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Fluorescence Enhancement of Terminal Amine Assembled on Gold Nanoclusters and Its Application to Ratiometric Lysine Detection

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ABSTRACT:

Ratiometric fluorescent sensors have emerged as an attractive tool for analytical sensing and optical imaging due to its providing a built-in self-calibration for environmental effects. However, cumbersome processes of nanoparticles modified with fluorophores for constructing traditional ratiometric sensors limit their further application. Herein, we reported a facile and label-free strategy for constructing ratiometric sensor based on aggregation-induced-emission (AIE) active amine-terminated small molecule on the surface of gold nanoclusters (AuNCs). Intrinsic fluorescence of terminal primary amine of small molecule lysine resulted from AIE was first observed in the presence of glutathione stabilized gold nanoclusters (GSH-AuNCs). Using lysine as both the fluorophore and the analyte, the synthesized GSH-AuNCs showed a good lysine-responsive ratiometric property. The AIE-active dual-emitting fluorescence property of GSH-AuNCs/lysine complex made it feasible to achieve ratiometrically detect of analyte without conjugated fluorogen. This AIE-active GSH-AuNCs based biosensor possesses high selectivity, rapid response, and excellent photostability. Moreover, the strategy opens a new pathway for the construction of label-free ratiometric fluorescent sensor with various applications.

INTRODUCTION

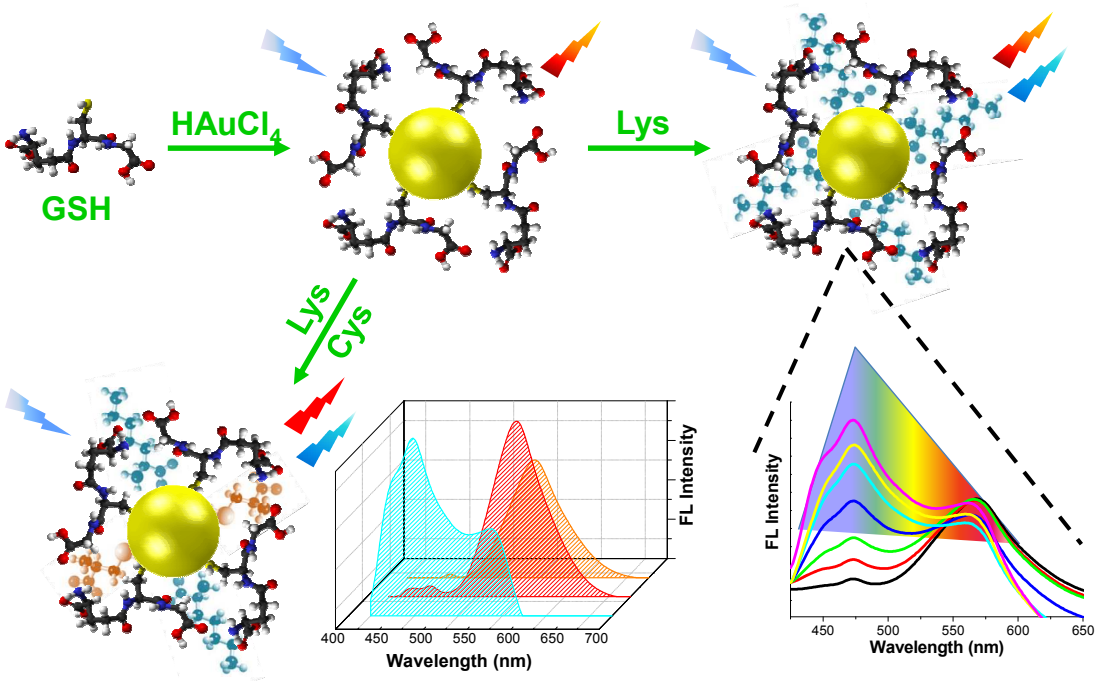
Compared with intensity-based fluorescent probes, ratiometric fluorescent probes are more precise measurement.[1] Ratiometric fluorescent methods utilize the simultaneous measurement of two fluorescence signals at different wavelengths followed by calculation of their intensity ratio and provide a built-in self-calibration for environmental effects.[2] To date, a wide variety of ratiometric sensing probes using rhodamine, naphthalimide, coumarin, quinolone, quantum dots (QDs) have been developed.[3] The present ratiometric fluorescence sensors are always built based on fluorescence resonance energy transfer (FRET) or photoinduced electron transfer (PET) mechanism.[4,5] In order to construct a fluorescence ratiometry system, fluorophores are covalently modified on the surface of the fluorescent nanoparticles or two fluorescent materials are embedded together by chemical reaction to form a nano-assembly, which suffers from cumbersome processes. The ideal ratiometry fluorescent nanoprobe should preferably possess their dual-emission centers without any conjugated fluorescent dye.

