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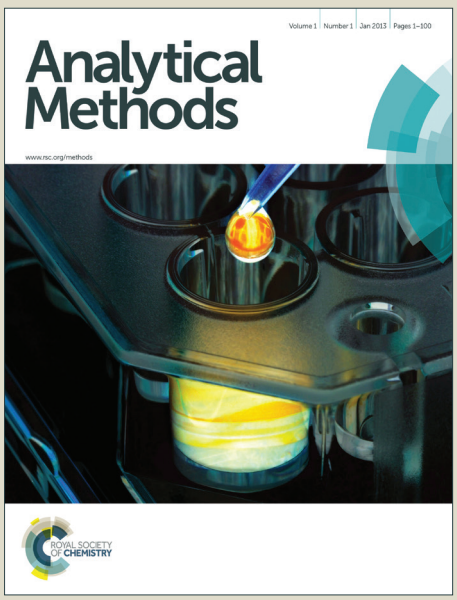

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A europium-based fluorescence probe for detection of thiols in urine

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Abstract

It is very necessary to develop a simple and sensitive analytical method for the detection of biothiol in biological fluids due to their vital biological functions. In this work, a fluorescent europium tetracycline complex (EuTc) was employed as a probe to detect biothiol, which is based on the enhancement and quenching of fluorescence of EuTc complex caused by H_2O_2 and biothiols. After the addition of H_2O_2, significant enhancement in the fluorescence of EuTc can be observed due to the replacement of coordinated water molecules and the formation of EuTc-H_2O_2 complex. In the presence of biothiols, however, the fluorescence of EuTc complex was quenched, which arise from the formation of disulfide bond caused by H_2O_2. The fluorescent intensity of EuTc was decreased linearly with increasing the concentrations of biothiols. The detection limits for Cys, GSH and Hcys in aqueous solution are 100 nM, 200 nM and 400 nM, respectively. The EuTc was successfully applied to detect total thiols in urine samples and showed a satisfactory result. Compared with other methods, the proposed method is advantageous because that it provides a simple and low-toxicity detection procedure for thiols and possesses the ability of eliminating background fluorescence.

Keywords: Europium; Fluorescence; H_2O_2; Tetracycline; Biothiols

Introduction

Biothiols are important in biological systems because their vital biological functions. Glutathione (GSH), homocysteine (Hcys) and cysteine (Cys) are three primary biological thiols (biothiols) in the cell. Among these three biothiols, GSH is the most abundant biothiol (1-15 mM). GSH is not only involved in regulating redox homeostasis between thiols and the corresponding disulfides in the cellular environment [1, 2], but also is responsible for some important cellular functions like detoxification and metabolism. It is well known that the ratio of glutathione (GSH) to glutathione disulfide (GSSG) is a key marker for monitoring the cellular oxidative stress, and its abnormal level can often lead to some diseases and cancers [3]. Cys and Hcy are essential biological components for the growth of cells and tissues, and the levels of Cys and Hcy are often used as indicators of disease. For instance, deficiency of Cys would be related with hematopoiesis reduction, retarded growth, hair depigmentation, liver damage, skin lesion development, and cancer [4]. Abnormal levels of Hcys have been linked to Alzheimer's disease [5], osteoporosis [6], and cardiovascular disease [7]. Therefore, the detection of biothiols in biological samples is of great importance for investigating cellular functions.

To date, various methods have been developed for the detection of biothiols, such as high performance liquid chromatography (HPLC) [8], capillary electrophoresis (CE) [8], electrochemical detection [9], laser-induced fluorescence detection [10], and mass spectrometry [11]. These methods are sensitive and highly specific, but certain drawbacks exist. For example, the HPLC and CE methods are time-consuming and require expensive and sophisticated instruments. Fluorescent methods are more desirable since they are simple, sensitive and convenient. In the past few years, various fluorescent probes based on organic dyes and nanomaterials, such as naphthalimide derivative [12], quantum dots [13] and Ag cluster [14], have been exploited for biothiols detection. Nevertheless, they still have some limitations. The organic dyes not only require complicated preparation procedure, but also have poor aqueous solubility and are prone to photobleaching. The high toxicity and difficult surface conjugation chemistry of quantum dots limits their wide application [15]. Moreover, some fluorescent methods for thiols detection involved the use of heavy metal ions, such as Hg^{2+} [16-18]. The most important is the fact that these fluorescent probes suffer

from the interference of background fluorescence and scattering light from biological tissues and matrice, which often leads to the decrease of detection sensitivity. Thus, it is highly desirable to develop fluorescent probes with the features including simple preparation procedure, low-toxicity, excellent emission stability, and the ability of eliminating background fluorescence for thiols detection.

