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Label-free colorimetric detection of biological thiols based on target-triggered inhibition of photoinduced formation of AuNPs

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Abstract

A label-free colorimetric method for the detection of biological thiols (biothiols) was developed. This method is based on prevention of the photoinduced reduction of auric ions (Au(III)) in the presence of amino acids (acting as a reducing agent) by biothiols; the photoinduced reduction is inhibited due to the strong interaction of the biothiols with Au(III). In this method, the sample was first incubated in an assay solution containing Au(III) and threonine; the sample solution was then exposed to 254 nm UV light. For samples without biothiols, this process led to the photoreduction of Au(III) followed by growth of gold nanoparticles accompanied by the visually detectable development of a red coloration typified by an absorption peak at ca 530 nm. Conversely, in the presence of biothiols, reduction of Au(III) to Au(0) was prevented by entrapment of Au(III) within the biothiols via the thiol group. The solution thus remained colorless even after UV irradiation, which was used as an indicator of the presence of biothiols. Using this strategy, biothiols were very conveniently analyzed by monitoring color changes of the samples with the naked eye or a UV–vis spectrometer. The strategy based on this interesting phenomenon exhibited high selectivity toward biothiols over common amino acids and was successfully employed for reliable quantification of biothiols present in human plasma, demonstrating its great potential for clinical applications.

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Keywords: colorimetric biosensor, biological thiols, photoreduction, gold nanoparticles, inhibition action

(Some figures may appear in colour only in the online journal)

1. Introduction

Amino acids containing thiol groups are termed biological thiols (biothiols). Cysteine (Cys), homocysteine (Hcy), and glutathione (GSH) are examples of biothiols; these species play significant roles in various biological processes [1]. For example, Cys is critically required for the growth of cells and regulates several biological pathways such as protein folding and detoxification [2]. Cys deficiency may present itself as slowed growth of children, depigmentation of hair, liver damage, muscle and fat loss, and skin lesions and weakness [3]. Hcy is a metabolic intermediate in methionine

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metabolism. An elevated level of Hcy in plasma is a risk factor for Alzheimer's disease [4], cardiovascular disease [5], neural tube defects [6], and osteoporosis [7]. GSH is the most abundant intracellular non-protein thiol and participates in a number of cellular antitoxic and defensive functions [8, 9]. An imbalance of GSH is observed in a wide range of pathologies including cancer, neurodegenerative disorders, cystic fibrosis, and human immunodeficiency virus (HIV)-related diseases [10, 11].

The criticality of biothiols for biological functions and their utility as biomarkers, as described above, has fueled the development of analytical methods for detection and quantification of these species in many clinical and research fields. Over the past several decades, numerous strategies have been developed for detection of biothiols. These strategies are based on various techniques such as high-performance liquid chromatography [12], fluorescence spectroscopy [13–17], capillary electrophoresis [18], and mass spectrometry [19, 20]. However, these methods require expensive instrumentation and high levels of technical expertise, which makes them unsuitable for point-of-care testing applications. Thus, there is a strong drive towards development of simple, convenient, and cost-effective protocols that are suitable for facility-limited environments.

Colorimetric methods fulfill most of the aforementioned requirements as they provide very convenient visual responses that do not require any detection instrumentation [21–23]. To date, several colorimetric methods for detection of biothiols have been developed. These are based primarily on chemical dyes (xanthene dyes [24], chromene derivatives [25], polythiophenes [26], etc,) and silver/gold nanoparticles (Ag/AuNPs) [8, 27–29]. Although substantial advancements in the determination of biothiols have emerged from these recent explorations, most of these methods require specialized and costly reagents or time-consuming and cumbersome manipulations for the synthesis of nanoparticles, surface functionalization, and for precise control of the detection conditions, such as the temperature and salt concentration. In this regard, the development of more facile and efficient detection methods is still required.