Intrinsic fluorescence phenomena of nitrogen-containing dendrimers without attached typical fluorophores cause extensive concern in recent years.[6] However, there are only a few reports on amino-containing small molecules in terms of their weak emission.[7] The fluorescence mechanism and properties of amine-containing compounds are quite different from those of the traditional fluorogen. The current research results revealed that their fluorescence emission attributed to the lone-pair electrons on their nitrogen atom. Especially, the fluorescence from primary amines is seldom observed due to loss of the hydrogen atom via predissociation.[8]

Gold nanoclusters (AuNCs), as one of new fluorescent nanomaterials, are attracting great attentions among multiple areas.[9-14] The most striking properties of AuNCs over organic fluorescent dyes are their size-tunable emission, broad excitation bands, biocompatibility, facile

synthesis, as well as resistance against photobleaching.[15-17] Compared with organic fluorescent dyes, exploiting of AuNCs in ratiometric fluorescent sensing is even more intriguing.[18,19]

Herein, we present the first example of glutathione (GSH) stabilized gold nanoclusters (GSH-AuNCs)-based fluorescent ratiometric nanosensor on the same particles without any conjugated fluorophores by aggregation-induced-emission (AIE) active amine-terminated lysine. Strong bluish green fluorescence of terminal primary amines was observed in the presence of GSH-AuNCs resulted from AIE, which offered us a great opportunity to achieve ratiometric detection lysine via non-covalently fluorophore. Moreover, this developed ratiometric fluorescent approach was applied to simultaneously detect cysteine and lysine.



Scheme 1. Schematic illustration for the synthesis of glutathione (GSH) stabilized gold nanoclusters (GSH-AuNCs), and the detection of lysine or cysteine utilizing pre-prepared GSH-AuNCs.

EXPERIMENTAL SECTION

Materials. L-Glutathione reduced (GSH, ⩾ 98.0%), and hydrogen tetrachloroaurate ($HAuCl_4 \cdot 3H_2O$) were purchased from Sinopharm Chemical Reagent Co., Ltd. (China). All chemicals were of analytical reagent grade and used as received without further purification. The phosphate buffer solution (PBS) was used to control the acidity of the reaction. Millipore Milli-Q ultrapure water (18.2 MΩ. cm) was used in all experiments.

Instrument. UV-vis absorption spectra were recorded on a Shimadzu UV-2450 UV–vis spectrophotometer (Shimadzu, Japan). Fluorescence spectra were obtained on Jasco FP-6500 fluorescence spectrometer (Jasco, Japan). Fourier transform infrared (FT-IR) spectra were determined from a NEXUS-670 FT-IR spectrometer (NICOLET, USA) in the spectral range of 400–4000 cm^{-1}. High-resolution TEM (HR-TEM) images were performed using a field emission transmission electron microscope at an accelerating voltage of 200 kV (JEM-2100, JEOL Ltd., Japan). pH values were measured with a PHS-3C pH meter (Leici, Shanghai, China).

Synthesis of water-soluble fluorescent GSH stabilized gold nanoclusters. GSH-AuNCs were prepared in one step by the reduction of $HAuCl_4$ with GSH as both reducing agent and stabilizer according to the literature with some modification.[20] In our experiment, 25 mL of freshly-prepared GSH (5 mM) was mixed with 25 mL of $HAuCl_4$ (5 mM) aqueous solution under vigorous stirring. Then, the mixture solution was incubated at room temperature and light irradiation for 4 days. Meanwhile, the color of the resulting solution changed from colorless to light yellow. After that, the obtained crude AuNCs containing solution was centrifuged at 15,000 rpm for 20 min to remove larger particles. The supernatants were further purified through a dialysis membrane tubing (12,000 Da, molecular weight cut-off). The final solution was stored in refrigerator at 4 °C for further research.

