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A graphene quantum dot-based method for the highly sensitive and selective fluorescence turn on detection of biothiols

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ABSTRACT

A facile assay method for the highly sensitive and selective sensing of biothiols based on graphene quantum dots (GQDs) has been developed. GQDs emitted strong blue fluorescence in an aqueous buffer solution. It was observed that mercury(II) ions could efficiently bind and quench the fluorescence of the GQDs. When a biothiol compound (glutathione, cysteine, or homocysteine) was added to the assay mixture of GQDs and mercury(II), it bound to mercury(II) ions. Hg^{2+}–GQD complex dissociated, and a fluorescence turn-on signal was detected. The emission intensity changes of the GQDs could be directly related to the amount of biothiol added to the assay solution. The assay is highly sensitive, the limits of detection (LOD) for GSH, Cys and Hcy were 5 nM, 2.5 nM and 5 nM, respectively. The assay is also highly selective, a number of amino acids and proteins were tested, and little interference was observed. In addition, GSH standard recovery in serum samples was also demonstrated. We envision that our assay method could facilitate the biothiol quantification related biological and biomedical research.

1. Introduction

Biothiol compounds such as glutathione, cysteine, and homocysteine play important roles in maintaining proper body functions. Glutathione (GSH) is a thiol-containing tripeptide (γ-glutamyl–cysteinyl–glycine). GSH is widely distributed in almost every cell [1]. It helps to maintain the normal functions of the immune system, and it is an essential endogenous antioxidant which has often been used against toxins and free radicals [2]. Abnormal GSH levels have been used for disease diagnosis and clinical therapies [3,4]. Cysteine (Cys) is one of the twenty amino acids required for protein biosynthesis. It is also a precursor required for the biosynthesis of GSH. The thiol functional group of Cys could be easily oxidized to give a disulfide bridge, which plays essential structural roles in many proteins [5]. Elevated levels of Cys have been associated with neurotoxicity [6]. Homocysteine (Hcy) is an amino acid that differs from cysteine by an additional methylene bridge (–CH_2–). The intracellular concentration of Hcy under normal conditions is kept low [7]. Elevated levels of Hcy are associated with Alzheimer's disease, cardiovascular diseases, and osteoporosis [8]. Therefore, quantitative detection of biothiol compounds is of great importance for human health.

Various biothiol sensing methods have been developed over the years, such as the electrochemical [9], colorimetric [10], fluorometric [11], high-performance liquid chromatography (HPLC) [12], surface-enhanced Raman scattering (SERS) [13], and mass spectrometry [14] techniques. And a number of novel biothiol sensing techniques have been developed in recent years based on the rationally designed small molecular probes [15], molecular beacon mediated fluorescence resonance energy transfer (FRET) [16], or advanced materials such as upconversion nanoparticles, quantum dots, nanoclusters, gold nanoparticles, etc. [17]. However, some of these methods offer low detection sensitivity, employ heavy instrumentation, complicated assay procedures, expensive or somewhat toxic materials. In addition, fluorophore labeling and the synthesis of organic fluorescent probes are technically demanding, time consuming, and expensive, and many of the organic dye molecules could be easily photobleached. GQDs have also been combined with silver nanoparticles for biothiol sensing. However, a fluorescence turn-off detection mode was used, which could increase considerably the likelihood of false positive signals [18]. Therefore, the development of a new sensitive and selective fluorescence turn on biothiol sensing technique is of great potential practical value.

Graphene is a new kind of carbon based material that draws increasing attentions in recent years [19]. It consists of a two dimensional network of sp^2 hybridized carbon atoms, with a number of unique properties, such as high surface area, good electronic conductivity, and superior mechanical stability. Graphene has found widespread applications in many diverse fields in

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physics, chemistry, material science, and biology. It has been shown that when a graphene sheet is small enough, its properties can vary significantly. At a size of smaller than 100 nm, a new kind of material called graphene quantum dots (GQDs) is produced [20]. GQDs usually contain one or two layers of graphene with a size distribution mainly in the range of 3–20 nm [21]. GQDs are highly luminescent as a result of the quantum confinement and edge effects [22]. The amounts of oxygen-containing groups, structural defects, and doping elements also contribute considerably to the luminescence properties of the GQDs [23]. They have higher surface area, larger diameter, and better surface grafting properties compared with the conventional quantum dots (QDs) and carbon dots (CDs) [24]. In addition, GQDs show excellent water solubility, low toxicity, high stability, and good biocompatibility. They are considered to be a promising material to replace the commonly used semiconductor nanocrystals for a number of biosensing and bioimaging related applications [25].