Fluorescence sensitization of lanthanide ions (Ln^{3+}), especially Tb^{3+} and Eu^{3+}, provide their analytical applications by its role as fluorescent probes. Due to f-f electronic transitions of Ln^{3+}, the lanthanide complexes have several outstanding optical properties, including a large Stokes shift (>150 nm), sharp emission (<10 nm full wave at half maximum) and long fluorescence lifetimes (millisecond) [19]. So, fluorescent assays based on lanthanide complexes offer a significant advantage over traditional fluorescent methods in eliminating the background interference and scattering fluorescence through time-delayed detection. In recent two decades, lanthanide-based fluorescent probes have widely applied to ultrasensitive analysis of metal ions [20], anions [21] and various biological molecules [22-25]. Particularly, a terbium-based fluorescent detection system for the detection of biothiols in cells has been recently reported by Tan and coworkers [24]. The terbium-based fluorescent detection system not only exhibited high sensitivity and selectivity for biothiols, but also can eliminate efficiently the interference of background fluorescence from biological samples. In spite of this, the terbium-based fluorescent detection system was designed on the basis of the high affinity of Hg^{2+} and thiols [24]. The use of Hg^{2+} with high toxicity limits its further application. Very recently, a lanthanide complex-based ratiometric fluorescent probe for biothiols was reported by Dai and coworker, which showed good specificity and high sensitivity toward biothiols [25]. However, complicated and time-consumed laborious procedures are required for the preparation of the antenna ligand.

In this work, we attempt to present a simple and sensitive analytical method for the detection of biothiols by using a europium (Eu^{3+})-based complex as a fluorescent probe. The Eu^{3+} complex (EuTc-H_2O_2) was prepared by using tetracycline (Tc) as atenna molecule and H_2O_2 as a fluorescence enhancer. Tc is a molecule containing β-diketonate configuration, which can coordinate with Eu^{3+} and transfer its excitation energy to Eu^{3+} to sensitize the Eu^{3+} emission in a

process known as the antenna effect [26]. However, the EuTc complex shows very weak fluorescence due to the quench effect of water molecules. In the presence of hydrogen peroxide (H_2O_2), enhanced fluorescence of EuTc can be observed due to the replacement of water molecules on the Eu^{3+} coordination sphere by H_2O_2 [27], which results in the formation of EuTc-H_2O_2 complex. On the other hand, it is well accepted that H_2O_2 acts as a signaling molecule in biological system and can lead to thiol oxidation, in particular disulfide bond formation, and resulting in the changes of the functional properties of affected proteins [28, 29]. With the addition of biothiols, thus, the coordinated H_2O_2 molecules would oxidize the thiols to disulfide. Consequently, the fluorescence of EuTc-H_2O_2 complex is expected to be quenched (**Scheme 1**).

Scheme 1

Experimental section

Chemicals and reagents

Europium nitrate (99.99%) and homocysteine (Hcys) was purchased from Sigma-Aldrich (Shanghai, China); 4-morpholine propanesulfonic acid (MOPS) was purchased from Sangon Biotech Co., Ltd. (Shanghai, China); Tetracycline (Tc), amino acids (Met, Lys, Gly, Glu, Asp, Arg, Ala, Trp, Phe, His, Tyr, Cys), and reduced glutathione (GSH) were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China); N-ethyl maleimide (>98%, NEM) was from J&K Scientific Ltd; MOPS buffer (10 mM, pH 6.9) was prepared by dissolving 0.23 g of MOPS in 100 mL of ultrapure water; concentrated HCl was used to adjust pH to 6.9. The pH value was calibrated with a pH meter (Sartorius). Ultrapure water (18 MΩ cm) was used for the preparation of all aqueous solutions. Unless otherwise stated, all chemicals are of analytical reagent grade and used without further purification.

