

Highly Sensitive and Naked Eye Dual-readout Method for L-Cysteine Detection Based on the NSET of Fluorophore Functionalized Gold Nanoparticles

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A simple, highly sensitive and selective method based on the rhodamine B-covered gold nanoparticle with dual-readout (colorimetric and fluorometric) detection for L-cysteine is proposed. A mechanism is that citrate-stabilized AuNPs were modified with RB by electrostatic interaction, which enables the nanometal surface energy transfer (NSET) from the RB to the AuNPs, quenching the fluorescence. In the presence of L-cysteine, it was used as a competitor in the NSET by the strongly Au-S bonding to release RB from the Au surface and recover the fluorescence, and the red-to-purple color change quickly, which was monitored simply by the naked eye. Under the optimum conditions, the detection limit is as low as 10 nM. The method possessed the advantages of simplicity, rapidity and sensitivity at the same time. The method was also successfully applied to the determination of L-cysteine in human urine samples, and the results were satisfying.

Key Words : L-Cysteine, Nanometal surface energy transfer, Colorimetric, Fluorometric

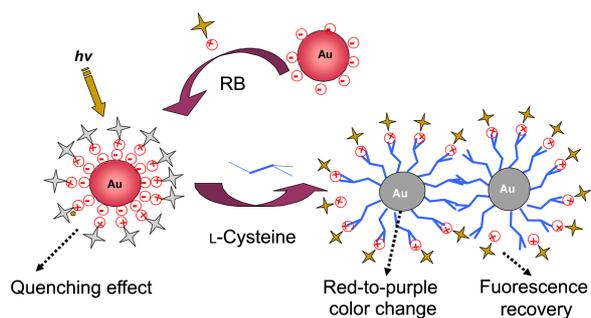
Introduction

L-Cysteine (Cys) is an important biologically active and thiol-containing amino acid which plays a critical role in biological systems because of its wide distribution in living cells.¹⁻³ Cys provides a modality for the intramolecular crosslinking of proteins through disulfide bonds to support their secondary structures and functions. It is also a precursor of coenzyme A and can easily be oxidized to cystine which serves as a model for the thiol group of proteins in a variety of biological media.⁴ Its deficiency is associated with a number of clinical situations, such as hair depigmentation, edema, skin damages, lethargy and loss of muscle and fat.^{1,5} Thus, pursuing an a sensitive, reliable, and convenient approach for trace analyzing L-cysteine is of great significance in biological, medical, and clinical studies and attract considerable interest in recent years. Various methods have been reported for the determination of L-cysteine, such as flow injection,⁶ electrochemistry,⁷⁻¹³ high-performance liquid chromatography,^{14,15} and capillary zone electrophoresis.¹⁶ Although some of these methods have more selective and sensitive, they either involve expensive biomolecule reagents, complicated pretreatment of samples, or/and require skilled operators. Thus the conventional methods could not ideally meet the requirements of L-cysteine detection. Nanoparticles, especially gold nanoparticles (AuNPs) have been widely used in the biosensor field because of large surface-to-volume ratio, high surface reaction activity, the higher extinction coefficient and strong adsorption ability.¹⁷⁻²⁰ Moreover, AuNPs provide strong affinity toward thiols and cause the aggregation of itself. Due to the color changes are highly sensitive to the size, shape, capping agents, and the aggregation states, AuNPs-based colorimetric assays have recently become useful for many types of analytes with the naked

eyes, without the aid of any advanced instruments.²¹⁻²⁵ Unfortunately, most of AuNPs-based colorimetric assays for L-cysteine still lack sufficient sensitivity, which significantly limited its application in real-life sample. In order to overcome these limitations, the improvement of their sensitivity is necessary.