Herein, we report a GQD-based method for the highly sensitive and selective sensing of biothiols. GQDs show strong blue photoluminescence in an aqueous buffer solution. It was observed that Hg^{2+} could significantly quench the fluorescence of GQDs. When a biothiol compound (GSH, Cys, or Hcy) was added to the assay solution, it could selectively bond to Hg^{2+} through Hg–S bonding interactions. As a result, the Hg^{2+}–GQD complex dissociated, and a turn on fluorescence signal was detected. The intensity enhancement of the GQDs could be directly related to the amount of biothiol added to the assay solution.

2. Experimental

2.1. Materials

Citric acid, glycine, glutamic acid, phenylalanine, alanine, and histidine were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Mercuric nitrate was purchased from Taixing Chemical Reagent Co., Ltd. (Taixing, Jiangsu, China). Sodium hydroxide was obtained from Beijing Chemical Works (Beijing, China). Glutathione and cytochrome *c* were obtained from Sangon Biotechnology Co., Ltd. (Shanghai, China). Cysteine and bovine serum albumin were obtained from Bio Basic Inc. (Markham, Ontario, Canada). Homocysteine and DTNB were obtained from Aladdin Chemistry Co., Ltd. (Shanghai, China). Cystine, trypsin, and lysozyme were obtained from Beijing Dingguo Biotechnology Co., Ltd. (Beijing, China). All other reagents were of analytical grade and used as received. Milli-Q ultrapure water was used in all experiments.

2.2. Instrumentation

UV–vis absorption spectra were obtained with a Cary 50 Bio spectrophotometer (Varian Inc., CA and USA). Emission spectra were recorded using a Fluoromax-4 spectrofluorometer (Horiba Jobin Yvon Inc., USA), with an excitation wavelength of 362 nm. Excitation and emission slit widths were 3 nm. Quartz cuvettes with 10-mm path length and 2-mm window width were used for the UV–vis and emission measurements. Transmission electron microscopy (TEM) images were obtained using a JEOL 1200 electron microscope operating at an accelerating voltage of 200 kV (JEOL Ltd., Japan). Atomic force microscope (AFM) was used to characterize the height of the GQD samples in the tapping mode (SPA-300HV with a SPI3800N Controller, Seiko Instruments Inc., Japan). A silicon microcantilever was used for the scanning (spring constant 2 N m^{-1}, resonant frequency ≈ 70 kHz, Olympus, Japan).

Table 1
Determination of biothiol content in diluted serum samples.

Determined biothiol/nM	Added GSH/nM	Measured GSH[a]/nM	Recovery[a] (%)	RSD (%, $n=3$)
19.8	10	10.99	109.94	3.36
	20	19.45	97.25	2.39

[a] Mean value of three independent measurements.

2.3. Synthesis of the GQDs

GQDs were prepared by the citric acid pyrolysis method following a reported literature procedure [26]. Briefly, 0.5 g of citric acid was put into a 5 mL beaker. The beaker was heated to 260 °C using a heating jacket for about 40 min. Citric acid was liquated and its color changed to orange. The liquid was transferred into a beaker containing 12.5 mL of NaOH aqueous solution (10 mg mL^{-1}) with continuous stirring. The sample solution was neutralized to pH 7.0 with NaOH, and the GQDs stock solution was stored at 4 °C before use.

2.4. Assay procedures

4 μL of GQDs (2.1 mg mL^{-1}) was mixed with 384 μL of water and 8 μL of Hg^{2+} (2 mM) in 10 mM phosphate buffer (pH 7.4). The sample solution was incubated at room temperature for 15 min. 4 μL of the biothiol compound (GSH, Cys, or Hcy) of various concentrations was added to the sample solution, and the emission spectra of the sample mixtures were recorded.

2.5. GSH recovery assay in serum samples

The detection procedures were similar to those described above for the biothiol assay, except that diluted calf serum (4 μL) was added to the assay mixture. Literature reports have shown that serum samples contained high concentrations of biothils [30], thus they had to be properly diluted before the analysis. The diluted serum stock solution was prepared by the addition of 0.1 mL of fresh serum into 49.9 mL of phosphate buffer (10 mM, pH 7.4), and mixed. The stock serum sample solution was stocked at 4 °C before use. The assay solutions were spiked with known concentrations of GSH following the standard addition method, the total quantities of GSH recovery, the recovery rate, and the standard deviation values were obtained (Table 1).