Fluorescence Experiments. The obtained GSH-AuNCs solution was diluted with an equal volume of phosphate buffer solution (50 mM PBS, pH 7.0) before the experiment. For the determination of lysine, various concentrations of lysine solution was added into 3 mL of the diluted GSH-AuNCs, and mixed thoroughly with gentle shaking. The fluorescence spectra of the mixture solution were recorded from 425 to 700 nm with the excitation wavelength at 402 nm.[21,22] The slit widths of the excitation and emission were both 10 nm. Meanwhile, for the detection of cysteine, series of cysteine solution were introduced into 3 mL of diluted GSH-Au NCs, and mixed thoroughly with gentle shaking. After 1 min incubation, the fluorescence spectra were recorded under the excitation at 402 nm. The whole process was carried out at room temperature.

Detection of lysine or cysteine in real sample. Human serum samples were collected from healthy laboratory volunteers and centrifuged. Then, 1.0 mL obtained supernatant was diluted to 200 mL. After that, 1.0 mL of the diluted solution, 1.5 mL GSH-AuNCs, and 0.5 mL phosphate buffer solution (50 mM PBS, pH 7.0) were mixed in a 4 mL centrifugal tube, and then appropriate amounts of lysine or cysteine were introduced. After 1 min, the mixture was used for the fluorescence determination. The amount of cysteine or lysine was determined by the standard addition method.

RESULTS AND DISCUSSION

Characterization of synthesized GSH-AuNCs. Gold nanoclusters were prepared by using a glutathione (GSH) as a scaffold and reducing agent in aqueous solution (see synthesis details in Experimental section).[20,23] As shown in Figure 1A, upon excitation at λ = 402 nm, the synthesized GSH-AuNCs exhibited an emission band centered at λ = 570 nm. Absorption

spectrum of synthesized GSH-AuNCs was displayed in Figure 1A. The high-resolution transmission electron microscopy (HR-TEM) image offered the morphological property of pre-prepared GSH-AuNCs (Figure 1B). The particles are well-dispersed, and the average diameter is 2.8 ± 0.5 nm. Fourier transform infrared (FT-IR) spectra of free GSH and GSH-AuNCs were carried out. The disappearance of S-H stretching band at 2526.5 nm on the surface of GSH-capped AuNCs reveals the formation of covalent bonds between thiols and AuNCs (Figure S1). In short, ultra-small fluorescent GSH-AuNCs were successfully synthesized.