Herein, we present a new method for detection of biothiols that exploits the photoinduced reduction of metal ions with the aim of overcoming the above-mentioned limitations of the conventional methods. Metal ion reduction induced by photoirradiation in the presence of biomolecules has been intensively studied over the past decade [30–34] because this technique could synthesize metal nanoparticles and nanowires in a simple and eco-friendly manner. However, this technique has been utilized in only a few biosensing application studies [35, 36]. Herein, for the first time, we utilize the photoinduced reduction of metal ions to develop a novel strategy for detection of biothiols in a label-free colorimetric manner.

2. Material and methods

2.1. Materials

Chloroauric acid ($HAuCl_4$), cysteine (Cys), homocysteine (Hcy), glutathione (GSH, reduced; GSSG, oxidized), the other 19 amino acids, human plasma (P9523), and triphenylphosphine (PPh_3) used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA). All reagents were of analytical grade and were used without further purification.

2.2. Instrumentation

UV–vis absorption spectra were recorded after photoinduced reduction of Au(III) by using an Infinite® 200 PRO microplate reader (Tecan Group Ltd., Switzerland). Field emission transmission electron microscopy (TEM) (Tecnai, FEI, Netherlands), performed at an acceleration voltage of 200 kV, was employed for detailed characterization of the structure of the synthesized AuNPs. The samples for TEM analysis were prepared by depositing the solutions onto a carbon-coated copper TEM grid followed by drying at room temperature.

2.3. Colorimetric assay based on photoinduced formation of AuNPs

For quantification of the biothiols, 0.5 mM of $HAuCl_4$ and 5 mM of threonine (Thr) were added to the sample solutions and thoroughly mixed. Photoirradiation was then conducted by placing the solutions under UV light ($\lambda = 254$ nm, power density $= 3000\ \mu W\ cm^{-2}$, Stratalinker® UV Crosslinker 1800 (Stratagene, USA)) for 10 min.

2.4. Determination of thiols in human plasma samples

For determination of the total biothiols in human plasma, the disulfide bonds were reduced in order to release the protein-bound thiols by addition of PPh_3 [37]. Briefly, a 500 μl aliquot of plasma was vigorously mixed with 40 μl of 0.2 M HCl and 20 μl of 0.4 M PPh_3 (in water–acetonitrile 20:80 v/v and 2 M HCl). After incubation for 15 min at room temperature, the hydrolyzed plasma was mixed with 500 μl of acetonitrile to precipitate the proteins, followed by centrifugation at 4000 rpm for 20 min. The supernatant, which contained the biothiols in plasma, was collected and appropriately diluted with acetonitrile/water (50:50 v/v) solution for further analysis [38, 39]. The unknown amount of thiols was determined via the standard addition method using Cys as a standard.

3. Results and discussion

3.1. The overall detection procedure

First, the effect of amino acids on the photoinduced synthesis of AuNPs was investigated by evaluating the colorimetric

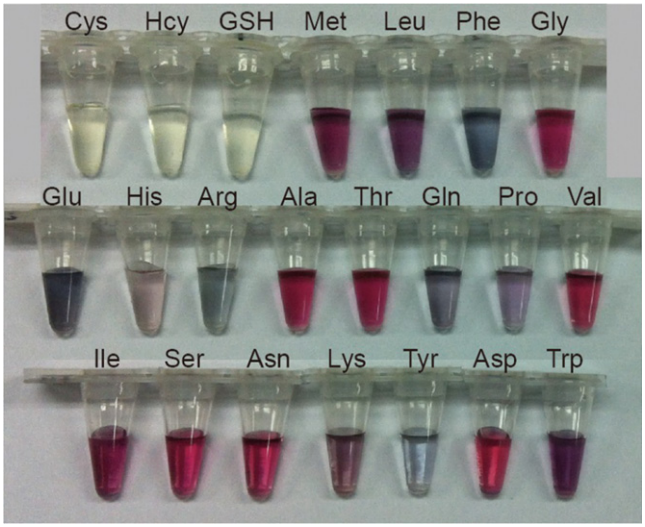
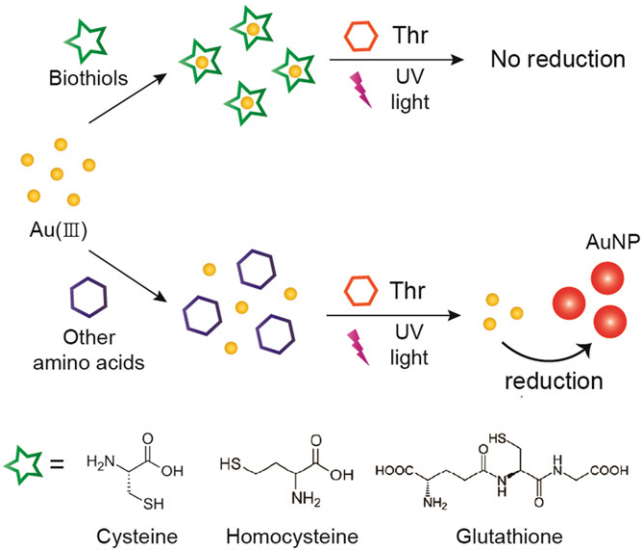