3. Results and discussion

3.1. Preparation and characterization of GQDs

The GQDs used in our experiments were prepared according to a pyrolysis method [26]. Citric acid was used as the starting material. It was a simple and efficient “bottom-up” approach to fabricate the GQDs. Transmission electron microscopy (TEM) study shows that the average diameter of the GQDs is about 9 nm (Fig. S1, Supporting Information). Atomic force microscopy (AFM) study shows that the GQDs are mostly single or bilayered with an average height of about 2 nm (Fig. S2). The UV–vis absorption and emission spectra of the GQDs are shown in Fig. S3. The GQDs show a well-defined absorption band centered at 362 nm with a narrow peak width at half height of 64 nm. The results suggest that the sp^2 clusters contained in the GQDs are mostly uniform in size [26].

3.2. The quenching effect of metal ions on the fluorescence of GQDs

We tested a number of metal ions. They were Mg^{2+}, K^{+}, Hg^{2+}, Ca^{2+}, Al^{3+}, Zn^{2+}, Fe^{3+}, Fe^{2+}, Mn^{2+}, Cd^{2+}, Co^{2+}, Ni^{2+}, Ba^{2+}, Pb^{2+}, Cu^{2+} and Ag^{+} ions. Fig. 1 shows that Hg^{2+} could quench the fluorescence of the GQDs very strongly. Fe^{3+} and Fe^{2+} ions have a slight quenching effect. Other ions show little quenching of the fluorescence of the GQDs. The high selectivity may be ascribed to the fact that Hg^{2+} ions have a stronger affinity towards the carboxylic groups on the GQDs surface than other metal ions as reported in the literature [27]. The selective quenching is presumably via electron or energy transfer from the GQDs to Hg^{2+} ions as suggested in the literature reports [28]. It is interesting to note that Fe^{3+} ions were previously reported to be able to selectively quench the fluorescence of GQDs@GSH, and Hg^{2+} ions did not exhibit any quenching effect [28]. The GQDs used in our experiments were prepared via a citric acid pyrolysis reaction, whereas the GQDs@GSH were prepared via a pyrolysis reaction using citric acid and glutathione. XPS measurements show that the GQDs@GSH contained 4.9% N and 1.51% S [28]. These doping elements may affect the structure and the emission properties of the GQDs significantly. It seems therefore not surprising that these two different GQDs show different response toward Hg^{2+} and Fe^{3+} ions.

The Hg^{2+} induced GQDs fluorescence quenching was studied in details. Fig. 2 shows that upon the addition of increasing concentrations of Hg^{2+}, emission intensity of the GQDs gradually decreased. A linear relationship was obtained when the maximum intensity of the GQDs at 460 nm was plotted against the Hg^{2+} concentration in the range of 0–70 μM. At 70 μM Hg^{2+} concentration, the fluorescence of the GQDs was efficiently quenched (with a quenching efficiency of about 93%).

The overall assay strategy is shown in Scheme 1. GQDs showed strong blue fluorescence in an aqueous buffer solution. When Hg^{2+} was added, it bound to GQDs and strong fluorescence quenching was observed. Upon the addition of a biothiol compound, the thiol functional group bound to Hg^{2+} through Hg–S bonding interactions. The Hg^{2+}–GQD complex dissociated, and restoration of the blue fluorescence of the GQDs was observed, which could be used to quantify the biothiol compound.

3.3. Optimization of experimental conditions

To get the best sensing response, the amount of Hg^{2+} added to the assay solution needed to be optimized. At a fixed GSH concentration of 5 μM, the I/I_0 value gradually increased when Hg^{2+} concentration was increased from 10 μM to 40 μM (Fig. 3). The maximum I/I_0 value was obtained at 40 μM Hg^{2+} concentration. Further increase of the Hg^{2+} concentration caused decreased I/I_0 value. Since binding of Hg^{2+} ions to the GQDs is an equilibrium process, there was always certain amount of free Hg^{2+} ions in the assay solution. The free Hg^{2+} ion concentration increased at higher total Hg^{2+} concentrations added to the assay solution, especially at near the saturation point (~70 μM Hg^{2+}, Fig. 2). The results suggest that at total Hg^{2+} concentration higher than 40 μM, there were too much free Hg^{2+} ions in the assay solution. The free Hg^{2+} ions would bind to GSH, thus much reduced GQDs emission intensity changes were observed. 40 μM of Hg^{2+} was therefore used for the biothiol sensing experiments. The buffer pH value of the assay solution was also optimized. Fig. 4 shows that at a buffer pH value of 7.4, maximum emission recovery of the GQDs was obtained.