Measurement

LS55 luminescence spectrometer (PerkinElmer, UK) was used for the recording of fluorescence spetra. A delay time of 0.05 ms and a gate time of 2 ms were used to obtain the time-resolved fluorescence spectra. The 390 nm excitation wavelength was used for the emission spectra. UV-vis spectra were measured with a UV 2600 spectrophotometer (Shimadzu, Japan) at room

temperature. The light path of the quartz cuvette is 10 mm. All the experiments were performed at room temperature. All error bars represent standard deviations from three repeated experiments.

Determination of thiols in aqueous solution

To form EuTc complex, 4 mg of Tc and 9.6 mg of Eu^{3+} were first dissolved in 200 mL of MOPS buffer solution. Then, the solution was reacted for 15 min at room temperature. To measure biothiols concentration in aqueous solution, 90 μL of 100 mM of H_2O_2 was added to 600 μL of EuTc solution and mixed well. After reacting for 10 min, different volumes of biothiols in the range of 0 - 100 μM were added to the above solution, MOPS buffer was used to remain the final volume of the mixture to 3 mL. The reaction was performed for 10 min. Then, the fluorescent intensity of the mixture was measured. To investigate the effects of foreign substances, 60 μL of 10 mM amino acids, GSH, and Hcys were added to the EuTc-H_2O_2 solution, respectively, and MOPS was added till the total volume reached to 3 mL. After reacting for 10 min, the emission spectra of these mixtures were recorded. For the experiment of pH effect, the mixture containing 600 μL of EuTc solution, 90 μL of H_2O_2 (100 mM) and 90 μL of thiols (100 μM) was added to MOPS buffer pH from 2 to 12 and incubated for 10 min. Then, these mixtures were transferred into quartz cell and measured their fluorescent spectra, respectively.

Determination of thiols in urine samples

All experiments were performed in compliance with the relevant laws and institutional guidelines, and the urine samples were collected from a healthy volunteer with the prior approval of ethics committee of the Beijing University of Chemical Technology. Informed consent was obtained from the volunteer prior to collection. The original urine was dilute 100-folds and treated with N-ethyl maleimide for 2 h at 37°C to block the thiol groups in urine sample. Different amount of thiols standard solution were added to obtain spiked urine samples. The final thiols concentrations in spiked urine samples ranged from 0 to 20 μM. Then, 600 μL of EuTc solution, 90 μL of H_2O_2 (100 mM) was added to the spiked urine samples, and MOPS buffer was used to make up the final volume of the reaction solutions to 3 mL. After 10 min incubation, the fluorescent spectra of these reaction solutions were examined. The fluorescence intensity of the urine samples at 615 nm was recorded using an excitation wavelength of 390 nm.

Results and discussion

Fluorescence characterization of EuTc complex

The emission spectra of EuTc were recorded were recorded by using an excitation wavelength of 390 nm. As shown in **Fig. 1**, the fluorescence of EuTc itself was very weak. Upon the addition of H_2O_2, however, the fluorescence of EuTc was enhanced more than 15 folds, which is consistent with previous reports [27, 30, 31]. The enhanced fluorescence of EuTc can be attributed to the removal of water molecules from the Eu^{3+} coordination sphere through the coordination of H_2O_2 with EuTc, which leads to the formation of EuTc-H_2O_2 complex [27]. The H_2O_2-induced enhancement in fluorescent intensity of EuTc is time-dependent and the enhancement reaction needs 10 minutes to complete. When the H_2O_2 concentration is more than 3 mM, the fluorescent intensity of EuTc reached to a constant (**Fig. S1**). To obtain the best sensitivity, thus, a concentration of H_2O_2 at 3 mM and a 10 min reaction time were used in the subsequent experiments. By contrast, the enhanced fluorescence of EuTc by H_2O_2 decreased drastically after the addition of Cys. It is well known that the H_2O_2 is a strong oxidizer, which can not only be used as a bleach or cleaning agent in daily life, but also can oxidize the thiols to form disulfide bond in biological system. So, the quenched fluorescence may result from the oxidization reaction of biothiols caused by H_2O_2.

