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Fluorimetric evaluation of glutathione reductase activity and its inhibitors using carbon quantum dots

Shuyun Zhu[a,*], Lei He[a], Fang Zhang[a], Mengyuan Li[a], Shulin Jiao[a], Yan Li[a], Mengyuan Chen[a], Xian-En Zhao[a,b,*], Hua Wang[a,*]

[a] Shandong Provincial Key Laboratory of Life-Organic Analysis, College of Chemistry and Chemical Engineering, Qufu Normal University, Qufu, Shandong 273165, China

[b] Key Laboratory of Tibetan Medicine Research, Northwest Institute of Plateau Biology, University of Chinese Academy of Sciences, Xining, Qinghai 810001, China

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ABSTRACT

A highly sensitive fluorimetric assay has been developed for the evaluation of glutathione reductase (GR) activity and the screening of its inhibitors by using carbon quantum dots (CQDs) as the signal reporter. The detection mechanism is based on the following facts: (1) the fluorescence of CQDs can be quenched by Hg(II) through the strong CQDs-Hg(II) coordination; (2) GR can catalyze the reduction of oxidized glutathione (GSSG) into reduced glutathione (GSH), so that the fluorescence recovery of CQDs would take place resulting from the strong GSH-Hg(II) interaction; (3) GR can lose its catalytic reduction of GSSG in the presence of its inhibitors, which will inhibit the recovery of the quenched fluorescence of CQDs. The developed CQDs-based fluorimetric method can facilitate the sensitive evaluation of GR activity in the range of 0.10–2.0 mU mL^{-1} with a detection limit of 0.050 mU mL^{-1}. In addition, other kinds of enzymes like myoglobin, thrombin, alcohol dehyrogenase, amylase, pepsin, and trypsin could show no significant effects on the evaluation of GR activity. This work may expand the biological applications of CQDs as the fluorescent probes with low cost, easy preparation, and high photostability.

1. Introduction

Glutathione reductase (GR) is an important enzyme that can catalyze the reduction of oxidized glutathione (GSSG) to reduced glutathione (GSH) with the help of β-nicotinamide adenine dinucleotide 2′-phophate hydrate (NADPH), a reduced tetrasodium salt of coenzyme II [1]. The GSH production plays a central role in buffering an intercellular redox condition and in activating the anti-oxidation system [2]. Thus, GR is known to play a key role in the response to oxidative stress and has been linked to several other diseases and conditions, including in the genesis of anxiety [3]. Also, a significant enhancement of GR activity can indicate the possible presence of malignant tumor cells [4]. Particularly, from a pharmaceutical perspective, GR inhibitors are shown to possess the anticancer and antimalarial activity per se [5]. Therefore, there exists an increasing need for the sensitive and selective determination of GR activity and the screening of its inhibitors.

Moreover, the classic spectrophotometric methods for the determination of GR activity are established based on the absorbance changes of NADPH [6,7], which are mostly insensitive and vulnerable. Alternatively, the fluorimetric analysis methods have been well recognized to present the higher sensitivity in monitoring GR activities but depending on the performances of the fluorescent probes to be chosen. As a result, many fluorogenic probes have been synthesized and applied for monitoring GR activity including organic fluorescent dye [8], anionic conjugated polyelectrolyte [9], CdS quantum dots (QDs) [10], DNA templated silver nanoclusters [11] and DNA bio-dots [12]. However, most of these fluorophores might suffer from some drawbacks, such as poor photostability and solubility in water for dyes, laborious synthesis procedure for fluorescent polyelectrolyte and CdS QDs, and high cost for metal nanoclusters and DNA bio-dots. Therefore, it is of great interest to develop new fluorescent probes for the determination of GR activity.

Carbon quantum dots (CQDs) are a new kind of fluorescent nanomaterials, which were first obtained during the purification of single-walled carbon nanotubes in 2004 [13]. Thereafter, CQDs have emerged as rising-star nanomaterials due to their great advantages over most of those conventional fluorescent probes, such as low toxicity, good aqueous solubility, easy preparation, low cost, environmental friendliness, and high photostability [14–17]. Also, CQDs are

* Corresponding authors.
E-mail addresses: shuyunzhu1981@163.com (S. Zhu), xianenzhao@163.com (X.-E. Zhao), huawangqfnu@126.com (H. Wang).

