Scientific paper

Thiol Reactive Probe Based on Fluorescence Resonance Energy Transfer between Fluorescein and Au Nanoparticles

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Abstract

Sensitive and selective fluorescent probe of thiols with lower limit of detection based on fluorescence resonance energy transfer (FRET) between fluorescein and Au nanoparticles (AuNPs) is presented. The fluorescein-AuNPs complex emits weak fluorescence. Upon chemically binding to organosulfur compound that contains a carbon-bonded sulfhydryl (-C-SH or R-SH) thiols, a stable enhancement of fluorescence is observed due to the competitive binding on AuNPs between thiols and fluorescein. The magnitude of fluorescence enhancement is linearly proportional to the logarithm of the thiols concentration. We use cysteine as an example to show how this useful analytical assay works selectively, which is closely nonresponsive to 20 other amino acids even though they are in solution at a concentration 10 times greater than the thiols. The detection limit for cysteine is 7.27×10^{-9} mol L^{-1}. The possible mechanism of this assay is discussed in details. The proposed method was successfully applied for the determination of Cys in urine.

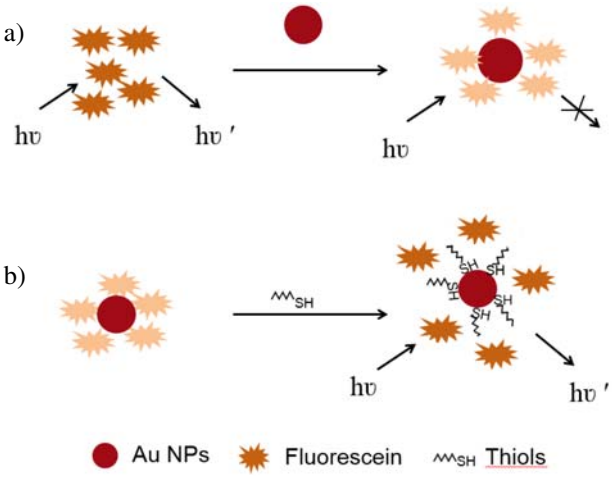
Keywords: Thiols, gold nanoparticles, fluorescein

1. Introduction

Over the decades, particular interest has been focused on the chemosensing of sulfhydryl-containing amino acids and peptides, the thiols, as they play crucial roles in biological systems. For example, thiol group in cysteine (Cys) residues is the active site of proteins is related to oxidative stress.[1] Cys is a member of essential amino acids in human body which also plays a vital role in food industry, pharmaceutical industry and cosmetics. It has a variety of important physiological functions, such as in metabolism, anti-aging and detoxification.[1–3] The deficiency of cysteine causes many diseases, such as delayed growth in children, depigmentation of hair, edema, lethargy, liver damage, loss of muscle and fat, and skin lesions.[4–5] In addition, glutathione (GSH) and homocysteine (Hcy) which belong to the thiol group, are also linked to various human diseases.[6–11] Therefore, selective detection and quantification of thiols is of growing importance. The majority of the reported methods have been focused on capillary electrophoresis,[12] high performance liquid chromatography,[13–14] electrochemical analysis[4] and mass spectrometry.[15]

In recent years, gold nanoparticles (AuNPs) have become highly studied materials and been widely used in a range of applications due to their excellent optical and electrical properties.[16–18] One feature that makes them particularly appealing is that AuNPs possess high absorption coefficients in the visible region, which ensures them to function as efficient quenchers for most fluorophores.[19,20] In order to take full advantage of the super-quenching ability of AuNPs, many high-performance fluorescence assays have been developed in recent years for optically sensing biologically important ions and molecules. Eunkeu Oh *et al.* designed an inhibition assay for avidin detection based on the modulation of FRET efficiency between biomolecule-conjugated QDs and AuNPs.[21] Dong reported a simple fluorescent method for cyanide detection based on the dissolution of Rhodamine B-adsorbed gold nanoparticles by cyanide.[22] Such methods are also used to determine melamine,[14] mercury ion,[23] copper ion,[24] cyanide,[22] and fenamithion.[25] It was reported that the FRET process between fluorescein and AuNPs was easily occurring because of a wide margin overlap between the absorption band of AuNPs and the emission band of fluorescein.[26] Based on this, a novel and simple

fluorescence assay for thiol determination was constructed, in which fluorescein acted as the energy donor and AuNPs served as acceptor. Upon addition of thiols such as Cys, Hcy and GSH, fluorescein was removed from the surface of AuNPs because of the stronger binding between the thiol group of Cys and gold to form Au-S bonds. As a result, the emission of fluorescein was restored. Herein, a simple and fast platform for the specific detection of thiols was presented and the proposed mechanism was depicted in Scheme 1.