Figure 1

Quenching of EuTc-H_2O_2 complex by biothiols

To identify the quenched fluorescence was resulted from the interaction of H_2O_2 and biothiols; we investigated the fluorescence of EuTc in the presence of various biothiols species. As shown in **Fig. 2**, the fluorescent intensity of EuTc remained unchanged upon the addition of GSH, Cys and Hcys, even the concentrations of these biothiols were increased to 1 mM. The results indicate that biothiols had no influence on the fluorescence of EuTc and the quenching of the fluorescence of EuTc-H_2O_2 complex is attributed to the oxidization reaction of biothiols with H_2O_2. Moreover, we examined the absorption spectra of EuTc-H_2O_2 complex in the presence of biothiols. As displayed in **Fig. S2**, free Tc exhibits two maximum peaks at 272 and 357 nm, while only an absorption

peak at 390 nm was observed when the coordination of Tc with Eu^{3+} was occurred. After the addition of H_2O_2, there are no changes in the absorption spectrum of EuTc. Compared with EuTc, the absorption spectrum of EuTc in the presence of Cys is also unchanged except their difference in the absorption intensity. The results not only reflect that the addition of H_2O_2 and/or Cys has no influence on the chemical structure of Tc and coordination of Eu^{3+} with Tc, but also further confirms that the quenched fluorescence of EuTc-H_2O_2 complex is originated from the occurrence of oxidization reaction between H_2O_2 and biothiols.

Figure 2

Time response and Effect of pH

To understand the fluorescent response rate of EuTc-H_2O_2 complex to biothiols, the fluorescent intensity of EuTc-H_2O_2 complex was measured at different time intervals. As displayed in **Fig. S3**, fluorescent response rate of EuTc-H_2O_2 complex to biothiols is fast and the fluorescence of EuTc-H_2O_2 complex was quenched completely within 10 min. On the other hand, the effects of pH on the biothiols detection reaction based on EuTc-H_2O_2 complex were investigated. As shown in **Fig. 3**, after the addition of Cys, the highest fluorescent intensity of EuTc-H_2O_2 complex was observed at pH 6.9. When the pH value of the reaction media decreased from 6.9 to 2.0 or excess 6.9, however, a gradually decreased fluorescence of EuTc-H_2O_2 complex can be founded. The decease of the fluorescence of EuTc-H_2O_2 complex with the decrease of the pH value may be attributed to the protonation of the Tc in acid environment, whereas the fluorescence quenching behavior under strong basic condition might be ascribed to the formation of europium hydroxide precipitation [32]. Therefore, a reaction time of 10 min and the MOPS buffer at pH 6.9 were choose for the detection reaction of biothiols.

Figure 3

Detection sensitivity

To examine the detection sensitivity of EuTc-H_2O_2 complex for biothiols, different concentrations of biothiols were added to an aqueous solution of EuTc-H_2O_2 complex. **Figure 4** is the fluorescent

response of EuTc-H_2O_2 complex to Cys with different concentrations. With the increase of Cys concentrations, the fluorescent intensity of EuTc-H_2O_2 complex decreased gradually. There is a good linear correlation between the fluorescence intensity of EuTc-H_2O_2 complex and the concentration of Cys in the range of 1 - 20 μM. The detection limit is about 100 nM on the basis of a signal-to-noise ratio of 3:1 (**Fig. 4 inset**). Additionally, when GSH and Hcys were used to conduct the same experiments with EuTc-H_2O_2 complex, the quenched fluorescence EuTc-H_2O_2 complex also can be observed (**Fig. 5a**). And the EuTc-H_2O_2 complex as a fluorescent probe for GSH and Hcys displayed 200 nM and 400 nM detection limit, respectively (**Fig. 5b** and **5c**). The detection limits are lower than the intracellular concentrations of GSH (millimolar range) [33] and the levels of Cys and Hcy in plasma or urine (micromolar range) [34]. Compared with other fluorescent methods for biothiols detection[13, 17, 35, 36], the presented method not only showed a comparable detection limit, but also possessed a simpler and low-toxicity detection procedure, which does not require the participation of heavy metal ions. Moreover, the chemicals for the preparation of EuTc-H_2O_2 complex are low-cost and easy obtained from commercial source without the requirement of complicated laborious preparation procedure. Furthermore, the water-soluble EuTc-H_2O_2 complex has an excellent fluorescent stability in aqueous solution and can eliminate efficiently the background through time-resolved mode.