Figure 1. Photoinduced AuNP synthesis (or prevention thereof) in the presence of amino acids. Colorimetric responses were obtained through photoreduction of 0.5 mM $HAuCl_4$ solution in the presence of 5 mM biothiols (Cys, Hcy, and GSH) or other 19 amino acids.



Scheme 1. Schematic illustration of colorimetric detection of biological thiols based on the target-triggered inhibition of photoinduced formation of gold nanoparticles (AuNPs).

responses produced upon UV irradiation of $HAuCl_4$ solutions in the presence of each type of amino acid presented in figure 1. After UV irradiation, a color change was observed for all solutions containing aqueous chloroauric acid ($HAuCl_4$) and 5 mM of the respective amino acids. As envisioned, however, the solutions containing biothiols did not produce a colorimetric response even after UV irradiation, indicating that photoinduced AuNP synthesis had not occurred. Interestingly, the color signals resulting from the photoreduction of Au(III) differed noticeably depending on the type of amino acid, with a range of colors from dark navy to pale red, which was also demonstrated by the ratio of UV absorption intensity at 530 nm before and after the

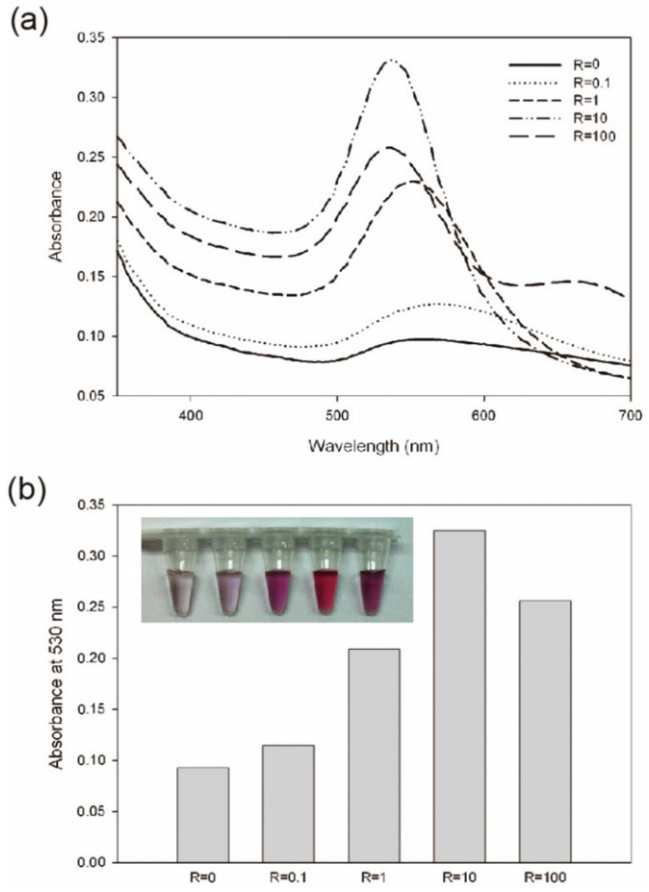


Figure 2. Effect of Thr concentration on the photoreduction of Au (III). (a) UV–vis absorption spectra of the solutions containing $HAuCl_4$ and Thr after photoirradiation. *R* is the molar concentration of Thr relative to 0.5 mM $HAuCl_4$. (b) Absorption intensities of the solutions at 530 nm. Inset shows images of the corresponding samples.