Scheme 1: Schematic representations of fluorescent probe for thiols detection

2. Experimental

2. 1. Apparatus

Fluorescence spectra were acquired on a F-4600 fluorescence spectrometer (Hitachi, Japan, www.hitachi-hitec.com) equipped with a xenon lamp source and a 1.0 cm quartz cell, and the scan speed was 1200 nm min^{-1}. Absorption spectra were recorded on a UV-vis 2550 spectrophotometer (Shimadzu, Japan, www.shimadzu.com) using a 1.0 cm quartz cell. Size distribution of AuNPs was measured on a Hydrosol Nanoparticle Size Analyzer and Zeta Potential Analyzer (PSA NANO2590, Malvern Companies, UK, www.malvern.com). The surface morphology of the AuNPs was characterized using Transmission electron microscope (TEM, JEM-2010 transmission electron microscope (JEOL Ltd.)) All pH measurements were made using a pHS-3 digital pH-meter (Shanghai REX Instrument Corp., Shanghai, China, http://en.leici.com) combined with a glass-calomel electrode. All optical measurements were carried out at room temperature.

2. 2. Materials

Chloroauric acid tetrahydrate ($AuCl_3 \cdot HCl \cdot 4H_2O$), trisodium citrate dihydrate ($C_6H_5O_7Na_3 \cdot 2H_2O$), and fluorescein were purchased from Sinopharm Chemical Reagent Co. Ltd., L-Cys, L-Arginine, L-Histidine, L-Leucine, Glutathione (Reduced) were purchased from Beijing Solarbio Science & Technology Co., Ltd., other amino acids were purchased from Chengdu Gray West Chemical Technology Co., Ltd. All other reagents were of analytical grade and used without any further purification. All solutions were prepared using doubly distilled water.

2. 3. Preparation of AuNPs

The monodisperse AuNPs were prepared through the classical citrate reduction method reported previously.[27] All glassware used in this experiment were cleaned with aqua regia (3:1 HCl/HNO_3) and rinsed with tap water and deionized water, and dried prior to use. Briefly, the mixture of 4.0 mL of 1% $HAuCl_4$ and 96 mL deionized water was brought to boiling. Then 2.0 mL 5% sodium citrate solution was quickly added into the solution with stirring. The mixture was refluxed for an additional 5 min, during this time the color of solution became deep red. Then the solution was cooled to room temperature and then stored at 4 °C. Additionally, the concentration of the AuNPs in the testing solution was determined to be 15 nM according to the Beer's law using an absorption coefficient of 2.7×10^8 cm^{-1} M^{-1} at 520 nm for Au NPs of 13 nm diameter.[27]

2. 4. Detection Procedures for Cys

0.2 mL 3.0×10^{-5} mol L^{-1} fluorescein and 4.0 mL of prepared AuNPs were added to an amber bottle, and then the solution was diluted with 0.01 mol L^{-1} (pH 8.0) phosphate buffer solution to a final volume of 10 mL. The mixture was maintained at room temperature for 1 h in the dark. Afterwards, different amounts of Cys were added to 0.5 mL of the prepared mixture, and each sample solution was diluted with 0.01 mol L^{-1} (pH 8.0) phosphate buffer solution to 2.0 mL. After 5 minutes' reaction, the fluorescence spectra were collected.

2. 5. Pretreatment of Human Urine Samples

Human urine sample (1.0 mL) collected from healthy adult female volunteers, and the analysis was conducted immediately after the sample collection. 1 mL of urine sample was added into a centrifuge tube, 1.0 mL of acetonitrile was added for removing proteins in urine. The mixture was centrifuged at 12,000 rpm for 10 min. The supernatant was filtered through a 0.22–μm filter, then dried by vacuum at 50 °C for 10 h, and diluted to 5 mL with deionized water before analysis.