Recently, fluorescence analysis was more favorable for rapid, technically simple, and efficient detection without any expensive apparatus.^{26,27} Fluorescence resonance energy transfer (FRET) is the energy interaction in which an energy donor transfers energy to an acceptor through nonradioactive dipole-dipole coupling. Normally organic dyes are used as the energy donor and the energy acceptor. Recently, the organic energy acceptor was replaced with gold nanoparticles to render the nanometal surface energy transfer (NSET) mechanism.^{21,28} Compared to FRET, NSET has a higher energy transfer rate from the organic donor to the AuNPs acceptor and a longer quenching distance.

In the present work, we designed a highly sensitive, simple and naked eye method using rhodamine B-functionalized gold nanoparticles (RB-AuNPs) coupled with NSET technique with dual readouts (colorimetric and fluorometric) for detection the level of L-cysteine. In this study, rhodamine B was used as an ideal model fluorophore because it has water-soluble, photostability, and positively-charged RB can be readily adsorbs onto surfaces of citrate-stabilized AuNPs *via* electrostatic interactions to result in highly efficient energy transfer from the fluorophore to AuNPs. When in the presence of L-cysteine, L-cysteine is used as a competitor in the NSET between fluorophores and AuNPs because of the Au-S bonding, which can bind more strongly onto surfaces of AuNPs than RB, thus removing RB from the Au surface to recover the fluorescence of RB and cause the red-to-purple color change quickly in that aggregation of AuNPs (Scheme



Scheme 1. Design of the dual-readout (colorimetric and fluorometric) assay for L-cysteine.

1). The dual-readout assay combining colorimetric characteristics of AuNPs with fluorometric sensitive measurement of NSET can be used to visually detect trace amounts of thiols. Owing to its simple, cost-effective, much better sensitivity and naked eye detection which is free from any preliminary treatment and specific instruments, this present dual-readout assay can thereby be a reliable option to determine the concentration of L-cysteine in biological samples.

Experimental

Apparatus. Absorption spectra were recorded by a UV-1800 UV-vis spectrophotometer (Shimadzu, Japan) using a quartz cell with the path length of 1.0 cm. Fluorescence measurements were performed with a LS-55 fluorescence spectrophotometer (PerkinElmer, USA) with a 1.0 cm quartz cell with 10 nm band width for both the excitation and emission monochromators. The pH was measured using a PHS-25 pH meter (Shanghai, China). All optical measurements were carried out at room temperature under ambient conditions.

Chemicals and Reagents. Trisodium citrate dehydrate (98%), Chloroauric acid ($\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$), RB (98%) and L-cysteine (98%) were purchased from Sigma-Aldrich Chemical Co. All chemicals used were of analytical grade. Mill-water used was purified using a Millipore filtration system (NANOPure, USA) with a resistivity of 18.0 M Ω cm. All chemicals and solvents were obtained from the commercial sources and used directly without further purification. All glassware used in this preparation was thoroughly cleaned in aqua regia (three parts HCl, one part HNO_3), rinsed in doubly distilled water, and oven-dried prior to use.

Preparation of Citrate-Capped AuNPs. Citrate-stabilized AuNPs were prepared following Frens' method with a minor modification.²⁸ Briefly, 2 mL 1% aqueous solution of HAuCl_4 , in 48 mL of water was heated to boil with vigorous stirring, and 5 mL 1% trisodium citrate was added quickly. The solution turned deep blue within 20 s and the final color change to deep-red. Boiling was pursued for an additional 10 min. The solution was cooled to room temperature with a continuous stir. The colloidal solution was stored in dark bottles at 4 °C. The resulting solution of colloidal particles was characterized by an absorption maximum at 520 nm.

The sizes of the nanoparticles were about 13 nm by TEM analysis (Figure 1(c)).