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expected as a promising biocompatible nanomaterial for the design of new fluorescent biosensors. Up to now, significant progresses have been achieved in the applications of CQDs. For example, CQDs have been applied to the detection of heavy metal ions [18–20], anions [21], small biomolecules [22–24], and DNA [25]. However, few researchers reported the applications of CQDs in the evaluation of enzyme activity. Feng's group employed CQDs to develop a real-time fluorescence assay for probing the catalysis activity of alkaline phosphatase as well as acid phosphatase [26–28]. Qian et al. developed a fluorometric assay for tyrosinase activity with CQDs [29]. Recently, a fluorometric assay for acetylcholinesterase activity and inhibitor screening has also been proposed using CQDs [30]. Liu et al. developed a surface energy transfer-based biosensor using amino-functionalized carbon dots and hyaluronate-stabilized gold nanoparticles [31]. To the best of our knowledge, CQDs have hardly been reported for the fluorescent determination of GR activity including the screening of its inhibitors.

Herein, we have developed a sensitive fluorescent detection assay for probing the GR activity based on the recovered fluorescence intensity of the CQDs-Hg(II) system. It has been established that the fluorescence intensity of CQDs can be quenched efficiently by Hg(II) due to their coordination with CQDs and then recovered gradually by the addition of GSH because of its stronger affinities with Hg(II) [23]. Moreover, GR can catalyze the reduction of GSSG to GSH in the presence of NADPH. By taking the advantages of these unique features, a CQDs-based fluorescent biosensor has been proposed for the first time for evaluating the GR activity (Scheme 1). This developed assay is not only simple and rapid avoiding the intricate preparation and surface modification but also can allow for the screening of the inhibitors of GR..

2. Experimental

2.1. Materials and reagents

Glutathione reductase (GR) from baker's yeast, reduced glutathione (GSH, 98%), oxidized glutathione (GSSG, 98%), and β-nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt hydrate (NADPH, 95%) were obtained from Sigma-Aldrich Chemical Co. The unit definition of GR is that one unit will reduce 1.0 μmole of oxidized glutathione per min at pH 7.6 at 25 °C. 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) was purchased from Tokyo Chemical Industry Co. Ltd. $HgCl_2$, sodium dihydrogen phosphate (NaH_2PO_4), disodium hydrogen phosphate (Na_2HPO_4), acetic acid (HAC), sodium acetate (NaAc), citric acid (CA), and sodium citrate (Na-Citrate) were obtained from Aladdin Chemical Co. Ltd. All reagents were of analytical reagent grade, and used as received. Phosphate buffer solutions (PBS) of various pH were prepared with different ratios of Na_2HPO_4, NaH_2PO_4, and ultrapure water. The stock solutions of GR and NADPH were both freshly prepared and cooled on ice before use.

2.2. Synthesis of CQDs

The CQDs was prepared as previously reported [23]. Briefly, a procedure is described as follows: triammonium citrate (5.0*g*) and Na_2HPO_4 (1.0 g) were dissolved in 10 mL distilled water to form a transparent solution. The mixture was sonicated for 5 min, and then heated in a microwave chemical reactor (WBFY-205, China) at 220 V for 10 min. A large number of brownish-yellow CQDs were obtained directly. The obtained powders were precipitated with ethanol-water (V/V, 80/20), and then centrifuged to remove impurities. After drying, CQDs were dissolved in double-distilled water to a final concentration of 1.0 mg/mL to be stored at 4 °C away from light.