3. Results and Discussion

3. 1. Characterization of AuNPs

The surface morphology of the prepared AuNPs was

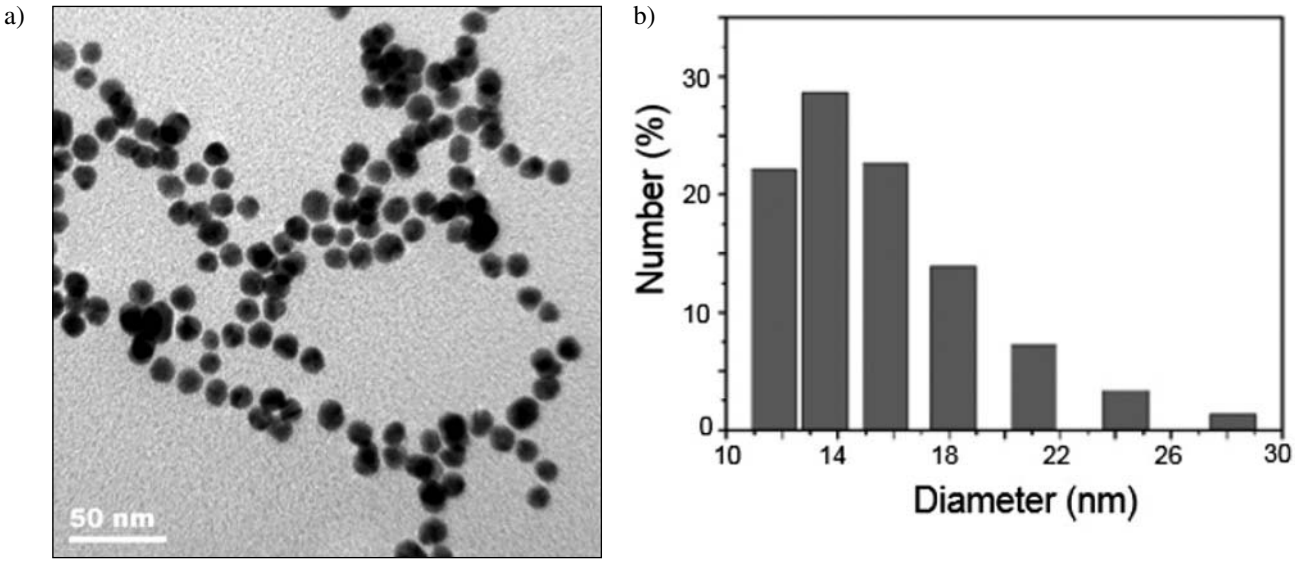


Fig. 1: TEM images of AuNPs (a) and size distribution of AuNPs (b)

studied by the Transmission electron microscope. As can be seen from Fig. 1 a, the prepared AuNPs were dispersed uniformly and close to spherical in shape. The size of 72% AuNPs was from 11 to 15 nm (seen Fig. 1 b) which was measured on a Hydrosol Nanoparticle Size Analyzerand Zeta Potential Analyzer. No obvious spectral change of size distribution was observed within 2 days, which suggested that the colloid solution of AuNPs was stable and homogeneous.

3. 2. Fluorescence Quenching Effect of AuNPs

Fig. 2 a depicts emission spectrum of fluorescein and the absorption spectrum of AuNPs. It can be seen that the maximum of emission band for fluorescein is at 517 nm, while the maximum of absorption band for AuNPs is at 520 nm. The spectral integral overlap between the fluorescein emission and the AuNPs absorption is excellent which promised the efficiency of FRET occurring. AuNPs might act as an absorber to attenuate the emission of fluorophore dye when the emission band of the fluorophore is superimposed on the absorption band of AuNPs[20].

The quenching effect of AuNPs on the fluorescence intensity of fluorescein was investigated. As seen from the Fig. 2 b, the fluorescence intensity of fluorescein decreases gradually upon addition of AuNPs, and the quenching process can be characterized by Stern-Volmer equation:

$$F_0/F = 1 + K_{SV}\,[\text{AuNPs}] \qquad (1)$$

F_0 and F denote the steady-state fluorescence intensities in the absence and presence of AuNPs, respectively.