Preparation of RB-functionalized AuNPs (RB-AuNPs). The RB-AuNPs were prepared according to the literature.^{28,29} Briefly, a stock solution of RB (2.0 mM, 5 μL), H_2O (1.5 mL), carbonate buffer solution (pH 9.6, 0.5 mL) was added with stirring into solutions of the as-prepared citrate-AuNPs (10 nM, 3.0 mL). The resulting solutions were stirred mildly in the dark at room temperature for 2 h. Subsequently, the RB-functionalized Au NPs were purified by centrifugation for 10 min at 6000 rpm to remove the excess RB. The fluorescence spectra of the RB-AuNPs solution were measured with excitation at 550 nm. The very low fluorescence of the RB-AuNPs solution indicated that all the RB had adsorbed effectively onto the surface of AuNPs.

Detection Procedure of L-Cysteine. A 0.1 M stock solution of L-cysteine was prepared in pH = 7.2 Britton-Robinson (B-R) buffer solution, from which various cysteine concentrations were prepared by serial dilution. Each of the working solutions (200 μL) was added to a solution of RB-AuNPs (2 mL) and B-R buffer solution (500 μL). The mixture was diluted to 5.0 mL with water and kept in the dark for 30 min. The pictures were taken, and at the same time, UV-vis absorption and fluorescence were measured respectively. All the measurements were repeated five times for each concentration.

Results and Discussion

Mechanism of the Dual-readout Assay for L-Cysteine.

To verify the mechanism which we have spoken above, the following experiments were conducted. We first synthesized RB-AuNPs by allowing an optimized concentration. Herein, positively charged amino groups of RB molecules can be readily adsorbed on the surface of negatively charged citrate-AuNPs *via* electrostatic interaction. The binding constant was calculated to be 5.7×10^9 using the equation: $\log[(F_0 - F)/F] = \log K + n \log [Q]$. (F and F_0 are the fluorescence intensities with and without the presence of the external quencher respectively, $[Q]$ is the concentration of the quencher, K is the binding constant, and n is the number of binding sites). We used fluorescence spectra and UV-vis absorption spectra to explain the mechanism of detection of L-cysteine. As shown in Figure 1(a), RB-AuNPs fluorescence almost near to zero mainly due to the efficient NSET from the RB to AuNPs, although the absorption band of RB-AuNPs was similar to that of the citrate-AuNPs and the color of RB-AuNP solution remained red. Upon the addition of L-cysteine into a RB-AuNP solution, RB is expected to be released due to -SH can bind more strongly onto surfaces of AuNPs than RB to produce specific Au-S bonding, accompanied with the fluorescence of RB was restored.

Simultaneously, the color change of the RB-AuNPs solutions from red to purple because of -SH attached onto different AuNP surfaces are able to cause the aggregation of AuNPs (Figure 1(b), insert). Figure 1(b) is the UV-vis absorption spectra of RB-Au NP nanoprobe before and after addition