2.3. Fluorescence experiments

For the evaluation of GR activity, 10 μL GSSG (2.5 mM), 10 μL NADPH (1 mM), and 10 μL GR of different concentrations were added into PBS buffer (10 mM, pH 6.0). After the reaction proceeded for 10 min at room temperature, 5.0 μL of 1.0 mg/mL CQDs and 12.5 μL of 2.0 mM Hg(II) were added into the above solution with the total volume of 500 μL. Subsequently, the mixture was shaken thoroughly and equilibrated for 8 min at room temperature. Then, the fluorescence intensities of CQDs were recorded. The emission intensity of CQDs at 434 nm was plotted against the concentrations of GR. The inhibitor experiments were carried out according to the same procedure mentioned above, except that GR was pre-incubated with BCNU for 10 min

2.4. Characterization methods of CQDs

Transmission electron microscopy (TEM) images were obtained using a Philips CM200 FEG microscope. The fluorescence measurements were conducted using a fluorescence spectrophotometer (Max-4, HORIBA, USA) operated at an excitation wavelength at 350 nm, with both excitation and emission slit widths of 5.0 nm. The fluorescence intensities were collected at 434 nm. UV–vis absorption spectra were obtained on a UV–vis spectrophotometer (UV-3600, Shimadzu, Japan).

3. Results and discussion

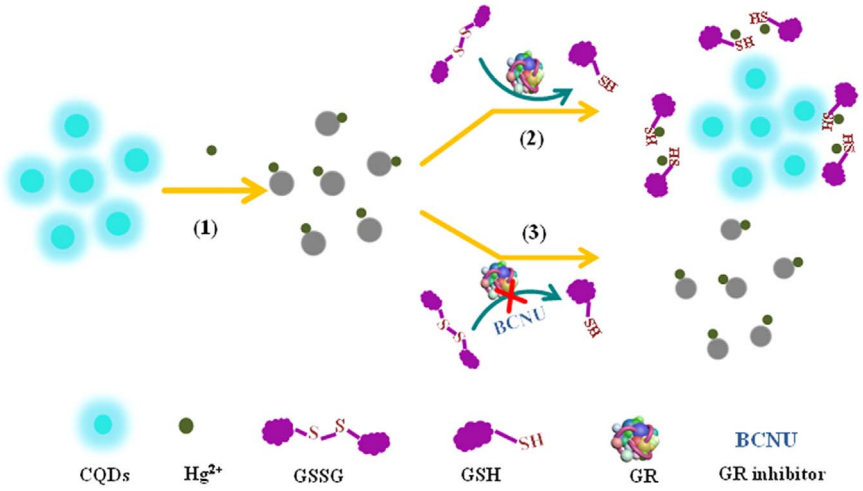
3.1. Characterization of CQDs

The TEM image showed that the as-prepared CQDs are uniformly arranged with the average size of about 6.5 nm (Fig. 1). The fluorescence emission spectra of CQDs are shown in Fig. 2. It could be found that a maximum emission at 434 nm was observed upon the excitation at 350 nm, which was consistent with that of the previous report [23]...

3.2. Main principle of the evaluation of GR activity

On the one hand, Hg(II) can efficiently quench the fluorescence of CQDs [24]. On the other hand, GSH, as a strong Hg(II) chelator, could form more stable complexes with Hg(II) [32–34]. As a result, Hg(II) was removed from the surface of CQDs, so as to recover the fluorescence of CQDs-Hg(II) system. In contrast, GSSG possesses a weak affinity with Hg(II), so that the fluorescence of CQDs-Hg(II) system can not be recovered. Based on these facts, a turn-on fluorescent biosensor has been developed for the evaluation of GR activity and the screening of its inhibitors. As shown in Scheme 1, the introduction of GR, GSSG, and NADPH into the CQDs-Hg(II) system brought about the substantial fluorescence recovery, owning to the fact that GR could catalyze GSSG to GSH in the presence of NADPH. Importantly, the enhanced fluorescence intensities could be correlated to varying GR levels to expect the feasible evaluation of GR activity. When a GR inhibitor was added into the system, GR activity would be inhibited with the loss of its capability of catalytic reduction from GSSG to GSH. Consequently, the fluorescence of the system remained quenched due to the absence of GSH. The inhibition effects on GR activity would be reflected on the fluorescence quenching degrees of the standard assays.