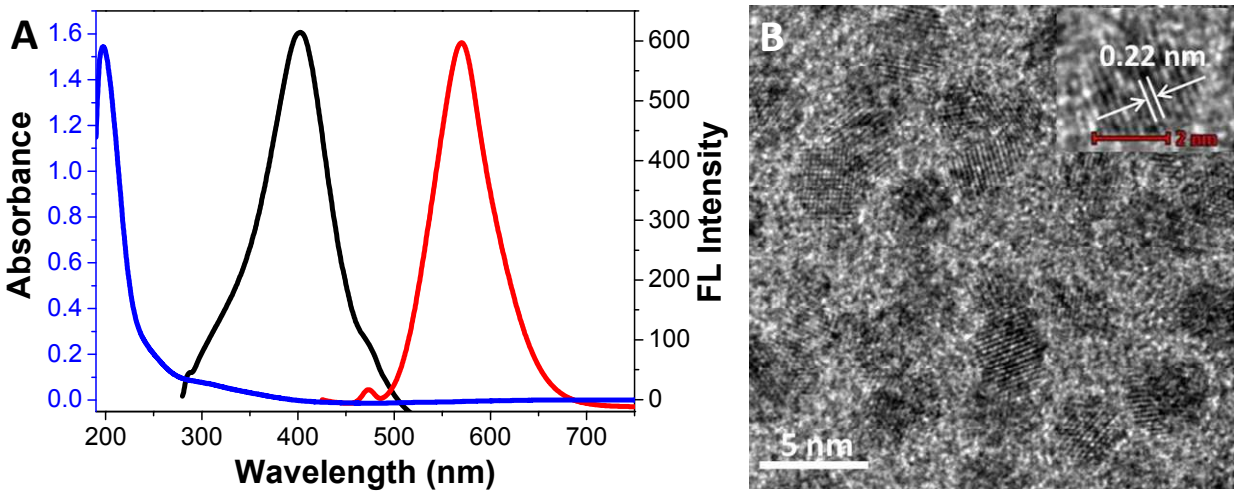


Figure 1. (A) Absorption spectrum (blue curve) of synthesized GSH-AuNCs, meanwhile fluorescence excitation (black curve) and emission spectra (red curve) of obtained GSH-AuNCs. (B) HR-TEM images of pre-prepared GSH-AuNCs, scale bar: 5 nm.

The design principle. The working principle of our study is represented in Figure 2A. The as-prepared GSH-AuNCs exhibit strong fluorescence emission peak located at λ = 570 nm due to

intraband transitions of free electrons of AuNCs (quantum yield = 8.5%, using quinine sulphate as the standard). Upon the addition of cysteine, the fluorescence maximum peak at λ = 570 nm enhanced significantly under the excitation wavelength at λ = 402 nm. More interestingly, after the addition of lysine, a new fluorescent emission peak located at λ = 473 nm turned up and increased gradually with increasing concentrations of lysine while the emission peak at λ = 570 nm almost kept constant under the same excitation wavelength at λ = 402 nm. Therefore, the pre-prepared GSH-AuNCs are promising as sole fluorophore for constructing ratiometric sensor. CIE1931 Chromaticity Coordinate Calculation was conducted to further illustrate the subtle difference among the emission peaks of synthesized GSH-AuNCs, GSH-AuNCs/Cys, and GSH-AuNCs/Lys system. As displayed in Figure 2B, the addition of cysteine induced emission intensity of GSH-AuNCs (see dot a and b) while the introduction of lysine led to blue shift of emission signal of GSH-AuNCs (see dot a and c), which revealed different mechanism of fluorescence enhancement. Moreover, GSH-AuNCs were demonstrated to be capable of simultaneously detecting Cys and Lys excitating at λ = 402 nm (Figure S2 and S3).

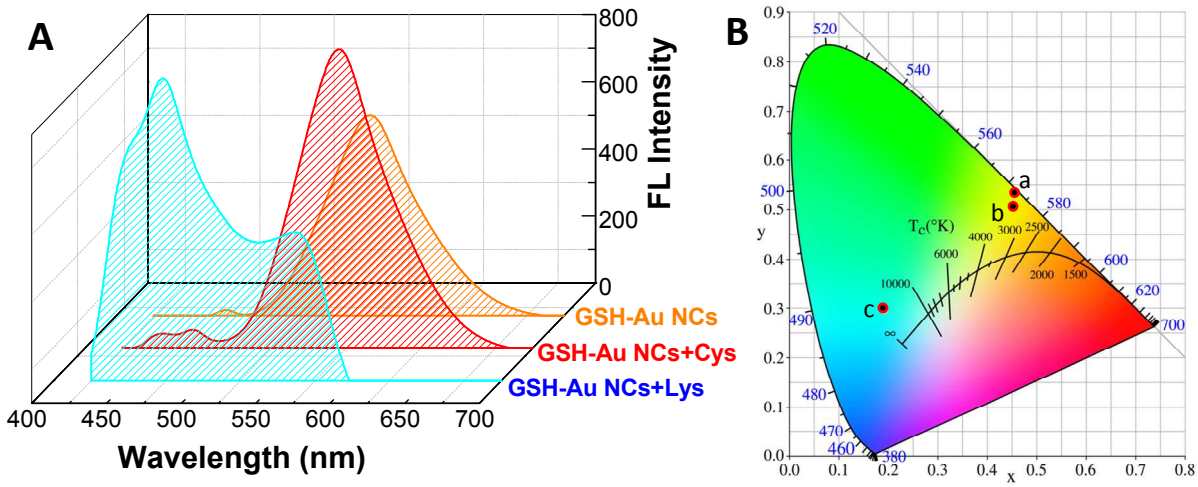


Figure 2. (A) Different fluorescent characteristics of as-prepared GSH-AuNCs, GSH-AuNCs/Cys, and GSH-AuNCs/Lys. Conditions: The concentration of cysteine (Cys) and lysine (Lys) is 100 and 6 μM, respectively. (B) The image of CIE1931 Chromaticity Coordinate Calculation of pre-prepared GSH-AuNCs (a), GSH-AuNCs/Cys (b), GSH-AuNCs/Lys (c).