Figure 4

Figure 5

Detection selectivity for thiols

To evaluate the selectivity of the EuTc-H_2O_2 complex towards thiol-containing amino acids, we investigated the influence of other natural nonthiol amino acids on the fluorescence of the EuTc-H_2O_2 complex. In this study, the amino acid with different chemical structures, such as Met, Lys, Gly, Glu, Asp, Arg, Ala, Trp, Phe, His, Tyr, Cys, were employed as potential interferences to evaluate the selectivity of EuTc-H_2O_2 complex as a fluorescent probe for thiol-containing amino acids. The reactions of the EuTc-H_2O_2 complex with these nonthiol amino acids were performed in MOPS buffer of pH 6.9. As shown in **Fig. 6**, only thiol-containing amino acids can result in a

significant decrease in the fluorescence of EuTc-H_2O_2 complex, and no remarkable changes in the fluorescence of EuTc-H_2O_2 complex were observed upon the addition of other nonthiol amino acids. This indicates that the EuTc-H_2O_2 complex as a fluorescent probe possesses a high specificity for thiol-containing amino acids detection. To further assess its utility as a thiol-selective probe, moreover, the EuTc-H_2O_2 complex was subjected to the interfering study by investigating its fluorescence response to Cys in the presence of typical nonthiol amino acids. The results showed that all of the nonthiol amino acids have little or no interference in the detection of Cys (**Fig. S4**), which reflects that the coexistence of nonthiol amino acids does not affect the fluorescent response of the EuTc-H_2O_2 complex to Cys. This may due to that these thiol-free amino acids cannot remove efficiently coordinated H_2O_2 in spite of the fact that they could bind with Eu^{3+} through their carboxyl groups. Therefore, the EuTc-H_2O_2 complex appears to be useful for selectively sensing thiol-containing amino acids at a physiological pH value.

Figure 6

It is known that the components of urine are complicated. Except amino acids, the substances including glucose, common metal ions and anions also exist in urine. The existence of these foreign substances could affect the fluorescence of EuTc complex by either reacting with Tc [37] or coordinating with Eu^{3+} [27]. So, the effects of these foreign substances, such as glucose, metal ions (Na^+, K^+, Ca^{2+}, Mg^{2+}), and anions (NO_3^- and Cl^-), on the fluorescence of EuTc-H_2O_2 complex were studied to detect the levels of thiols in urine by employing the EuTc-H_2O_2 complex as a fluorescent probe. As shown in **Fig S5**, no changes in the fluorescence of EuTc-H_2O_2 complex were observed in the presence of these foreign substances. This result indicates the EuTc-H_2O_2 complex can be used as a fluorescent probe to detection thiols in urine samples.

Detection thiols in urine samples

In biological system, thiols exist in many forms in biological system, such as protein-bound, oxidized and reduced. Nevertheless, a total levels of thiols was usually measured in most clinical tests due to thiol groups are easily oxidized. Thus, we measure the concentrations of total thiols in urine by employing the EuTc-H_2O_2 complex as a fluorescent probe. The urine samples used in this

work were collected from a healthy volunteer. The analyzed urine samples containing different concentrations of biothiols were made by adding different amounts of standard Cys. The results of EuTc-H_2O_2 complex as a fluorescent probe for detection thiols in urine were presented in **Table 1**. The recoveries of biothiols in urine samples were between 97.07 % and 101.2 %. The relative standard deviations (RSD, n = 3) are all less than 8.43 %. The results indicate that EuTc-H_2O_2 complex as a fluorescent probe for the detection of biothiols in urine samples showed good recovery and precision.

