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Enzyme-coupled fluorescence sensor for sensitive determination of uric acid and uricase inhibitor

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Abstract

In this work, an enzyme-coupled fluorescence sensor was developed successfully for the sensitive detection of uric acid (UA) and uricase inhibitor based on graphene quantum dots (GQDs). UA is first oxidized by uricase and produced hydrogen peroxide (H_2O_2), which could oxidize phenol to benzoquinone in the presence of horseradish peroxidase (HRP) as the catalysis. Benzoquinone is an efficient quencher and can cause the fluorescence quenching of GQDs. It is found that the quenching degree was proportional to UA concentration and was liner to UA concentration in the ranges of 0.2-14 μmol/L. The detection limit was 0.06 μmol/L for UA. In addition, the proposed sensor was further utilized to the UA detection in human serum samples with satisfactory reproducibility and accuracy. The proposed strategy provides may be widely utilized to the sensing of H_2O_2 or H_2O_2 generating process in related biological environment.

Keywords: Uric acid detection, Graphene quantum dots, Enzyme hybrid biosensor, Inhibitors screening

1 Introduction

Uric acid (UA) is a basic final product of purine metabolism and exists in human's serum and urine.[1] The normal UA concentration in human serum is 0.13 - 0.46 mM.[2] An abnormal UA concentration in either serum or urine can lead to related diseases like gout, hyperuricemia, kidney stone and preeclampsia.[3-5] Thus, the sensitive detection of UA is of significant importance, especially for the evaluation of health status.[6] Previous researches contain a variety of detection methods for UA in recent decades, such as high-performance liquid chromatography (HPLC),[7-9] gas chromatography/mass spectrometry (GC/MS),[10,11] Raman spectroscopy,[12,13] capillary zone electrophoresis (CZE),[14,15] absorption and emission spectroscopy.[16-18] All such methods relatively present some drawbacks like complicated detection procedures or high-cost instrumentation. Compared with other methods, fluorophotometry is widely applied for its operational simplicity, high sensitivity and rapid response.[19,20]

Graphene quantum dots (GQDs), as a recent developed carbon-based fluorescent nanomaterial,[21] are superior to common carbon dots due to the unique single or few-layer graphene sheet characteristics with sizes smaller than 100 nm,[22] which have attracted considerable attentions these years. GQDs possess many good properties such as low toxicity, high fluorescence activity, good biocompatibility, excellent solubility, and long-term resistance to photo bleaching as well as the feasibility of functionalization at their edges.[22-25] Owning to such outstanding characteristics, GQDs have been widely applied in many fields,[26-28] such as fuel cells, drug delivery, bioimaging, especially biosensors.

In this study, an enzyme-coupled fluorescence sensor for sensitive and selective determination of uric acid and uricase inhibitor was developed. In the presence of benzoquinone, the fluorescence quenching of GQDs was obviously took place. In addition, benzoquinone can be generated by the reaction of phenol and H_2O_2. H_2O_2 is usually a critical intermediate product in some biological processes. For UA detection, UA can be catalyzed to produce H_2O_2 with the presence of uricase. Therefore, monitoring UA can be indirectly regarded as determination of H_2O_2. Then a dual-enzyme (HRP and uricase) fluorescent biosensor based on GQDs for UA was established (Scheme 1). The proposed sensing strategy

may also give a basis fluorescent method for detecting targets which generating H_2O_2 in the process of oxidation. With the effect of dual enzyme, the selectivity of biosensors is especially high. This method may widely be used in detecting H_2O_2 or H_2O_2 generating process based on GQDs in future biochemical field.

2 Experimental

2.1 Chemicals and Instrumentation

HRP was obtained from Dingguo Biotechnology (Beijing, China). Citric acid, sodium hydrate, NaH_2PO_4, Na_2HPO_4, and H_2O_2 were obtained from Beijing Chemical Works (Beijing, China). UA and uricase were purchased from Sangon Biotech (Shanghai, China). All chemical reagents are analytical grade and do not require further purification. All solutions were prepared with purified water obtained from Wahaha Group Co. Ltd. (Hangzhou, China).

Fluorescence spectra were collected on a Shimadzu RF-5301 spectrometer at an excitation wavelength of 370 nm and emission wavelength of 380 - 650 nm. A 1 cm path length quartz cuvette was used and the spectra were carried out at 25 °C under ambient conditions.

2.2 Synthesis of GQDs

GQDs were prepared according to previous publications.[29,30] The preparation route is: 2.0 g citric acid in a 250 mL beaker first heated to 260 ºC and then held for about 20 min. 100 mL of 250 mM sodium hydroxide (NaOH) aqueous solution was then added. The light yellow GQDs solution was obtained after full mixing.

2.3 Assay of H_2O_2

For the H_2O_2 detection, a mixed solution was first prepared by mixing 0.5 μg/mL HRP, 0.5 mM phenol, 10% GQDs, and different concentrations of H_2O_2. After shaking evenly for 5 min, the spectral data were collected using the Shimadzu RF-5301 spectrometer.

2.4 Assay of UA

For UA detection, a mixed solution was first prepared by mixing 0.5 μg/mL HRP, 0.5 mM phenol, 0.5 mU/mL uricase, 10% GQDs, and different concentrations of UA. After shaking evenly for 5 min, the solution was incubated at 37℃ for 1.5h. The spectral data were then collected at room temperature using the Shimadzu RF-5301 spectrometer.

2.5 Determination of UA in human blood serum

Normal human serum samples were obtained from a local hospital in Changchun, China (China Japan Union Hospital). For UA detection in human serum samples, a mixed solution was first prepared by mixing 0.5 μg/mL HRP, 0.5 mM phenol, 0.5 mU/mL uricase, 10% GQDs, 1% serum, and different concentrations of UA. After shaking evenly for 5 min, the solution was incubated at 37℃ for 1.5h. The spectral data were then collected at room temperature.