Accordingly, the feasibility of CQDs for the qualitative evaluation of GR activity was first assessed with the results shown in Fig. 3. It was noted that CQDs themselves exhibited a strong fluorescence (curve a) that could be quenched in the presence of Hg(II) by the decrease in 80% fluorescence intensity of CQDs (curve b). While GSH was added into the CQDs-Hg(II) system, about 84% fluorescence intensity was recovered due to the stronger affinity of CQDs with Hg(II) (curve c). However, when GSSG was added into the CQDs-Hg(II) system, a slight change in fluorescence intensity was observed due to the weak interaction between Hg(II) and GSSG (curve d). The results indicate that Hg(II) can possess the different recognition abilities towards GSH and GSSG. These phenomena were also verified by the UV–vis



Scheme 1. Schematic illustration of the principle and procedure for the evaluation of GR activity and its inhibitor based on the CQDs-Hg(II) interaction system.

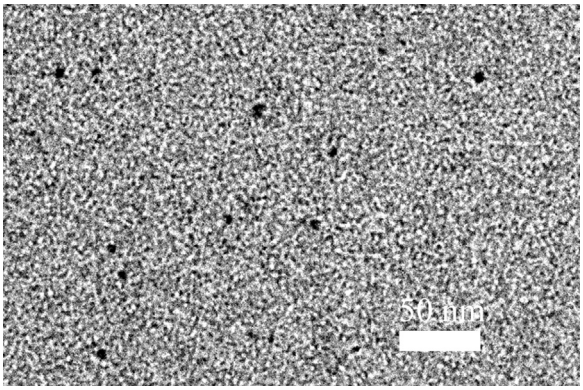


Fig. 1. HRTEM images of CQDs.

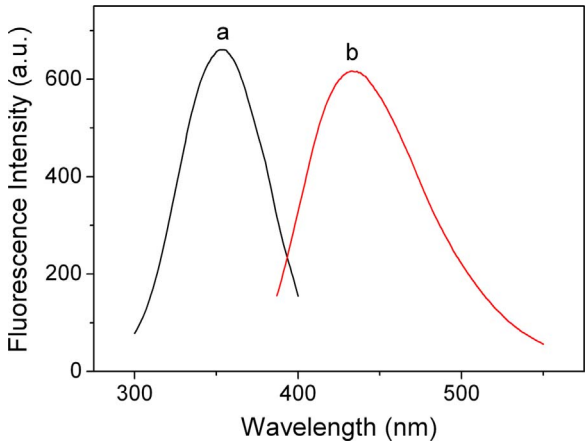


Fig. 2. The fluorescence excitation (a) and the maximum emission (b) spectra of as-prepared CQDs.

spectrometry (Fig. S1). Obviously, CQDs had a typically strong absorption peak at about 335 nm, which implied the formation of a graphitic structure (curve a) [35]. Upon the addition of Hg(II), furthermore, the absorption peak intensity decreased seriously (curve b). In addition, when GSH was added into the CQDs-Hg(II) system, the absorption peak intensity at 335 nm increased obviously (curve c), while the presence of GSSG caused a slight change in the absorption peak intensity (curve d)..

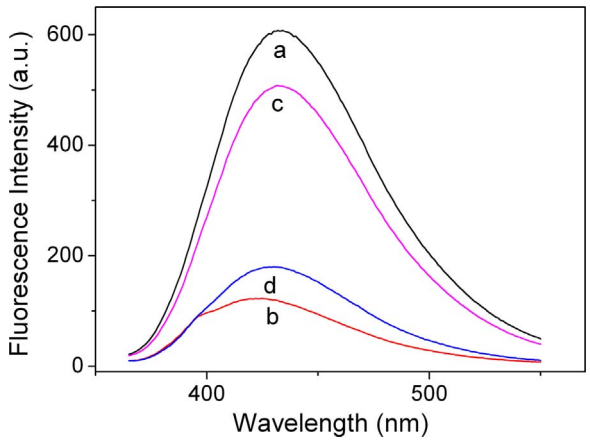


Fig. 3. Comparison of fluorescence spectra of CQDs among the presence of different compositions: (a) mere CQDs, (b) CQDs and Hg(II) (50 μM), (c) CQDs, Hg(II) (50 μM) and GSH (50 μM), (d) CQDs, Hg(II) (50 μM) and GSSG (50 μM).