Table 1

Conclusion

In summary, we have developed a simple and sensitive analytical method for biothiols by using a Eu^{3+}-based fluorescent probe. The EuTc-H_2O_2 complex emits strong fluorescence due to the sensitization effect of Tc and the enhancement effect of H_2O_2. Upon the addition of biothiols, however, quenched fluorescence of EuTc-H_2O_2 complex can be observed, which is ascribed to the formation of disulfide bond caused by the oxidization of H_2O_2. The fluorescent intensity of EuTc-H_2O_2 complex was decreased linearly with the biothiols (Cys) concentration from 1 to 14 μM. The detection limits for Cys, GSH and Hcys in aqueous solution are 100 nM, 200 nM and 400 nM, respectively. The presented fluorescent probe for biothiols exhibits the advantages of simple and low-toxicity detection procedure, excellent fluorescent stability, and the ability of eliminating background fluorescence. The total levels of biothiols in urine samples can be measured accurately by using the EuTc-H_2O_2 complex as a fluorescent probe. We believe that the appearance of the detection method based on the Eu^{3+} fluorescence may provide an alternative method for monitoring the levels of biothiols in biological system.

Acknowledgement

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Figure captions

Scheme 1 Illustration of EuTc complex for detection of thiols.

Figure 1. Fluorescent spectra of EuTc (a), EuTc-H_2O_2 (b) and EuTc-H_2O_2 in the presence of 14 μM of Cys (c).

Figure 2. The effects of GSH, Hcys and Cys with different concentrations on the fluorescent intensity of EuTc at 615 nm.

Figure 3. The effects of pH of MOPS on the fluorescent intensity of EuTc at 615 nm.

Figure 4. Fluorescence spectra of EuTc-H_2O_2 complex in the presence of different concentrations of Cys. The inset is the linear relationship between the fluorescent intensity of EuTc-H_2O_2 complex and Cys concentration.

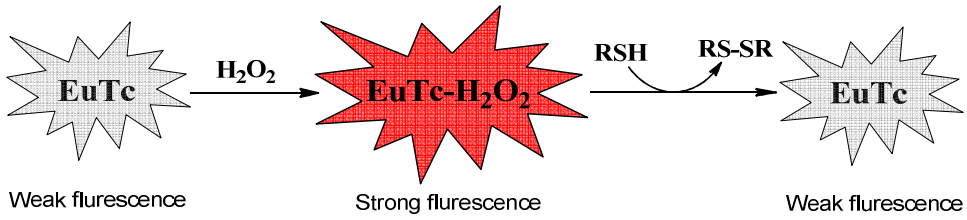
Figure 5. Fluorescence spectra of EuTc-H_2O_2 complex alone and in the presence of GSH and Hcys (a); The linear relationship between the fluorescent intensity of EuTc-H_2O_2 and GSH (b) and Hcys (c) concentration.

Figure 6. Fluorescent responses of EuTc-H_2O_2 to biothiols (Cys, GSH and Hcys, 20 μM) and various amino acids at concentrations of 20 μM.

Table 1. Determination of total biothiols in urine samples.

Figures and Schemes

Scheme 1



Tetracycline (Tc)