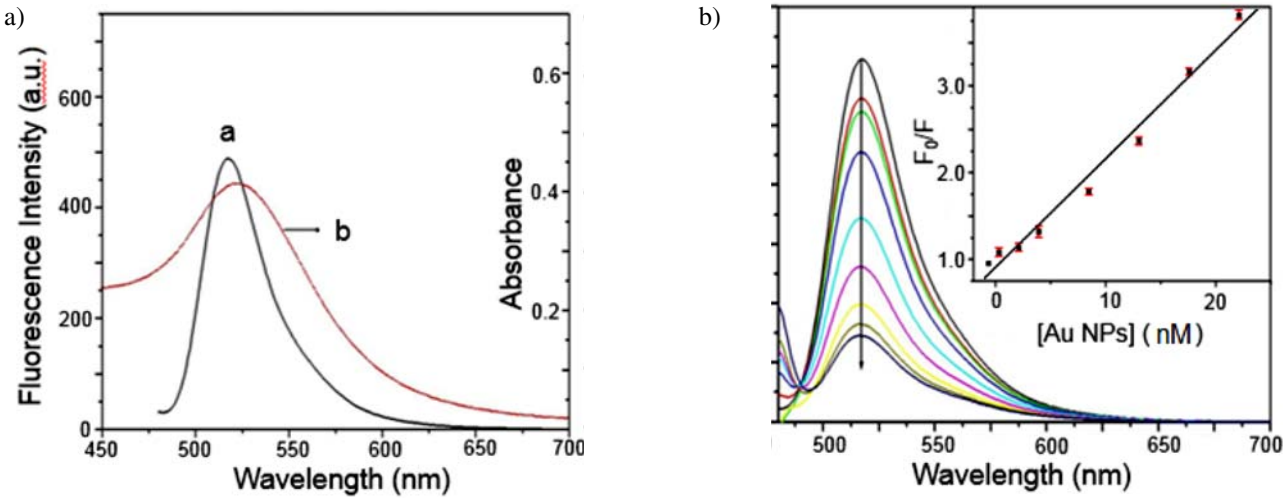


Fig. 2: a) Emission spectrum of fluorescein (curve a) and absorption spectrum of AuNPs (curve b); b) Fluorescent spectra of fluorescein upon addition of AuNPs with various concentrations. The inset shows the relationship between F_0/F versus [AuNPs]. Fluorescein: 1.5×10^{-7} mol L^{-1}; AuNPs (from top to bottom): 0.0, 0.88, 2.6, 4.2, 8.5, 13, 17, 21, 23×10^{-10} mol L^{-1}

[AuNPs] represents the concentration of AuNPs. The Stern-Volmer equation is fitted as: $F_0/F = 0.879 + 1.26 \times 10^9 \times$ [AuNPs], the correlation coefficient r is 0.992, and the K_{SV} is calculated to be 1.26×10^9 mol L^{-1}. Obviously, the quenching effect shows the good linear relationship which suggests the quenching caused by single effect of FRET.