Fluorescence ratiometric nanosensor of lysine detection using GSH-AuNCs. The excellent fluorescence properties of synthesized GSH-AuNCs prompted us to apply it to target-responsive analysis. Interestingly, a ratiometric fluorescent sensing lysine based on GSH-AuNCs was observed. Upon the addition of 10 μM lysine, lysine molecules assembled on the surface of the fluorescent GSH-AuNCs probe. The assembly process induced the intensity of new emission peak located at λ = 473 nm significant enhancement while the fluorescence emission intensity at λ = 570 nm almost stayed constant (Figure 3A). Furthermore, the lysine concentration was detected by measuring the ratio of the intensities of the λ = 473 nm and 570 nm emissions (I_{473}/I_{570}). The dual-emission fluorescence intensity ratio (I_{473}/I_{570}) value increased linearly with the concentration of lysine in the range of 0.02~5 μM with a detection limit of 2 nM (*S/N* = 3) (Figure 3B).

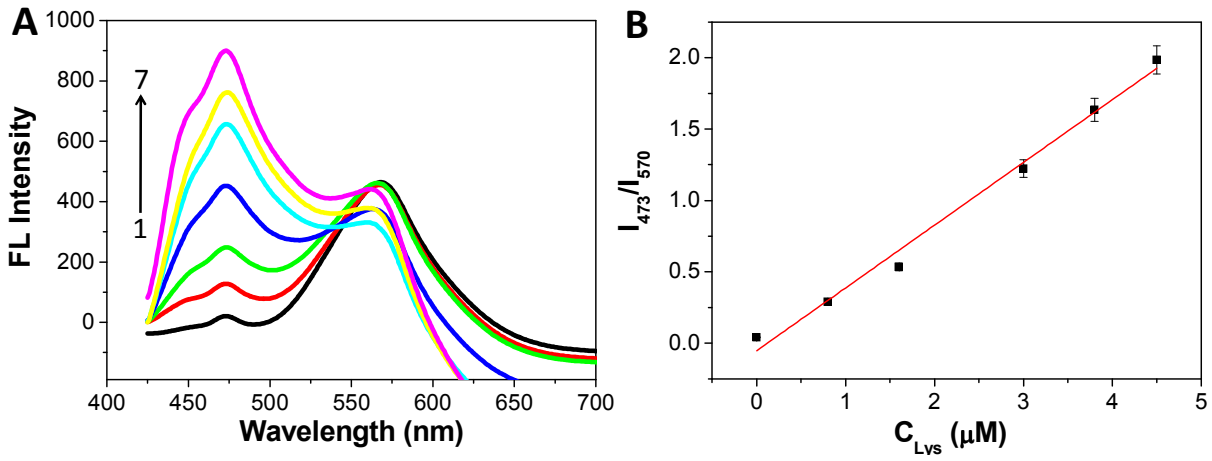


Figure 3. (A) Fluorescence spectra of synthesized GSH-AuNCs in the presence of increasing lysine concentrations. Conditions: 1, GSH-AuNCs; 2-7, 1 + lysine (μM): 0.8, 1.6, 3, 4.5, 6, and 10. (B) Fluorescence ratio (I_{473}/I_{570}) of the proposed sensor as a function of different lysine concentrations.

Mechanism of lysine-responsive ratiometric fluorescence of GSH-AuNCs. The mechanism of the ratiometric fluorescence response of GSH-AuNCs toward lysine has been investigated. It has been reported that lysine exhibited fluorescence signal in the visible region and its absorption was also relatively high.[24] The experimental assays revealed that a unique fluorescence property of lysine was due to its special 6-amino functional groups compared with other amino acid. It is recognized that emission signal of lysine was assigned to the intrinsic fluorescence of nitrogen atom. As lysine does not have conjugated functional group, aggregation of terminal 6-primary amino appears and induces the approaching of the electron transition level and produced red-shift of fluorescence emission peak.[25] Special terminal 6-primary amino functional groups of lysine is the key to its generation of fluorescent centers and chromophores. Thus, we proposed that terminal 6-primary amino functional groups of lysine aggregated on the surface of GSH-AuNCs and acted as fluorescent moieties,[6] which were responsible for the appearance of new