3.3. Optimization of reaction media for the evaluation of GR activity

The choice of reaction media is a crucial factor for the evaluation of GR activity. Herein, we investigated the influences of pH, molarity, and composition of the media on the fluorescence intensity of CQDs, Hg mobility, and even the GR activity. Fig. S2A shows the fluorescence intensity of CQDs in the absence and presence of Hg(II) in different pH media. The fluorescence intensity of pure CQDs did not change apparently in the pH range of 4.0–9.0. While in the presence of Hg(II), the quenching efficiency of Hg ions towards CQDs increased with increasing the pH from 4.0 to 6.0 and then decreased. The reasons may be that the CQDs and Hg(II) has weaker affinity in acidic medium because of the protonation of carboxylic groups on the surface of CQDs, while Hg(II) would give priority to the combination with the alkaline groups in solution in basic medium [24]. The influence of pH on the GR activity was also investigated. As shown in Fig. S2B, the enhancement factor $(F\text{-}F_0)/F_0$ increased with pH in the range of 4.0–7.0 and then decreased, where F_0 and F is the fluorescence intensity of CQDs-Hg(II) system in the absence and presence of GR, respectively. Moreover, the GR activity in pH 6.0 is comparable with that in pH 7.0. Thus, after overall consideration, we chose the buffer with pH 6.0 in the following experiments.

The molarity of media is another crucial factor for the evaluation of GR activity. Fig. S3A shows the influence of molarity of PBS buffer with pH 6.0 on the fluorescence intensities of CQDs in the absence and presence of Hg(II). The results indicated that the molarity of PBS buffer

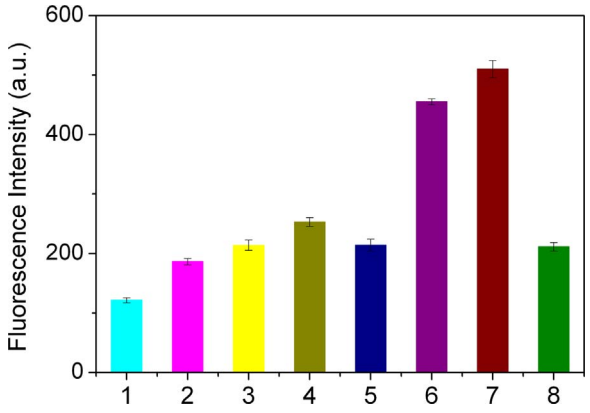


Fig. 4. Fluorescence intensities of (1) CQDs-Hg(II), and CQDs-Hg(II) in the presence of (2) 50 μM GSSG, (3) 20 μM NADPH, (4) 50 μM GSSG and 20 μM NADPH, (5) 50 μM GSSG and 2.0 mU/mL GR, (6) 50 μM GSSG, 20 μM NADPH and 2.0 mU/mL GR, (7) 50 μM GSH, and (8) 50 μM GSSG, 20 μM NADPH and heat-activated GR.

has little influence on both the fluorescence intensities of GQDs and the interaction between GQDs and Hg(II). The influence of the molarity of PBS buffer on the GR activity was also investigated. As shown in Fig. S3B, the enhancement factor $(F\text{-}F_0)/F_0$ decreased slightly with the increasing concentration of PBS buffer, where F_0 and F is the fluorescence intensity of CQDs-Hg(II) system in the absence and presence of GR, respectively. Therefore, in the following experiments, we chose a moderate concentration of 10 mM in order to guarantee both the high enough ion strength and the high enough GR activity.

Lastly, we investigated the buffer of pH with different compositions on the evaluation of GR activity. As shown in Fig. S4, the compositions of media has little influence on the fluorescence intensity of CQDs, the interaction between GQDs and Hg(II), and the GR activity. Therefore, the PBS buffer was chose due to its highest buffer capacity in the following experiments.

3.4. Fluorimetric evaluations of GR activity

Considering that the coenzyme NADPH exhibits a fluorescence at 460 nm [36,37], which is close to the fluorescence emission peak of CQDs, here, the effect of the NADPH dosages were explored. As shown in Fig. S5, NADPH with a concentration up to 20 μM might show no significant effect on the fluorescence intensity of CQDs. In addition, the fluorescence intensity of CQDs-Hg(II) system was investigated under different conditions. As shown in Fig. 4, the fluorescence intensities of CQDs-Hg(II) system changed negligibly in the presence of GSSG, NADPH, GSSG with NADPH, GSSG with GR, or heat-inactivated GR with NADPH. In contrast, the fluorescence intensities of CQDs-Hg(II) system increased obviously in the presence of GSH or GSSG, GR with coenzyme NADPH. It indicates that this fluorimetric sensor could respond to GR through the enzyme catalysis transformation from GSSG to GSH. Therefore, the activities of GR could be readily monitored by recording the changes of the CQD fluorescence intensities..