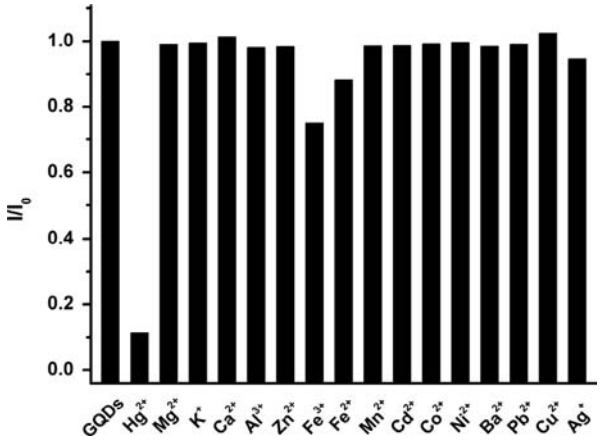


Fig. 1. Quenching of the fluorescence of the GQDs by different metal ions. All ions were at 100 μM concentration. I and I_0 were the emission intensities of the GQDs at 460 nm in the presence and absence of the metal ions. Conditions: 10 mM phosphate buffer (pH 7.4).

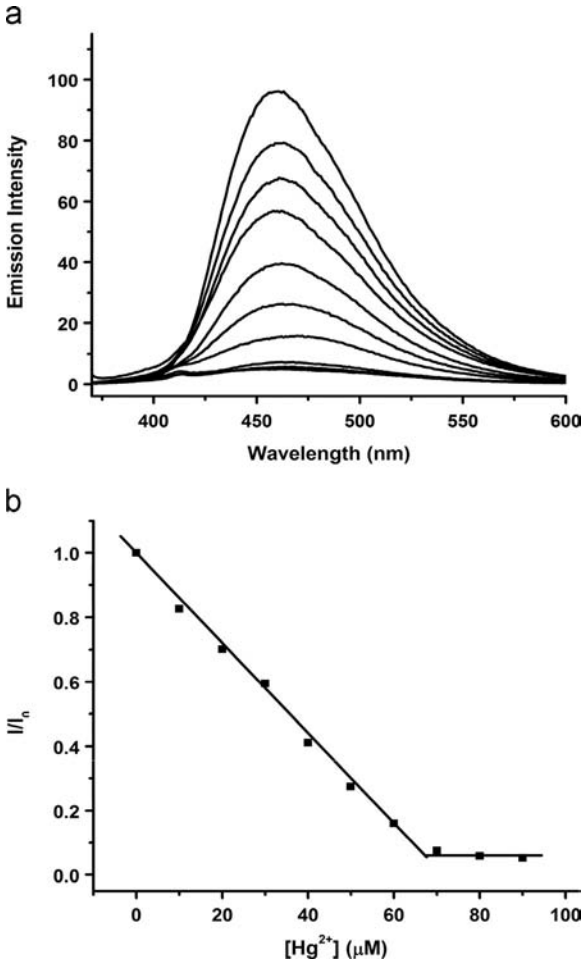
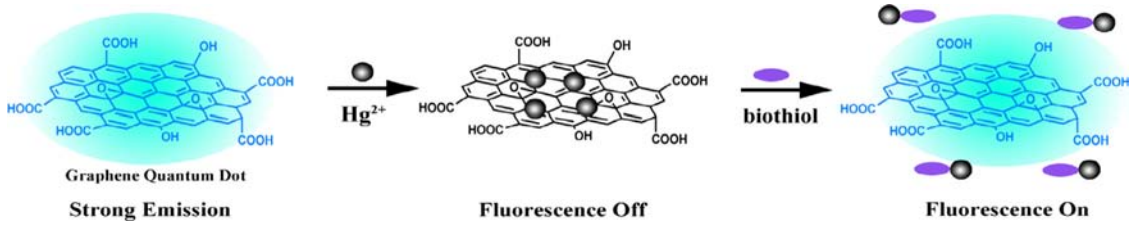


Fig. 2. (a): Changes in emission spectra of the GQDs at different Hg^{2+} concentrations (0–90 μM). (b): Changes in emission intensity at 460 nm versus Hg^{2+} concentration. Conditions: 10 mM phosphate buffer (pH 7.4).

