris–HCl buffer (pH 6.5) was incubated at 30 °C for 1 h. The enzymatic reaction was stopped every 10 min by inactivation using boiling water. The reaction solution was added to the AuNPs, and then, the absorption spectra were measured. The absorbance of AuNPs at 520 nm gradually increased, and the absorbance at 670 nm gradually decreased with the incubating time from 0 to 60 min. As shown in Fig. 4, the A_{670}/A_{520} ratio of the reaction

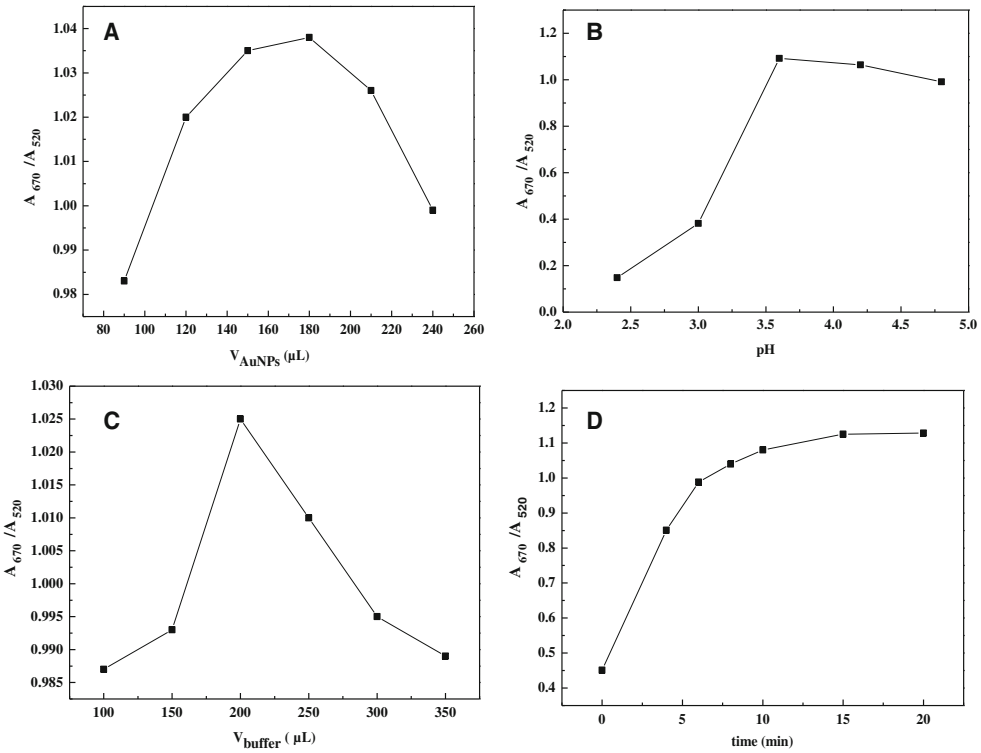

Fig. 3 **a** Effect of AuNP amount on the absorption ratio (A_{670}/A_{520}). Experimental conditions: *CDNB*, 3.0 mM; *GSH*, 2.5 mM; *BR buffer*, pH 4.0, 180 μL; *AuNP binding time*, 30 min. **b** Effect of media pH on the absorption ratio. Experimental conditions: *CDNB*, 3.0 mM; *GSH*, 2.5 mM; *(+)AuNPs*, 180 μL; *BR buffer*, 180 μL; *AuNP binding time*, 30 min. **c** Effect of buffer volume on the absorption ratio. Experimental conditions: *CDNB*, 3.0 mM; *GSH*, 2.5 mM; *(+)AuNPs*, 180 μL; *BR buffer*, pH 3.6; *AuNP binding time*, 30 min. **d** Effect of binding time of GSH on the absorption ratio. Experimental conditions: *CDNB*, 3.0 mM; *GSH*, 2.5 mM; *(+)AuNPs*, 180 μL; *BR buffer*, pH 3.6, 200 μL

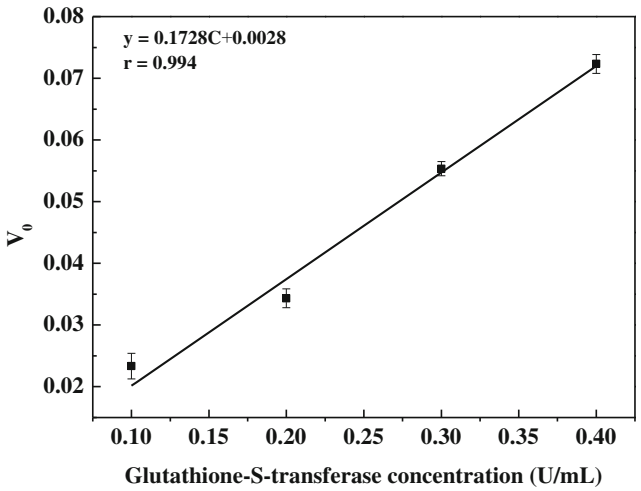

Fig. 5 Linear relation between the initial rate of enzyme reaction and enzyme activity. Experimental conditions: *CDNB*, 3.0 mM; *GSH*, 2.5 mM; (+)*AuNPs*, 180 μL; *BR buffer*, pH 3.6, 200 μL

system decreased with increasing incubation time form 0 to 60 min. The A_{670}/A_{520} ratio of the system did not decrease any more after 60 min, which indicated that the enzyme reaction was nearly completed within 1 h under the condition.