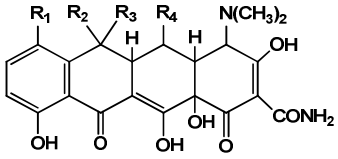


Figure 1

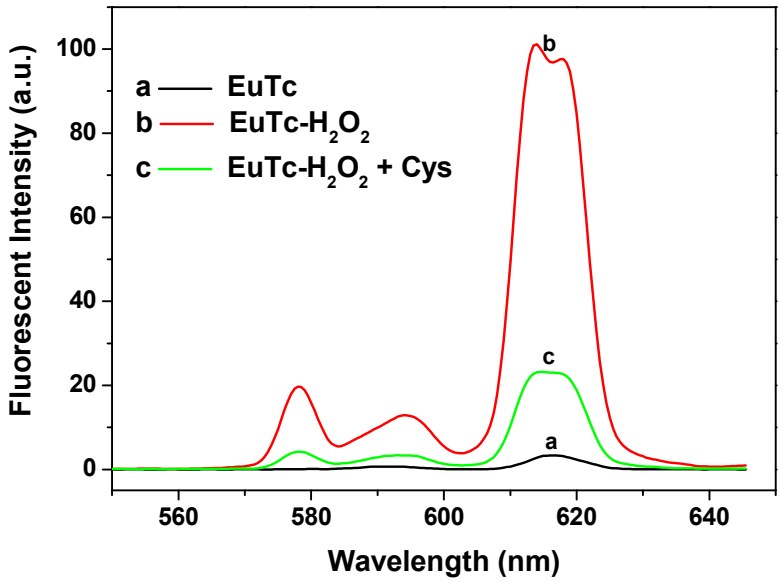


Figure 2

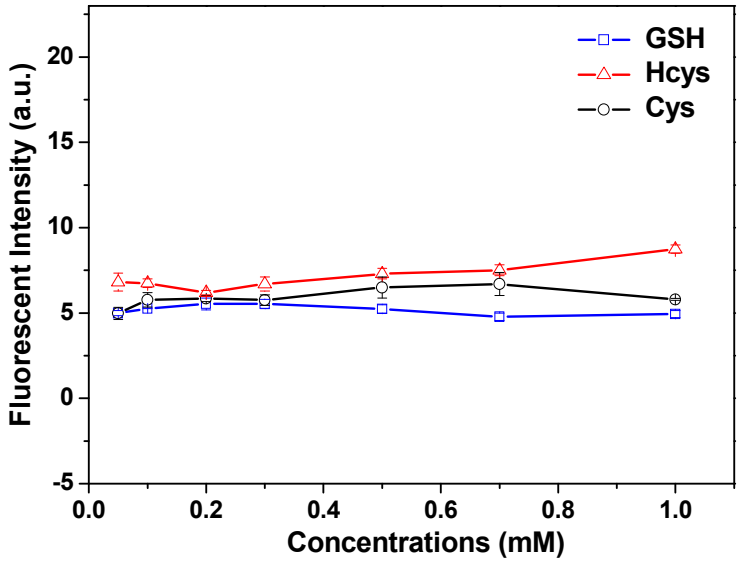


Figure 3

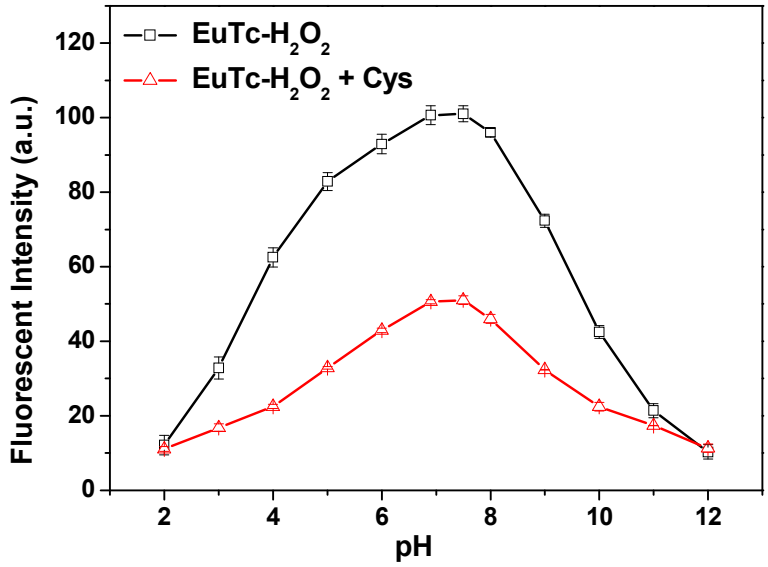


Figure 4

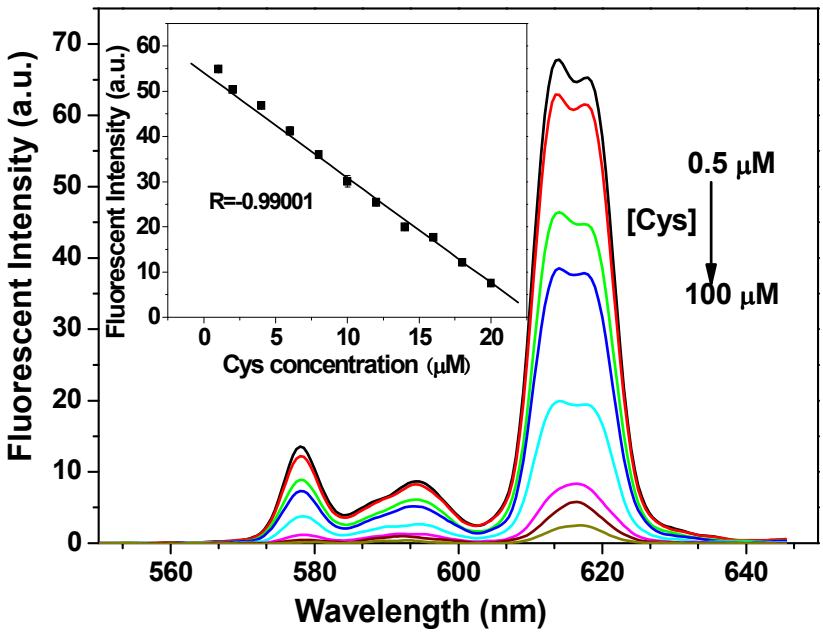


Figure 5