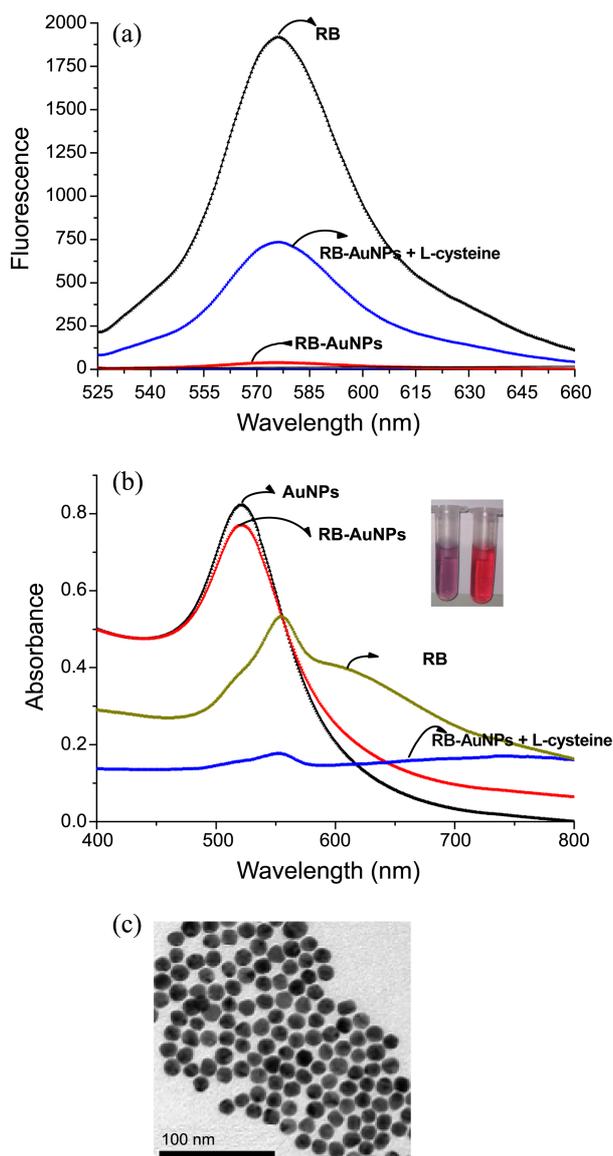


Figure 1. Fluorescence spectra of the solutions (a), their corresponding UV-vis absorption (b) and TEM image of Au NPs (c).

of L-cysteine. It could be seen that the absorption band of RB-AuNPs and the absorbance was nearly invariant, but the absorbance underwent a significant decrease by addition of L-cysteine. To further obtain sensitively detection, the effect of parameters such as concentration of RB, pH and buffer, time and the influence of interferent were investigated by the fluorescence spectra.

Effect of Concentration of RB. Concentration of RB has great effect on the sensitivity of the method. we carried out reactions where all parameters were fixed with the exception of the concentration RB: For each reaction, 5 μ L RB solutions with a concentration ranging from 0.5 mM to 4 mM, 1.5 mL H₂O, 0.5 mL carbonate buffer solution (pH 9.6) was added to 3.0 mL solutions of the as-prepared citrate-AuNPs (10 nM). The fluorescence spectra of the RB-AuNP solutions were measured with excitation at 550 nm. The results were displayed in Figure 2. It could be seen that the position

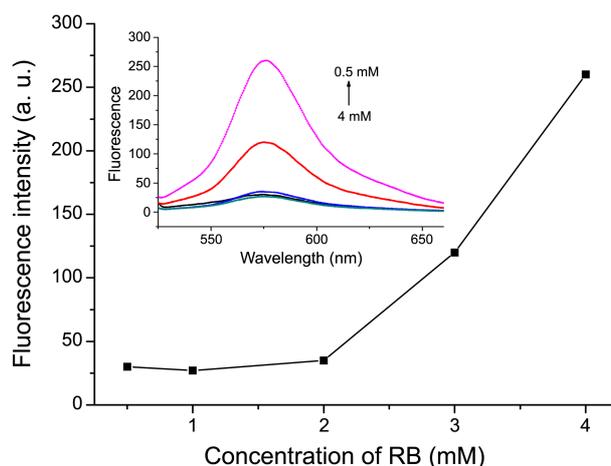


Figure 2. Effect of concentration of RB upon the fluorescence intensity.

of the PL emission peak was nearly invariant and the response increased with the concentration of RB over 2 mM. This indicates that the excess RB not only can't contribute to the NSET between RB and AuNPs, but also increase the background value. On the contrary, when the concentration of RB was below 2 mM, there was no obvious change in fluorescence intensity. Considering the relatively low sensitivity caused by low concentration of RB, the RB concentration of 2 mM was selected for all of the experiments. That is to say, we first synthesized RB-AuNPs by allowing an optimized final concentration of RB (1 μ M) to adsorb on the surfaces of citrate-AuNPs (3 nM).

Effect of Reaction Time. The reaction rate of Au-S bonding is very quickly. The influence of reaction time was investigated by monitoring the fluorescent intensity of RB-AuNPs in the presence of L-cysteine. As shown in Figure 3, at a reaction time within 10 min, the fluorescence was multiple enhanced, then increased slowly with the reaction time between 10 and 25 min and the response started to level off above 30 min. This indicated that the interaction of RB-Au NPs with L-cysteine had reached equilibrium after 30 min, so the reaction time of 30 min was selected for all of the experiments.