3. 3. Fluorescence Recovery Fluorescein-AuNPs in the Presence of Thiols

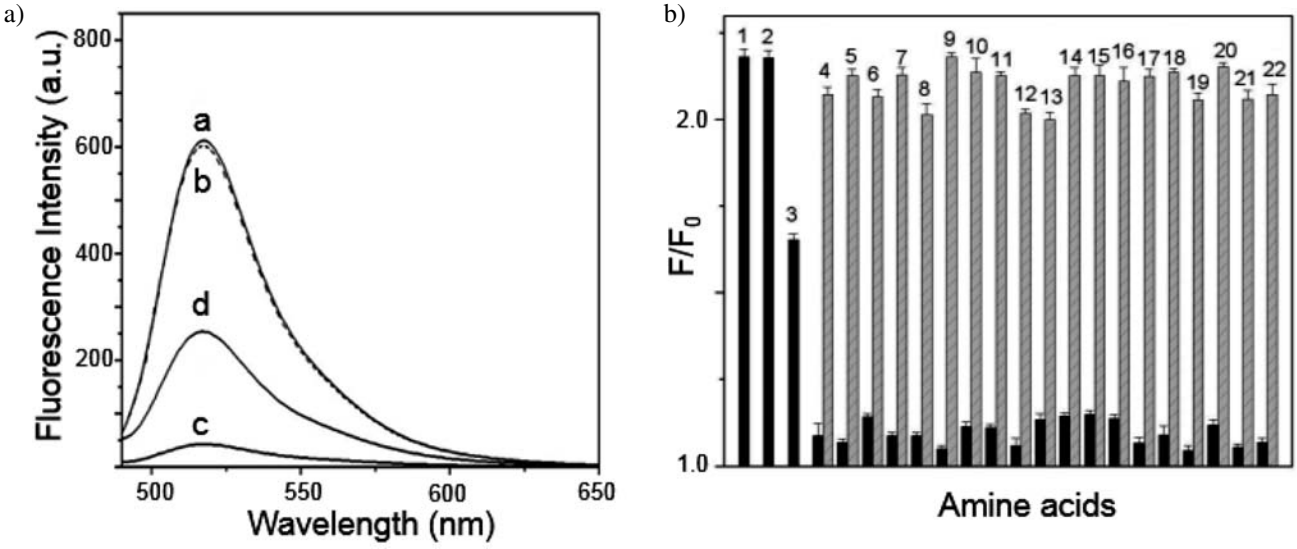


Fig. 3: (**a**) Fluorescence spectra of fluorescein (a), fluorescein with Cys (b), fluorescein-AuNPs complex (c), fluorescein-AuNPs complex and Cys (d). The concentrations of fluorescein,Cys and AuNPs complex are 1.5×10^{-7} mol L^{-1}, 7.5×10^{-7} mol L^{-1} and 3.0×10^{-9} mol L^{-1}, respectively. **(b):** Effects of different amino acids on the enhancement of fluorescence. Black bars represent the addition of a single amino acid; gray bars are the addition of Cys (2.5×10^{-7} mol L^{-1}) with another acid (2.5×10^{-6} mol L^{-1}). Cys, Hcy, GSH: 2.5×10^{-7} mol L^{-1}, other acids: 2.5×10^{-6} mol L^{-1}; 1–22: Cys, Hcy, GSH, Phe, Glu, His, Ser, Val, Thr, lle, Asp, Arg, Met, Pro, Trp, Leu, Asn, Tyr, Gly, Lys, Gln, Ala.

The introduction of thiols to the fluorescein-AuNPs complex enhances the fluorescence intensity because of the strong affinity of thiols to AuNPs as shown in Scheme 1. Fig. 3 shows the emission spectra of fluorescein, fluorescein-Cys, fluorescein-AuNPs complex and fluorescein-AuNPs-Cys under the same experimental conditions. The Hcy and GSH behave in similar way. Fluorescein exhibits a strong fluorescent signal at 517 nm (see curve a in Fig. 3 a), and no obvious changes were observed after adding 7.5×10^{-7} mol L^{-1} Cys to the solution (curve b in Fig. 3 a), which indicates that no interaction between Cys and fluorescein occurred. However, the fluorescence intensity of fluorescein was remarkably decreased in the presence of AuNPs, which was attributed to the highly efficient energy transfer between fluorescein and AuNPs (curve c in Fig. 3 a). The addition of Cys into the fluorescein-AuNPs solution resulted in the fluorescence enhancement (curve d in Fig. 3 a). As shown in Scheme 1, strong affinity of thiols to AuNPs released or freed fluorescein and therefore restored its fluorescence. As a result, the energy transfer between fluorescein and AuNPs was blocked and the emission of fluorescein was turned on.