fluorescence emission peak located at λ = 473 nm. The ratiometric response of the dual-emissive GSH-AuNCs/Lys complex was attributed to energy transfer from 570 nm emission center to 473 nm emission center. In order to further investigate the fluorescence source, the control assays were also carried out (Figure 4). The photographs of GSH-AuNCs, GSH-AuNCs/Cys, GSH-AuNCs/Lys under UV light are displayed in Figure 4A. Three different colors suggested the occurrence of reaction and different reaction mechanisms. The fluorescent experimental results revealed that lysine even at high concentration (6.8 mM) only displayed weak emission peak located at λ = 440 nm under the excitation wavelength at λ = 402 nm (Figure 4B). The differences of fluorescence assays confirmed that the dual emissions with ratiometric Lys response originated from the aggregation-induced-emission of the amine-terminated lysine on the surface of GSH-AuNCs.[26] In the meanwhile, GSH stabilized AuNCs served as the "570 nm emission center". In addition, the aggregation process was verified by dynamic light scattering (DLS) analysis (Figure S4). Those phenomena demonstrated the potential of as-prepared GSH-AuNCs for ratiometric Lys sensing.

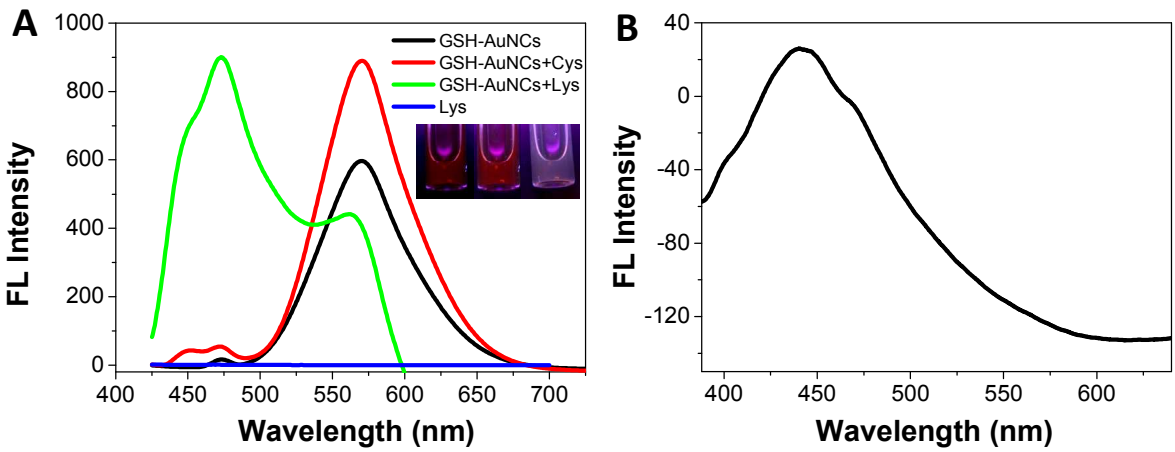


Figure 4. (A) Fluorescence emission spectrum of synthesized GSH-AuNCs (black curve), GSH-AuNCs/Cys (100 μM) (red curve), and GSH-AuNCs/Lys (6 μM) (green curve), and lysine (Lys, 6800 μM) excited at 402 nm. The inserted figures are the photographs of synthesized GSH-AuNCs, GSH-AuNCs/Cys, and GSH-AuNCs/Lys under UV light (from left to right). (B) Fluorescence emission spectrum of lysine (Lys, 6800 μM) excited at 402 nm.