photoirradiation (A/A_0) (figure S1). This result implies that the degree of reduction of Au(III) varies based on the reducing power of the amino acids having different functional groups [40]. Among the 22 amino acids, threonine (Thr) yielded the most intense color signal and the highest A/A_0 ratio after the photoreduction process. Therefore, Thr was selected as the Au(III) reductant in the further strategy for detection of biothiols.

The strategy for detection of biothiols is illustrated in scheme 1. Sample solutions were first incubated with $HAuCl_4$ solution containing Thr as a reducing agent; the solutions were then irradiated with UV light at 254 nm. In the absence of biothiols, UV irradiation of the colorless suspension led to nucleation of Au(III) to Au(0) followed by the growth of AuNPs with consequent generation of a red suspension, for which a surface plasmon resonance (SPR) peak of the AuNPs was detected at ca. 530 nm. On the other hand, in the presence of biothiols, Au(III) could neither be reduced to Au(0) nor form AuNPs upon UV irradiation, leaving the solution in its initially colorless state. The detailed mechanism has not been fully elucidated, but it is assumed that formation of the AuNPs might be prevented by the formation of Au(III)-

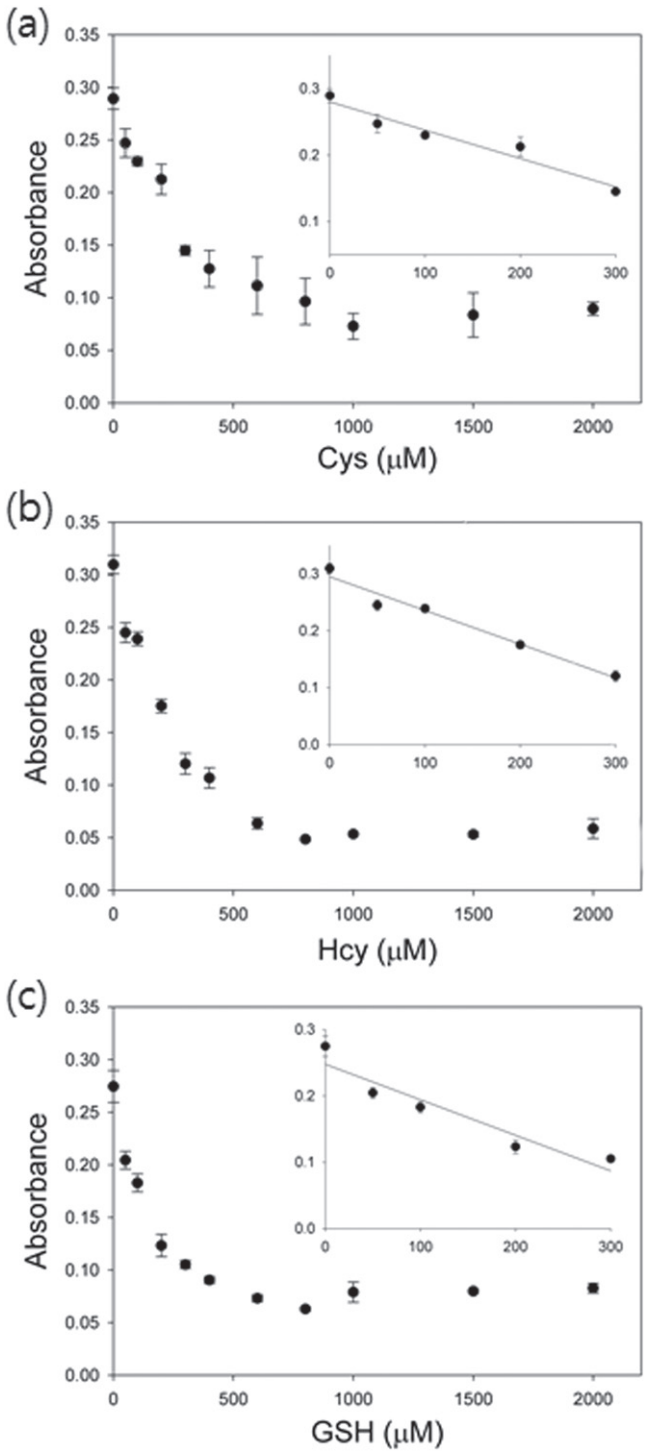


Figure 3. Colorimetric detection of biothiols ((a) Cys, (b) Hcy, and (c) GSH) based on the target-triggered prevention of AuNP formation. The absorbance intensities at 530 nm were plotted as a function of the concentration of the biothiols. Data points are averages of three independent experiments.

biothiol complexes [40, 41]. Liu *et al* also recently reported that Cys prevented the photoreduction of Ag^+ thus precluding formation of AgNPs especially when the concentration of Cys is higher than that of Ag^+ [40].

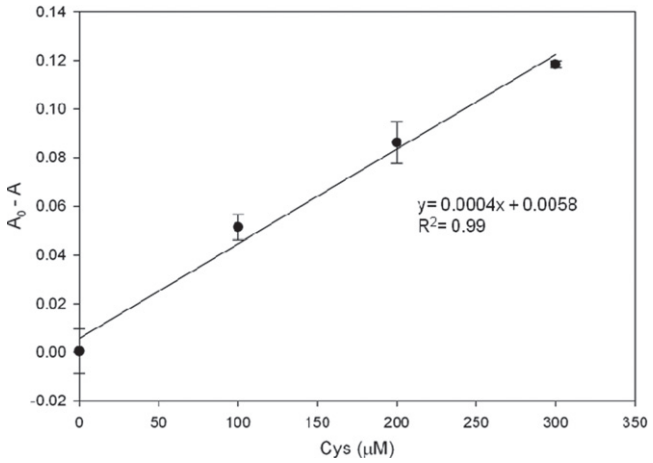