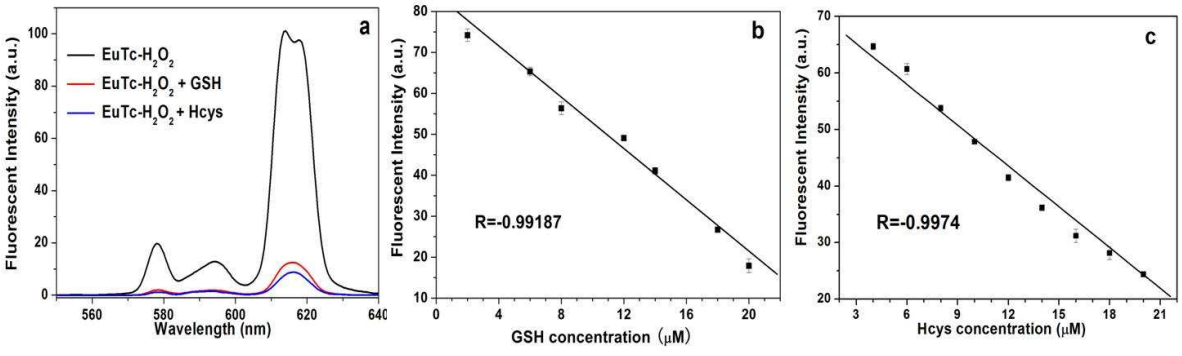


Figure 6

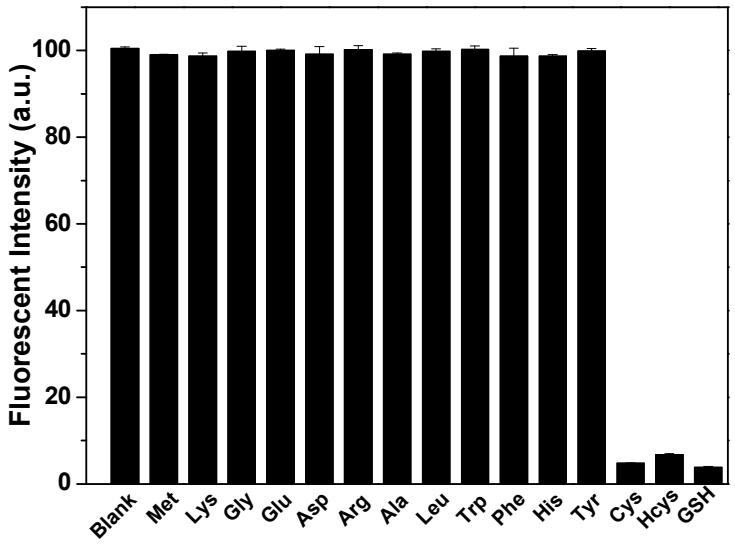


Table 1

Samples	Added (μM)	Founded (μM)	Recovery (%)	RSD (n=3, %)
Urine - 1	1	1.102 ± 0.13	101.20	11.78
Urine - 2	3	2.912 ± 0.26	97.07	8.92
Urine - 3	5	4.985 ± 0.42	99.70	8.43

RSD: Relative standard deviation.