Fluorescence turn-on detection of cysteine using GSH-AuNCs. Meanwhile, the respective investigation of synthesized GSH-AuNCs upon cysteine treatment indicated that the emission intensity of GSH-AuNCs located at λ = 570 nm enhanced significantly (Figure 5A). The corresponding calibration curve showed that the enhanced fluorescence intensity value of GSH-AuNCs was correlated linearly with cysteine concentrations in the range of 1~165 μM and the detection limit was 50 nM (S/N = 3) (Figure 5B). This enhancement effect was attributed to the change of microenvironment of gold nanoclusters by thiol after the addition of cysteine.[27,28] Considering the existence of thiol group in cysteine and the observed change in fluorescence spectra of glutathione stabilized gold nanoclusters, we guessed that the added cysteine interacted directly with gold clusters via the strong Au-S interaction and amide bond. The interaction

process effectively protects the chromophore from fluorescence quenching effect of the solvent, which induced enhanced fluorescent signal at 570 nm.[29]

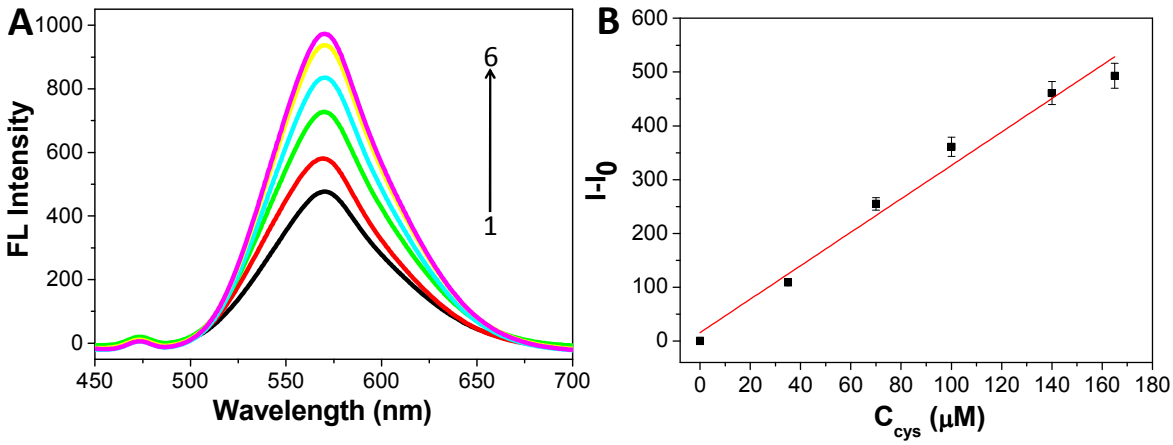


Figure 5. (A) Fluorescence spectra of pre-prepared GSH-AuNCs in the presence of increasing cysteine concentrations. Conditions: 1, pre-prepared GSH-AuNCs; 2-6, 1 + cysteine (μM): 35, 70, 100, 140 and 200. (B) The linear calibration curve between ($I-I_0$) and the concentrations of cysteine.

Selectivity assay. To investigate the selectivity of our present detecting systems, the response of the assay to other 19 essential amino acids in a concentration of 100 μM was carried out under the same conditions as in the case of detecting lysine (Figure 6). The results suggested that the new developed approach had quite high selectivity for lysine detection compared with other amino acids by the present ratiometric strategy (green histogram). It was guessed that the activity of 2-amino decreased due to its in close proximity to carboxyl for other 19 amino acids. Whereas, lysine possesses its special 6-primary amine functional groups that stays away from carboxyl group, which are thought to be responsible for fluorescence enhancement at λ = 473 nm based on aggregation-induced-emission.[8] In the meanwhile, the present assay displayed

effectively competition against all amino acids tested other than cysteine by enhanced fluorescence strategy (red histogram). It dued to the fact that cysteine possessed unique thiol functional group compared with other 19 amino acids. Thiol changed the microenvironment of gold nanoclusters, leading to the enhancement fluorescence signal at λ = 570 nm.[27-29]