After we noticed the potential of fluorescence probes for detecting thiols, we optimized the working conditions. Because the emission of fluorescein was steady in basic solution and the intensity was enhanced with the increased pH, the pH effect to this probe was studied (Figure S1A). In pH 8.0 phosphate buffer solution, the emission enhancement of fluorescein reached the maximum on addition of Cys, therefore the pH 8.0 phosphate buffer solution was chosen. The effect of AuNPs concentration on fluorescence enhancement (F/F_0) was also investigated. The intensity ratio F/F_0 was increased with an increasing concentration of AuNPs, while it was decreased when the concentration of AuNPs was over 3.0 nM (Figure S1 b). It was reported that a lower concentration of AuNPs could increase the background fluorescence, and a higher concentration of AuNPs might decrease the detection sensitivity. The experimental results showed that the concentration of 3.0 nM AuNPs was the optimal. Additionally, the inference of reaction time on fluorescence enhancement (F/F_0) was also examined (Figure S1 c). The fluorescence initially increased and then stabilized at 3 min after the addition of Cys. In order to be given enough reaction time, the fluorescence intensities were measured after 5 min incubation when Cys was added.

Under the optimal conditions, the selectivity and recognition ability of fluorescein-AuNPs complex probes for different amino acids were investigated. Fig. 3 b shows the fluorescence intensity change upon addition of 20 different amino acids (black bar). The changes for sulphur-

containing amino acids including Cys, Hcy, and GSH were strikingly bigger than for the other amino acids. Furthermore, the fluorescence enhancement provided by the thiols was not affected (less than 10%) by the presence of these extraneous amino acids. The results above suggested the thiol functionality in amino acids is essential for the fluorescence recovery. In the present paper, Cys was selected as a representative sulphur-containing amino acid. Interference of the amino acids was evaluated on the present system (Fig. 3 b gray bar). The results showed that 10-fold excess of other amino acid did not interfere with the determination of Cys with AuNPs.

3. 4. Detection of Cys

Fig. 4 shows the fluorescence emission spectra of fluorescein-AuNPs complex in the presence of different concentrations of Cys. As shown, the increase of fluorescence intensity can quantitatively reflect the amount of Cys added and a good linear relationship between the increase of fluorescence intensity at 517 nm and the concentration of Cys is obtained in the range of 2.50×10^{-8} to 3.25×10^{-7} mol L^{-1}. The linear equation is $(F–F_0)/F_0 = 0.01041 + 6.76 \times 10^6$ [Cys] (mol L^{-1}), and the data fitting gives the correlation coefficient of 0.998. The limit of detection (LOD) is given as 7.27×10^{-9} mol L^{-1} by equation $LOD = K \times S_0/S$, where K is a numerical factor chosen according to the confidence level desired, S_0 is the standard deviation (SD) of the blank measurements (n = 13, K = 3), and S is the slope of the calibration curve. It is also found that the color of the solution did not change significantly when the Cys was in this concentration range, but it changed to blue when the concentration of Cys increased to 3.0×10^{-5} mol L^{-1}. This phenomenon indicated that a higher concentration of Cys might cause the aggregation of gold nanoparticles. Therefore, the presented assay was optimal for detecting low concentrations of Cys via fluorescence assay. Some methods for determination Cys are listed in Table 1. Compared to the approaches developed previously, the presented method possesses advantages such as low detection limit and a longer determination wavelength for Cys analysis. Moreover, the method of fluorescence resonance energy transter (FRET) is simple and easy to construct. Most important of all, AuNPs has an excellent biocompatibility and is environmentaly friendly compared to the assays based on cadmium quantum dots.

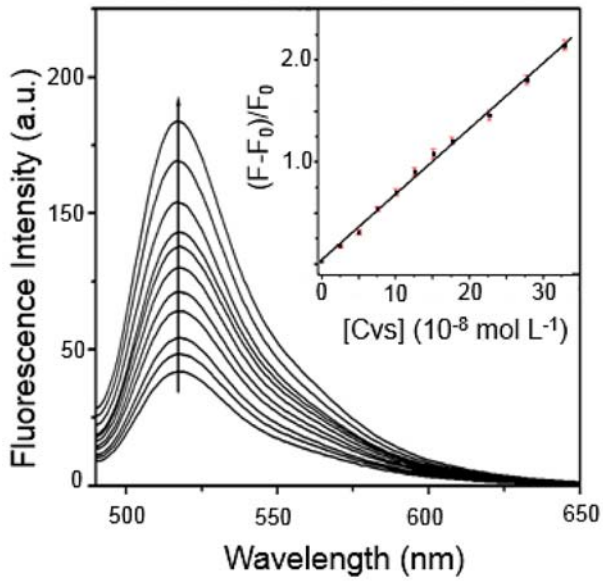


Fig. 4: Fluorescent spectra of fluorescein-AuNPs complex in the presence of different concentrations of cysteine. Inset is the calibration curve of $(F-F_0)/F_0$ versus the concentration of cysteine. The concentrations of cysteine (from bottom to top) were: 0, 2.5, 5.0, 7.5, 10.0, 12.5, 15.0, 17.5, 22.5, 27.5, 32.5×10^{-8} mol L^{-1}