Figure 4. Determination of total biothiols in human plasma via standard addition method. A_0 is the absorbance of the AuNPs generated by photoinduced formation in acetonitrile/water (50:50 v/v) solution without biothiols while A is the absorbance of the AuNPs by photoinduced formation in plasma samples spiked with Cys at different concentrations.

3.2. Optimization of the Thr to Au(III) ratio and UV irradiation time

The effect of the concentration of a reducing agent (Thr) on the photoreduction of Au(III) was next investigated (figure 2). Photoreduction of the solutions containing Thr resulted in an obvious colorimetric signal indicated by the development of a red color. Concomitantly, the intensity of the absorption at 530 nm increased as the concentration ratio (R) of Thr to Au (III) increased from $R = 0$ to $R = 10$ (R is the molar concentration of Thr relative to 0.5 mM Au(III)), indicating that a higher concentration of Thr within the stated range was favorable for photoreduction of Au(III). However, in the case of $R = 100$ the intensity of the absorption at 530 nm decreased relative to that of $R = 10$ and a broad SPR band between 600 and 700 nm appeared, suggesting that some of the synthesized AuNPs underwent aggregation (figure 2(a)). Based on these results, 5 mM Thr ($R = 10$) was employed for photoreduction of Au(III) to generate the AuNPs in this study. Formation of the AuNPs via photoreduction of the employed solution ($R = 10$) was further confirmed by TEM analysis (figure S2).

The effect of the UV irradiation time on the colorimetric response induced via photoreduction was examined by measuring the absorption intensities at 530 nm after photoirradiation of the solutions containing Au(III) and Thr. Based on the results presented in figure S3, the absorbance at 530 nm increased as the UV irradiation time increased up to 10 min; no further increase in the absorbance was observed after 10 min. Therefore, 10 min was selected as the optimal UV irradiation time. Even with prolonged irradiation, no photoreduction of the solutions was observed in the presence of biothiols.