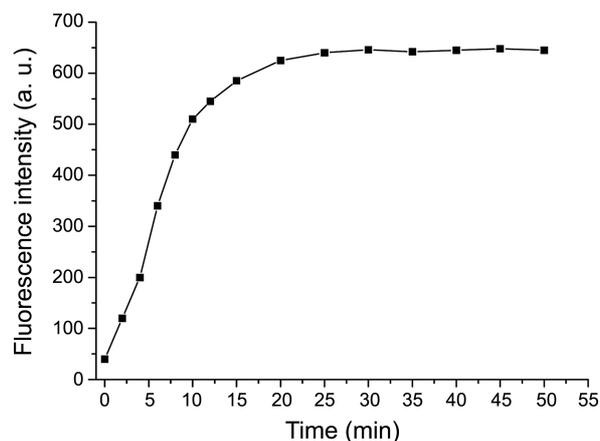


Figure 3. Effect of reaction time upon the fluorescence intensity.

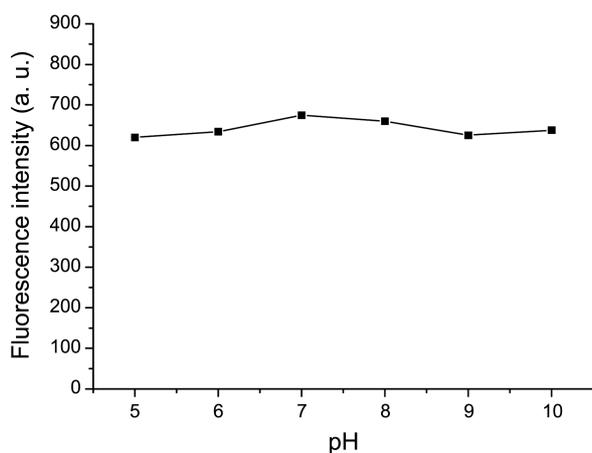


Figure 4. Effect of pH upon the fluorescence intensity.

Effect of pH and Buffer. The influence of pH value on the luminescence intensity was examined in the range of 5–10. It can be seen that the changes of fluorescence intensity are small (Fig. 4). It is explained that (1) the fluorescence emission of RB is relatively stable and the fluorescence intensity has remained broadly consistent in this range of pH. (2) the influence of the specific Au-S covalent interaction upon pH is also small. Furthermore, the effect of buffer solutions, carbonate buffer, phosphate-buffered saline, Tris-buffered saline, Britton-Robinson (B-R) buffer solution, on the luminescence intensity was subsequently optimized. The results indicated that the B-R buffer solution offered the highest sensitivity.

The Influences of Coexisting Substances. In order to examine the selectivity of the method toward L-cysteine, we tested some metal ions as the interferent, including NH_4^+ , Na^+ , K^+ and Cl^- . The results were also shown in Table 1. If the coexisting compounds caused a relative error of less than $\pm 5\%$ in the fluorescence intensity, they were considered to have no interference in the detection of L-cysteine in relatively high concentrations of coexisting ions. Moreover, various amino acids that make up proteins were also studied. Results indicated other 10 amino acids had almost no interference on the detection of L-cysteine, and further confirmed the mechanism that only -SH can be used as a competitor in the NSET to bind onto surfaces of AuNPs and recover the fluorescence of RB.