3.4. Detection of biothiols

Our results show that under the optimized assay conditions, with the increase of the assay solution GSH concentration, a gradual recovery of the GQDs emission was observed. The results suggest that when GSH was added to the assay solution, it competed with the GQDs for the binding of the Hg^{2+} ions. Depending on the amount of GSH added to the assay solution, certain amount of Hg^{2+} ions were removed from the surface of the



Scheme 1. Schematic illustration of the mechanism of the graphene quantum dot-based biothiol sensing.

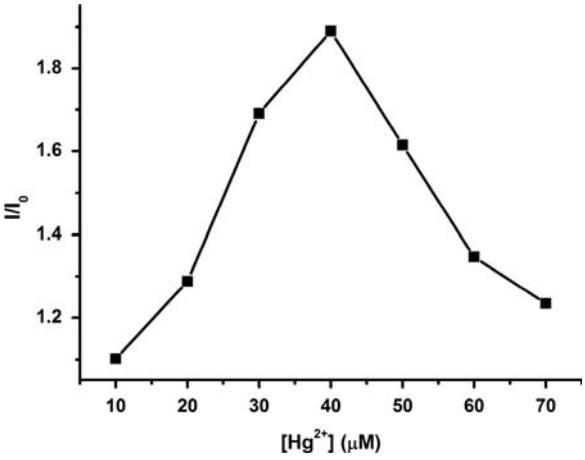


Fig. 3. Changes in the I/I_0 value as a function of the Hg^{2+} concentration. Conditions: 5 μM GSH, 10 mM phosphate buffer (pH 7.4).

GQDs, thus fluorescence recovery of the GQDs was observed. The I/I_0 value was plotted against the GSH concentration, and a linear relationship was observed in the range of 0–50 nM GSH [Fig. 5, S4-(a)]. The linear regression equation is $Y=0.0046C+1.0012$ (correlation coefficient $R^2=0.992$), where "Y" is the I/I_0 value and "C" is the concentration of GSH in nM. When Cys and Hcy were used instead of GSH, similar results were obtained (Figs S4 and S5). Our assay is very sensitive. The detection limits for GSH, Cys and Hcy were 5, 2.5, and 5 nM respectively, which are among the best biothiol sensing methods reported in recent years (Table S1, Supporting information). [9–18,29] The linear detection could be further expanded at higher biothiol concentrations (Fig. S6).

3.5. Selectivity

The selectivity of the assay was studied. Five common amino acids (histidine, glycine, phenylalanine, alanine, and glutamine), one disulfide bond containing amino acid (cystine), and four proteins (bovine serum albumin, trypsin, lysozyme, and cytochrome *c*) were tested. Fig. 6 shows that none of these amino acids and proteins gave noticeable interferences, only the free thiol containing biomolecules caused significant recovery of the fluorescence of the GQDs.

3.6. Determination of biothiols in serum samples

Our assay could be used to determine the biothiol content in complex sample mixtures (diluted serum samples) (Table 1). In addition, known amounts of GSH were added to the diluted serum samples, and satisfactory GSH recovery values were obtained, with the RSD values of less than 4%. Literature reports have shown that GSH and Cys concentrations in serum are much higher than Hcy [30]. And since the linear regression equations for GSH and Cys are almost identical [Fig. 5 and S5-(a)], the biothiol content in serum could therefore be estimated as the sum of GSH and Cys. The