Fig. 5(A) displays the fluorescence emission spectra of CQDs-Hg(II) in the presence of different concentrations of GR. The dynamic response range of GR in buffer solution was measured through the fluorescence changes of CQDs-Hg(II) system. As the GR levels increased from 0.0 to 2.0 mU mL^{-1}, the fluorescence intensities of CQDs-Hg(II) system continually increased, implying a gradual reduction of GSSG to GSH by GR. Fig. 5(B) presents the enhancement factor $(F\text{-}F_0)/F_0$ versus different concentrations of GR, where F_0 and F is the fluorescence intensity of CQDs-Hg(II) system in the absence and presence of GR, respectively. The calibration curve in the range from 0.10 to 2.0 mU mL^{-1} was obtained. The regression equation for the calibration curve can be expressed as y =0.268+0.354x, with R^2=0.992.

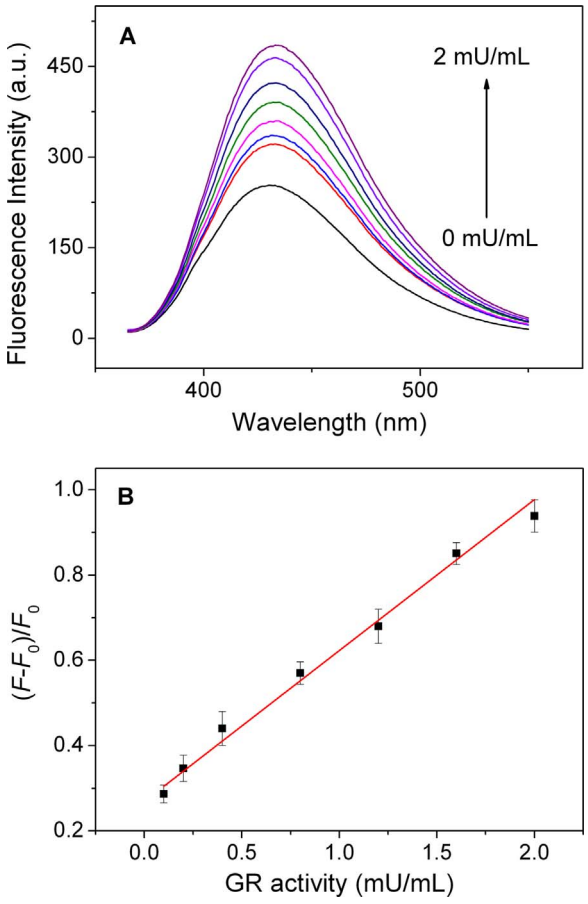


Fig. 5. **(A)** Fluorescence emission spectra of CQDs-Hg system in the presence of different GR concentrations (from bottom: 0, 0.10, 0.20, 0.40, 0.80, 1.2, 1.6, and 2.0 mU/mL). **(B)** Enhancement factors $(F\text{-}F_0)/F_0$ versus the concentrations of GR. F_0 and F are the fluorescence intensities of CQDs in the absence and presence of GR at 434 nm, respectively; [GSSG]=50 μM, [NADPH]=20 μM.

Table 1
Comparison of different fluorescent probes for the determination of GR.

Fluorescent probe	Linear range (mU/mL)	Detection Limit (mU/mL)	References
DNA-AgNCs[a]	0.2–2.0	0.2	[11]
DNA-biots	4–40	0.5	[12]
conjugated polyelectrolyte	0.2–2.0	0.2	[9]
CQDs	0.1–2.0	0.05	This work

[a] DNA-protected Silver nanoclusters.

This assay could allow for the detection of GR down to 0.050 mU mL^{-1} as estimated from its calibration curve (S/N =3). These analytical parameters are comparable or even better than those reported in the literature (Table 1)..