Table 1. Tests for the interference of coexisting substances^a

Coexisting substance	Coexisting concentration (mM)	Relative error (%) ^a	Coexisting substance	Coexisting concentration (mM)	Relative error (%) ^a
Na^+	≥ 0.1	-1.2	L-His	0.005	4.8
NH_4^+	0.05	3.0	L-Pro	0.01	-2.4
K^+	≥ 0.1	1.5	L-Leu	0.01	-1.6
Cl^-	≥ 0.1	2.2	L-Ala	0.02	2.1
L-Lys	0.02	4.5	L-Arg	0.002	3.3
L-Asp	0.02	3.1	L-Glu	0.01	1.9
L-Ser	0.01	-1.8	L-Try	0.01	-4.0

L-cysteine: 5 μM ; RB-AuNPs: 0.3 mM; pH 7.2. Note: "a" denotes average of three determinations.

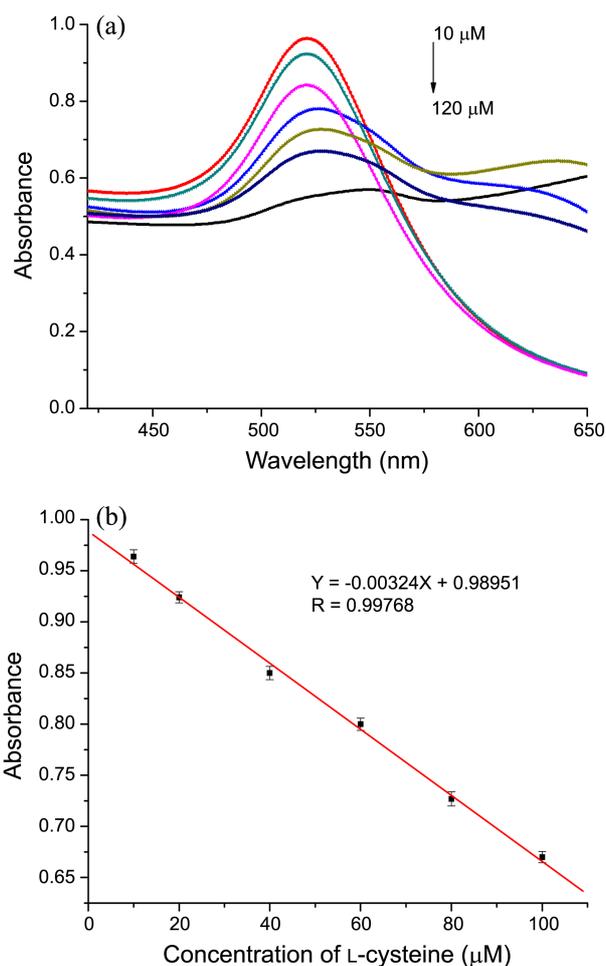


Figure 5. the absorbance of RB-AuNPs at 520 nm (a) the linear calibration data (b) versus the concentration of L-cysteine.

Analytical Performance. Under the optimum experimental conditions, the calibration graph for L-cysteine, detection limit and precision were obtained. Figure 5 shows A_{520} values versus various concentrations of L-cysteine, respectively. The absorbance signal at 520 nm gradually decreases with the L-cysteine concentration from 10 to 120 μM (Fig. 5(a)), a linear relationship of the absorbance of RB-AuNPs at 520 nm and the concentration of L-cysteine over the range of 10–100 μM was established using the equation $Y = -0.00324X + 0.98951$, and the linear correlation coefficient was 0.99768