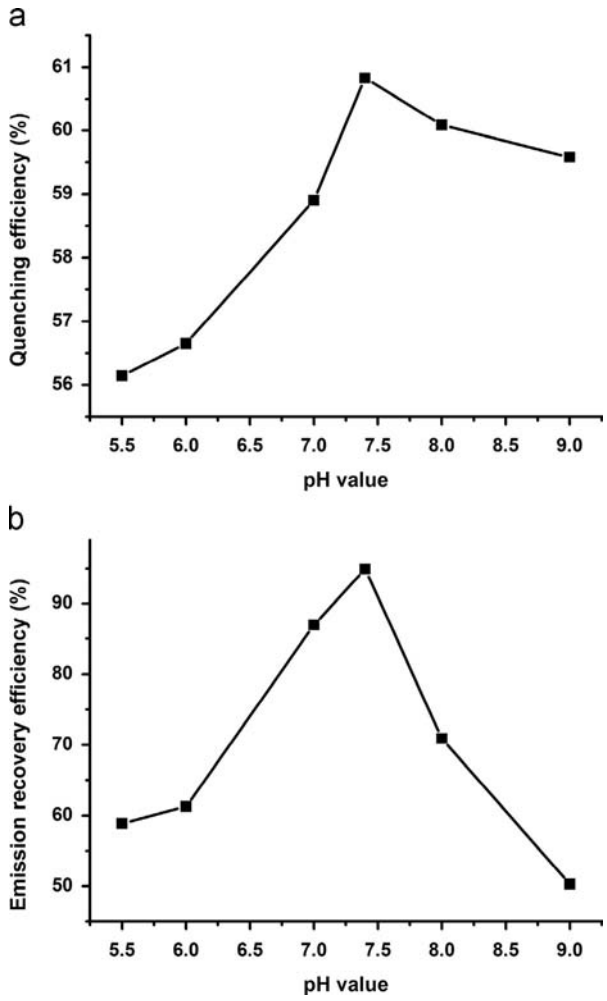


Fig. 4. (a): Changes in quenching efficiency of the GQDs in the presence of 40 μM Hg^{2+} at different buffer pH values. (b): Changes in emission recovery efficiency $[(I-I_0)/I_0]$ of the GQDs in the presence of 5 μM GSH at different buffer pH values.

results clearly show that our assay could be used for the detection of biothiols in complex assay mixtures. Ellman's reagent [5,5'-dithiobis-(2-nitrobenzoic acid) or DTNB] was also used to quantify the biothiol content in the serum samples [31], and consistent results were obtained (Table S2).

4. Conclusions

In summary, a novel GQD-based assay for the facile fluorescence turn on detection of biothiols with high sensitivity and selectivity has been developed. The sensing strategy rooted in the selective quenching of the fluorescence of the GQDs by Hg^{2+} ions, the dissociation of the Hg^{2+}–GQD complex, and the restoration of the GQDs fluorescence upon the addition of the biothiol

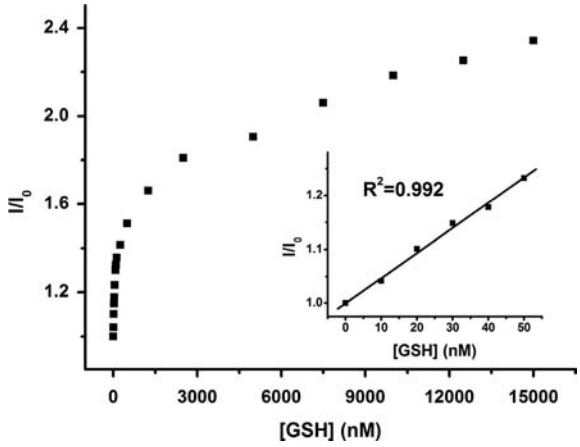


Fig. 5. Changes in I/I_0 value as a function of the GSH concentration. Conditions: 40 μM Hg^{2+}, 10 mM phosphate buffer (pH 7.4).

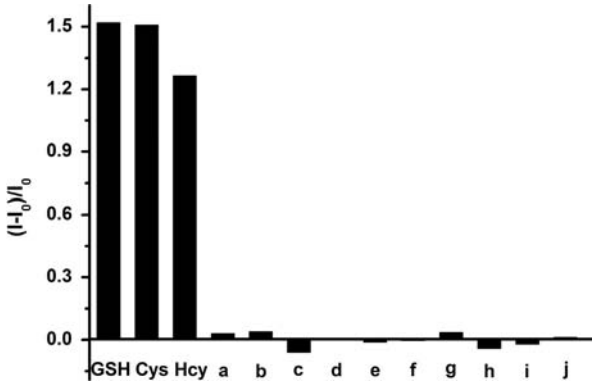


Fig. 6. Selectivity study. Columns a–j: histidine, glycine, phenylalanine, alanine, glutamine, cystine, bovine serum albumin, trypsin, lysozyme, and cytochrome *c*, respectively. Conditions: GSH, Cys, Hcy, and the other amino acids each at 20 μM concentration, proteins each at 200 nM concentration; Hg^{2+} concentration: 40 μM; buffer: 10 mM phosphate buffer (pH 7.4).

compounds. Our assay has several important features. First, a fluorescence "turn-on" detection could avoid the possible false-positive signals associated with the "turn-off" mode. Second, the assay is simple in design and offers a convenient approach for rapid biothiol detection. Third, the assay is highly sensitive, the limits of detection of 5 nM GSH, 2.5 nM Cys and 5 nM Hcy could be easily achieved. Fourth, the assay is also very selective, a number of amino acids and proteins were tested, and little interference was observed. Fifth, all materials used are inexpensive, and no further chemical modification of the GQDs is required, thus the assay is fairly cost effective. We envision our assay could be used for the biothiol sensing related biological applications.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2013.11.065.

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