3. 5. Recovery from Urine

The recovery from urine at three different concentrations (0.5, 1.0, and 1.5×10^{-7} mol L^{-1}) of Cys was determined. Mean recovery was 95% for urine, with a range 89%–104% (n = 9). The relative standard deviation (RSD%) were calculated and listed in Table 2.

Table 1: Comparison of methods for the determination of Cys

Method	Reagent(s)	Analytical ranges (μM)	Detection limit (nM)	Determination index wavelength (nm)	Ref
Fluorimetry	Mercaptoacetic acid-capped CdSe/ZnS QDs	0.01–8.0	3.8	F_{565}	16
Spectrophotometry	Ag NPs in the presence of Cr^{3+}	Not given	1.0	$A_{590/390}$	28
	AgNPs in the presence of Ca^{2+}	0.25–10	85	$A_{524/396}$	
Spectrophotometry	Triangular silver nanoprisms	Not given	160	509	29
Spectrophotometry	Triiodide ion and hexadecylpyridinium chloride	0.0082–0.12	4.9	500	30
Spectrophotometry	Ferric ions and ferrozine	0.17–50	Not given	562	31
Voltammetry	Carbon-paste electrode	0.5–100	200		32
Fluorimetry	Thiazole orange /DNA/Hg^{2+}	0.0029–0.11	5.1	540	33
	Cu^{2+}-morin complex	0.65–22	65.2	539	34
Electrochemical determination	polymers/gold nanoparticles hybrid nanocomposites	0.5–200	50		35
Fluorimetry	Fluorescein and Au nanoparticles	0.025–0.325	7.27	517	This method

Table 2: Recovery of Cys from Urine

Urine sample	Added (10^{-7} M)	Average (10^{-7} M, n = 3)	Recovery (%)	RSD (%, n = 3)
1	0.5	0.4429	89	2.1
2	1.0	1.0462	104	1.7
3	1.5	1.3825	92	6.3

4. Conclusions

In summary, we developed a novel, fast, sensitive, selective and easily operated fluorescent probe for thiols, which are important in health and disease diagnostics, based on FRET between the fluorescein and AuNPs. By engineering the Au-NPs and fluorescein complex strategy, thiols were identified due to the strong binding affinity of the thiol groups with AuNPs. A stable enhancement of fluorescence is observed after 3 min. The magnitude of fluorescence enhancement is linearly proportional to the concentration of thiols, at an optimized nanoparticle concentration (3 nmol L^{-1}) and pH 8.0. The linear relationship was observed from cysteine concentration of 2.50×10^{-8}–3.25×10^{-7} mol L^{-1}, with a detection limit of 7.27×10^{-9} mol L^{-1}. This analytical assay is found to respond only to thiols, and is closely nonresponsive to other amino acids that do not possess a thiol chemical moiety, even when their concentrations are 10 times higher than thiols concentration in the solution. Cys concentrations were measured in three urine samples before and after addition of pure Cys at 0.5, 1.0, and 1.5×10^{-7} mol L^{-1}. Mean recovery was 95%.

5. Acknowledgments

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Povzetek

Predstavljamo občutljivo in selektivno fluorescentno probo za tiole z nižjo mejo zaznave, ki je osnovana na fluorescenci po resonančnem prenosu energije (FRET) med fluoresceinom in Au nanodelci (AuNP). Kompleks fluorescein-AuNP šibko fluorescira. Po kemični vezavi na organožveplovo spojino, ki vsebuje na ogljik vezano sulhidrilno skupino (-C-SH ali R-SH), t.j. tiol, opazimo stabilno povečanje fluorescence zaradi kompetitivne vezave AuNP na tiole ali fluorescein. Velikost povečanja fluorescence je linearno sorazmerna logaritmu koncentracije tiolov. Cistein smo uporabili za primer, kako selektivno deluje ta uporabni analizni test, ki je bil skoraj neodziven na 20 drugih aminokislin, čeprav so bile prisotne v 10-krat višji koncentraciji kot tioli. Meja zaznave za cistein je $7{,}27 \times 10^{-9}$ mol L^{-1}. Podrobno razpravljamo o možnem mehanizmu delovanja tega testa. Predlagano metodo smo uspešno uporabili za določitev Cys v urinu.