Table 1. Determination of total thiols in human plasma (diluted 40-fold). The concentration of total biothiols in undiluted plasma was determined to be ca. 580 μM.

Determined thiol compounds (μM)[a]	Added Cys (μM)	Measured (μM)	Recovery (%)	CV[b] ($n = 3$, %)
14.5	50	63.9	99.1	3.4
	100	119.5	104.4	3.7
	150	176.1	107.1	3.3

[a] 40-fold dilution.
[b] Coefficient of variation.

3.3. Demonstration of its clinical applicability

To verify the detection capability of the proposed strategy based on the target-triggered inhibition of AuNP formation, the absorbance at 530 nm originating from the generation of photoreduced AuNPs was measured as a function of the concentration of biothiols (figure 3). As envisioned, the absorption intensity decreased as the biothiol concentration increased. When the concentration of biothiols was higher than that of Au(III) (= 0.5 mM), formation of the AuNPs was almost completely inhibited. The extent of the reduction in the absorbance was linearly dependent on the biothiol concentration within the range of 0–300 μM; the detection limits for Cys, Hcy, and GSH were 76 μM, 44 μM, and 65 μM, respectively, based on a signal-to-noise ratio (S/N) of 3. The detection levels suggest that the proposed method may prospectively be applicable for the diagnosis of several diseases associated with biothiols in clinical applications [42, 43]. Considering that Hcy level higher than 100 μM in blood is recognized to be an indicator of severe hyperhomocysteinemia, our assay system could be directly applicable to diagnose this disease. We also applied our system to determine several predetermined ratios of GSH/GSSG, which is an indicator to evaluate cellular health and potential therapeutics efficacy [44], including GSH 100%, GSH 50% and GSSG 50%, and GSSG 100%. As a result, the absorption intensity increased as the GSSG ratio increased, demonstrating the potential of our system to be used for the measurements of GSH/GSSG ratio (figure S4).

To further demonstrate the clinical applicability of this system, the proposed method was employed for assay of the total thiols in human plasma. The determination of total biothiols in human plasma is not directly associated with specific human disease but the higher levels of total biothiols could indicate the abnormal health state [2–11]. Therefore, it is of great interest to develop an efficient method for detection and quantification of total biothiols for early stage monitoring of several health problems. In this experiment, human plasma was appropriately diluted with a buffer to fall within the linear range and the amount of spiked thiol was quantitatively determined. The total biothiol content was determined to be 580 μM by the standard addition method using Cys as a standard [45]. This result is within the acceptable range compared with those reported in previous studies [46, 47]. As presented in figure 4 and table 1, this method yielded good recovery rates of 99.1%–107.1%, confirming the reliability of the present method for the determination of biothiols in clinical applications. The precision of the procedure was also excellent as confirmed by the CVs which were less than 3.7%.

The linear range and the detection limit of the previous methods to detect biothiols were summarized and compared with those of this study (table S1). Although the detection limit for biothiols of the current method was in a range of several tens of μM, the proposed method successfully eliminated the use of specialized or costly reagents and time-consuming and cumbersome manipulations for nanoparticle synthesis, surface functionalization, and precise control of reaction conditions such as temperature and salt concentration, given that the proposed system only requires an aqueous Au(III) solution and UV light for operation.

4. Conclusions

In summary, a novel strategy for colorimetric detection of biothiols was successfully developed. The assay relies on the inhibition of photoinduced formation of AuNPs, which is triggered by target biothiols. To the best of our knowledge, this is the first study to exploit the interesting tendency of biothiols to prevent the photoinduced reduction of metal ions for their own (biothiol) detection. This assay can be easily conducted by monitoring the colorimetric transition of the samples from colorless to red with the naked eye or a UV–vis spectrometer. Moreover, the proposed method has several advantages over previous colorimetric detection strategies, including the use of readily available and inexpensive materials and an easily executed protocol. The clinical utility of this simple yet highly efficient colorimetric strategy was successfully verified by reliably quantifying the total biothiol content in human plasma. The present study may provide a new avenue for the rapid, convenient, and cost-effective detection of thiol-containing biomolecules in practical applications.

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