3.5. Screening of GR inhibitors

To further demonstrate the potential applications of the developed fluorimetric assay in screening GR inhibitors, 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), a commonly used irreversible GR inhibitor, was chosen as an example. As shown in Fig. 6, the fluorescence intensity of the sensing system gradually decreased as the increasing the concentrations of BCNU. These results indicated that the GR activity decreased with the increase in BCNU concentrations, illustrating the feasibility of the as-established assay for the screening of GR inhibitors..

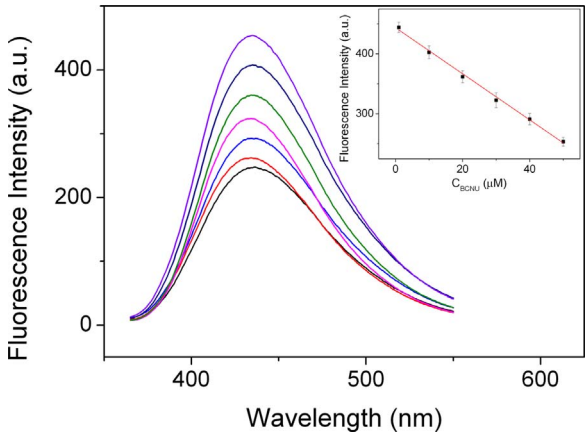


Fig. 6. Fluorescence emission spectra of CDs-Hg(II) system in the presence of GR and different concentrations of BCNU (from up: 1.0, 5.0, 10, 20, 30, 40, and 50 μM). Inset: fluorescence intensity versus the concentrations of BCNU. [GR]=2.0 mU mL^{-1}, [GSSG] =50 μM, [NADPH]=20 μM.

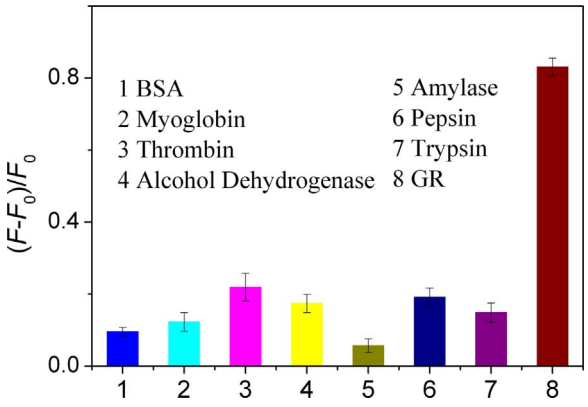


Fig. 7. Changes of fluorescence intensities of CQDs-Hg(II) system caused by various proteins in the presence of 50 μM GSSG and 20 μM NADPH. The concentrations of these proteins are 5.0 μg mL^{-1} with the exception of [GR]=2.0 mU mL^{-1}.

3.6. Selectivity of GR activity evaluation

The detection selectivity of the developed fluorescent GR biosensor was examined. The fluorescence responses of CQDs-Hg(II) system to GR were monitored in comparison with those to seven other proteins/enzymes (Fig. 7). Herein, the experimental procedures were conducted mostly according to the procedure of GR activity assays except for replacing GR with each of those proteins. As shown in Fig. 7, no obvious fluorescence changes were observed for these proteins. The facts indicated that the present fluorescent sensor could exhibit the favorable evaluation selectivity for the GR activity, which is thought to be related to the high catalysis of GR toward GSSG in the presence of NADPH..

4. Conclusions

In summary, a CQDs-based fluorescent biosensor has been successfully developed for the evaluation of GR activity and the screening of its inhibitors based on the Hg(II)-mediated fluorescence quenching of CQDs. Herein, the well-known reactions between GSH and Hg(II) and the effective reduction of GSSG to GSH by GR catalysis play the key roles. Remarkably, CQDs can present some advantages over other fluorescent probes, such as low cost, easy preparation, and good photostability. Compared with most of the previously reported fluorimetric assays, the as-developed CQDs-based method can exhibit a high sensitivity and selectivity in the evaluation of GR activity. Moreover, the screening of the typical GR inhibitors (i.e., BCNU) can also be expected.

Acknowledgements

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.talanta.2016.09.048.

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