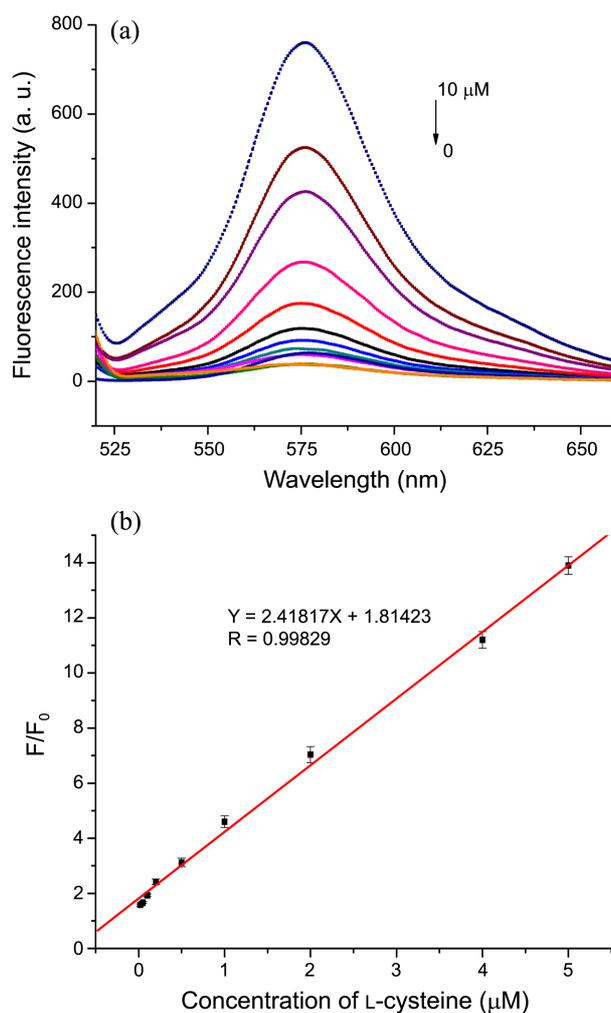


Figure 6. Fluorescence emission spectra of RB-AuNPs (a) and the linear calibration data (b) versus the concentration of L-cysteine.

(Fig. 5(b)).

Moreover, the quantitative detection was monitored by testing the F/F_0 values (the ratio of fluorescence intensity at 575 nm in the presence and absence of L-cysteine) to various concentrations of the L-cysteine. As shown in Figure 6(a), with increasing L-cysteine concentrations from 0 to 10 μM , the fluorescence intensity enhanced progressively, leading to the increase of F/F_0 values. A good linear relationship with the relative coefficient of 0.99829 is obtained in the range of 0.02–5 μM for the concentration of L-cysteine (Figure 6(b)). The detection limit (LOD) is estimated to be 10 nM (according to IUPAC, $\text{LOD} = 3\sigma/b$, where σ is the known standard deviation of ten measurements for the reagent blank's signal and b is the slope of the calibration curve at low analyte concentration).

Sample Determination. To demonstrate the applicability of the proposed method, three human urine samples detection were quantified. The results are shown in Table 2. From Table 2, it can be seen that the values found for the three samples are identical with the true values, and the recovery and relative standard deviation are satisfactory. Therefore,

Table 2. Results of analysis of three human urine samples

Sample	L-cysteine in samples/ (μM)	Concentration found ($\mu\text{M})^a$ (mean \pm SD)	Recovery ^a /%	RSD ^a /%
1	1.50	1.52 \pm 0.07	101.3%	4.6%
2	2.60	2.54 \pm 0.10	97.7%	3.9%
3	4.00	4.20 \pm 0.18	105.0%	4.3%

Note: "a" denotes average of three determinations.

this proposed dual-readout assay for detecting L-cysteine is applicable.

Conclusion

In this paper, A simple, highly sensitive and selective method based on the rhodamine B-covered gold nanoparticle with dual-readout (colorimetric and fluorometric) detection for L-cysteine is proposed. The dual-readout assay combining colorimetric characteristics of AuNPs with fluorometric sensitive measurement of NSET can be used to visually detect trace amounts of thiols. Under the optimum conditions, the detection limit is as low as 10 nM. Furthermore, the concentration of L-cysteine in three synthetic samples was determined. The results of determination were identical with the true values, and the recovery (97.7%–105.0%) and relative standard deviation (RSD, 3.9%–4.6%) are satisfactory. Owing to its simple, cost-effective, much better sensitivity and naked eye detection which is free from any preliminary treatment and specific instruments, this present dual-readout assay can thereby be a reliable option to determine the concentration of L-cysteine in biological samples.

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