When the concentrations of GSH and CDNB substrate were fixed, the enzyme reaction rate is dependent on the concentration of the used GST. Five concentrations of GSH (0, 0.1, 0.2, 0.3, and 0.4 U/mL) were prepared to react with GSH–CDNB solution. The enzyme reaction was followed by measuring the absorption spectrum of AuNPs with the reaction mixture. The experimental results showed that the absorbance ratio rapidly decreased for the enzyme reaction with a high concentration of GST. The initial enzyme reaction rate (V_0) was estimated from the absorbance ratio of the mixture before and after reaction of 10 min, and a nearly linear plot of V_0 versus the concentration of GST was observed (Fig. 5). These results also validate the possibility of using the AuNPs as a colorimetric probe for GST activity. These results validate the possibility of using the AuNPs as colorimetric probe for GST activity. The enzymatic activity in the range 0.1–0.4 U/mL can be measured using a simple colorimetric reader. The detection limit that is taken to be three times the standard derivation in blank solution is found to be 0.06 U/mL. Furthermore, the semi-quantitative analysis of GST activity could be performed

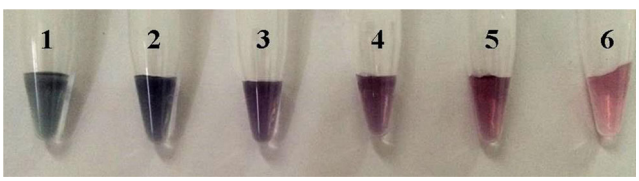

Fig. 6 Photographs of the reaction system in different conditions: (*1*) blank, (*2*) 0.3 U/mL GST, (*3*) 0.4 U/mL GST, (*4*) 0.6 U/mL GST, (*5*) 0.8 U/mL GST, and (*6*) 1.0 U/mL GST. Experimental conditions: 3.0 mM CDNB, 45 μL; 2.5 mM GSH, 25 μL; 20 mM Tris-HCl, 200 μL; (+)AuNPs, 50 μL; pH 3.6 BR buffer solution, 100 μL; AuNP binding time, 15 min

with the naked eye using (+)AuNPs as colorimetric probes. From Fig. 6, it can be seen that the proposed method can allow the naked eye detection of GST in the range of 0.3~0.8 U/mL without resorting to any spectroscopic instrumentation. Compared with the method in the common GST assay method, this method is simple and easily operated.

GST can be used as targets for cancer therapy [2]. Molecules that inhibit GST are therefore considered candidates for new anticancer drug [23]. The GST-catalyzed GSH–CDNB reaction can be followed with AuNPs as colorimetric probes, and one can easily use the A_{670}/A_{520} ratio to evaluate whether the molecule can inhibit GST activity and compare the inhibition efficiency among different inhibitors. Dihydroquercetin, a known GST inhibitor [24], was used in our proof-of-concept experiment. A certain amount of dihydroquercetin was added to the substrate solution before the enzyme reaction. As shown in Fig. 7, with increasing dihydroquercetin concentration, an obvious decrease in the absorption peak at 520 nm and a strong increase in the absorption peak at 670 nm were observed. Dihydroquercetin (0.26 μM) could inhibit half of GST activity. The control experiments indicated that dihydroquercetin itself could not result in the spectra change of the AuNPs. In addition, the color of the AuNP solution gradually changed from its initial red color to blue (Fig. 7, inset). The color change of the AuNP solution can reveal experimental results more conveniently, and the color-based assay can be more easily operated. Hypochlorous acid is also the potent inhibitor of GST [25]. We studied the inhibition efficiency of hypochlorous acid with different concentrations, and 10 μM hypochlorous acid could inhibit half of GST activity. At the same time, ascorbic acid was used as a model negative compound. The results showed that ascorbic acid could not change the absorption ratio of $A_{670}/$

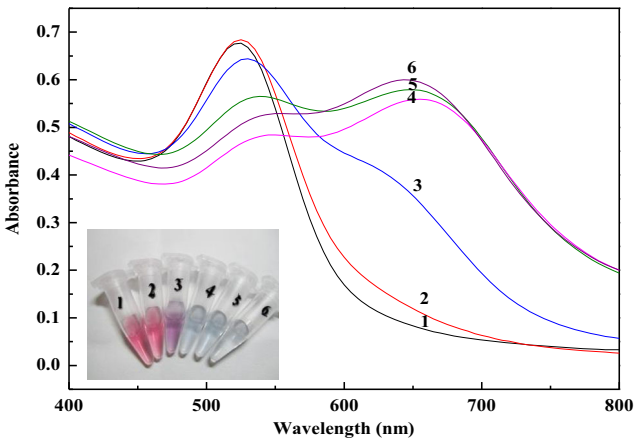

Fig. 7 Absorbance spectra of different systems: (*1*) AuNPs, (*2*) AuNPs + CDNB + GSH + GST, (*3*) AuNPs + CDNB + GSH + GST + dihydroquercetin (0.1 μM), (*4*) AuNPs + CDNB + GSH + GST + dihydroquercetin (1.0 μM), (*5*) AuNPs + CDNB + GSH + GST + dihydroquercetin (10.0 μM), and (*6*) AuNPs + CDNB + GSH. The *inset* shows the corresponding photographs. Experimental conditions: *GST*, 0.3 U/mL; *CDNB*, 3.0 mM; *GSH*, 2.5 mM; *(+)AuNPs*, 180 μL; *BR buffer*, pH 3.6, 200 μL

A_{520}. These results of the inhibition assay also confirm the results for the GST activity.

Conclusions

In conclusion, a new colorimetric assay was developed for detecting GST activity. The assay is based on GSH-induced aggregation of cysteamine-capped AuNPs. The most important characteristic of the assay is direct visualization of the GST activity by the "naked eye", which makes it more convenient than other methods that rely on advanced instrumentation. The method is homogeneous, making it easy to automate by standard robotic manipulation of microwell plates. So, this simple and easily operated method can be adapted to high-throughput screening of GST inhibitors (potential drug candidates) from large combinatorial libraries.

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