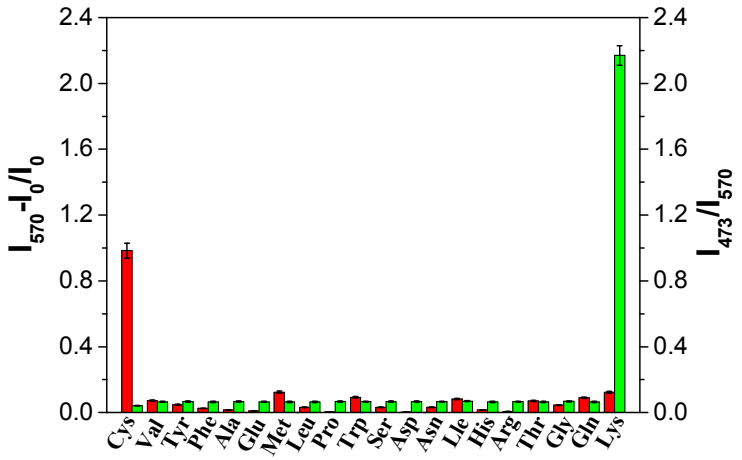


Figure 6. Selectivity of proposed GSH-AuNCs approach for analyzing cysteine (100 μM) (A, red histogram) or detecting lysine (10 μM) (B, green histogram) over other 19 amino acids (100 μM).

Analysis of real samples. In order to further assess the applicability, the present approach was further applied on lysine determination or cysteine detection in real samples. The detected lysine or cysteine content in real serum samples was derived from the standard curve and the regression equation. Moreover, the average recovery tests were performed by using the standard addition method. The detection results are listed in Table 1 and Table 2. The results provided by the developed GSH-AuNCs sensor were compared with those obtained by the standard HPLC method. As displayed in Table 1 and Table 2, good consistency was obtained between these two

methods, suggesting the excellent behavior of our proposed method in real samples. It has been reported that GSH-AuNCs probe have no acute toxicity to cells by methylthiazolyldiphenyl tetrazolium bromide (MTT) assay and apoptosis assay.[30] Therefore, our present GSH-AuNCs based probe possesses the potentiality of monitoring lysine and cysteine in life systems.

Table 1

Application of the proposed ratiometric method for the determination of lysine in human serum samples (n = 3) by GSH-AuNCs probe.

Sample No.	Blank	The developed GSH-AuNCs approach in this paper				HPLC method			
	Found[a] (μM)	Amount Added (μM)	Amount Found[b] (μM)	R.S.D. (%)	Recovery (%)	Amount Added (μM)	Amount Found[b] (μM)	R.S.D. (%)	Recovery (%)
1	-	1.5	1.426 ± 0.007	1.3	95.1	1.5	1.475 ± 0.005	1.0	98.3
2	-	3.0	2.969 ± 0.009	1.5	98.9	3.0	2.978 ± 0.96	1.4	99.3
3	-	5.0	5.102 ± 0.013	1.9	102.1	5.0	5.065 ± 1.80	1.7	101.3

[a] - Not Found.

[b] Average value of three determinations ± standard deviation.

Table 2

Application of the proposed enhancement method for the detection of cysteine in human serum samples (n = 3) by GSH-AuNCs probe.

Sample No.	Blank	The developed GSH-AuNCs approach in this paper				HPLC method			
	Found[a] (μM)	Amount Added (μM)	Amount Found[b] (μM)	R.S.D. (%)	Recovery (%)	Amount Added (μM)	Amount Found[b] (μM)	R.S.D. (%)	Recovery (%)
1	-	35	33.892 ± 0.023	1.7	96.8	35	34.585 ± 0.019	1.5	98.8
2	-	80	80.932 ± 0.035	1.9	101.2	80	81.212 ± 0.038	1.8	101.5
3	-	120	124.3 ± 0.057	2.1	103.6	120	123.5 ± 0.046	2.0	102.9

[a] - Not Found.

[b] Average value of three determinations ± standard deviation.

CONCLUSIONS

In summary, we demonstrated a facile method to construct ratiometric fluorescent sensor without conjugated chromophore. The AIE-active amine-terminated small molecule on the surface of synthesized GSH-AuNCs made it feasible to fabricate ratiometric probe for analyte. The present approach effectively overcomes unavoidable drawbacks of traditional ratiometric probes such as complicated dual-emission element linking or synthesis of nanohybrid. Moreover, the proposed

assay was demonstrated for detecting lysine and cysteine simultaneously with the same excitation wavelength. In addition, the present strategy will bring a promising path for the design of ratiometric sensors for various applications.

ASSOCIATED CONTENT

Supporting Information

Fourier transform infrared spectra, fluorescence emission spectrum, the image of CIE1931 Chromaticity Coordinate Calculation, and dynamic light scattering (DLS) analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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Graphical abstract