Supporting Materials

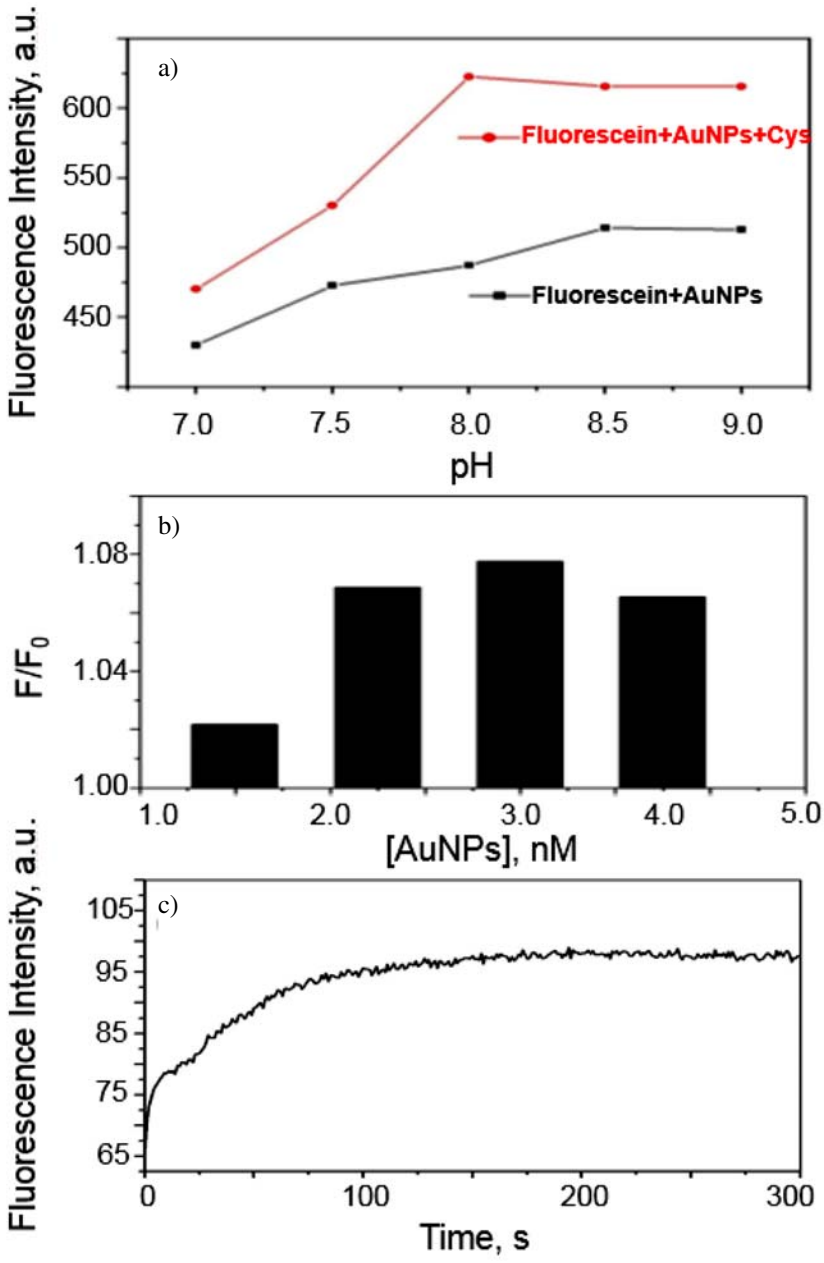

Fig. S1. Effects of pH (a), AuNPs concentration (b), and reaction time (c) on the enhancement of fluorescence.