3 Results and discussion

3.1 H_2O_2 induced fluorescence quenching of GQDs

Effect of H_2O_2 concentration on the fluorescence intensity of GQDs was first studied. The prepared GQDs emits strong blue fluorescence with the maximum emission peak at 466 nm at the excitation of 370 nm as the previous reported.[30] H_2O_2 do not quench the fluorescence of GQDs. However, in the presence of HRP as a catalyst, H_2O_2 can oxidize phenol to quinone which can quench the fluorescence intensity of GQDs efficiently.[30] Fig. 1 shows the fluorescence spectra of GQDs in the presence of 0.5 mmol/L phenol, 0.5 μg/mL HRP, and different concentrations of H_2O_2. The fluorescence intensity of GQDs decreased with enhanced the concentration of H_2O_2, and a linear relationship between I/I_0 and H_2O_2 concentration was observed as the following form:

$$I/I_0=0.988-0.0663C\ (\mu mol/L)$$

where I and I_0 are the fluorescence intensities of GQDs in the presence and absence of H_2O_2, respectively. C represents H_2O_2 concentration; the linear range for H_2O_2 is from 0.2 to 12 μmol/L with the correlation coefficient of $R^2 = 0.997$. The limit of detection for H_2O_2 was

0.08 μmol/L, calculated following the 3σ IUPAC criterion. According to the experiment result above, this quenching method may be widely utilized to the sensing of H_2O_2 and the H_2O_2 generating process in related biological environments.

3.2 The strategy for UA sensing

Abundant of indispensable biological process accompany H_2O_2 generation.[31-35] For example, uricase oxidizes UA to allantoin with the concomitant generation of H_2O_2. As the shown in Scheme 1, the produced H_2O_2 could oxidized phenol to benzoquinone. And the final oxidation product (benzoquinone) could quench the fluorescence of GQDs, which can quantify the concentration of glucose. The UA detection processes will be selectivity due to the two-enzyme effect. The fluorescence of GQDs decreased with enhancing UA concentration in GQDs-HRP/uricase system. Therefore, the quantitative analysis of UA could be realized via detecting the fluorescence quenching of GQDs based on enzymatic catalyzed oxidation of phenol. Fig. 2 shows the fluorescence spectra of GQDs-HRP-uricase-phenol system in the absence and presence of UA. Significant fluorescence quenching of GQDs was observed in the presence of 10 μmol/L UA, which suggested that the possibility for the detection of UA.

3.3 Optimization of the experimental parameters

The concentration of uricase was first optimized. The normalized PL intensity of UA biosensor with different respective uricase concentration was studied in Fig. 3. The biosensor for UA was indicated with the increasing concentration of enzyme caused remarkable fluorescence quenching of GQDs. Uricase concentration (0.5 mU/mL) at plain is adopted in the further study.

The reaction time was then optimized. The normalized fluorescence intensity of UA biosensor changed with reaction time was studied in Fig. 4. The results indicated that the increasing time from 0 to 80 min caused obvious fluorescence quenching of GQDs. However, further prolonging reaction time (after 80 min), the fluorescence intensity decreased slowly. The above results indicated that the reaction almost finished in 80 min. Therefore, 80 min was chosen as the optimized incubation time in the further study.

3.4 UA assay

The effect of UA concentration on the fluorescence intensity of GQDs was then investigated at the optimized conditions. As shown in Fig. 5a, the fluorescence intensity of GQDs-HRP/uricase system decreased obviously as increasing the concentration of UA. Furthermore, Fig. 5b shows that the fluorescence intensity of GQDs at 466 nm decreased gradually as increasing the concentration of UA. The regression equation between UA and relative fluorescence intensity is I/I_0= 0.976-0.0572 C_{UA} (μM) in the range of 0.2-14 μM. The corresponding regression coefficient is 0.998, and the limit of detection (LOD) for UA was 0.06 μM. As shown in Table 1, our method showed very good sensitivity comparing with the developed UA detection methods in previous publication.

3.5 Selectivity study

The specificity and selectivity of the UA detection strategy was studied in Fig. 6. Several representative ions and molecules which may existing in organism were utilized, including human serum albumin, alkaline phosphatase, glucose, Ca^{2+}, Na^{+}, and K^{+}. Among the potentially interfering substrates, the tolerance limits were still high enough for UA selective detection. This is due to the high selectivity of biological enzyme. The detection system for UA is also in this situation. The above results suggested that our method for UA is acceptable and reliability.

3.6 Determination of UA in dilute human serums

In order to investigate the applicability of the UA detection method in real samples, the UA determination in two human serum samples were carried out. Table 2 shows that recoveries of two human serum samples were in the range 98.5-102.5%, and RSDs were no more than 3.9%. And the detection results were close to the data obtained by a commercial UA meter. The above results suggested that our work was applicable for UA detection in practical sample and achieved satisfying results.

3.7 Uricase inhibitor screening

Oteracil potassium (OP) as a common uricase inhibitor (UI) was usually used to acute animal model of hyperuricemia on rat.[44] And in this work, UI detection was realized in the proposed enzyme-coupled fluorescence sensor. As Fig. 7a shows, the increasing concentration of OP has little effect on the fluorescence intensity in a range of 0 - 5 mM. This means the UI addition cannot affect the system fluorescence intensity in this concentration range.

From the above results, we found out that the fluorescence intensity could be quenched by the additions of UA, uricase, HRP and phenol. When UI was introduced to the detection system, a reduced quenching degree of the fluorescence intensity was observed. And with the increasing of the concentration of UI, a fluorescence turn-on will be expected. As shown in Fig. 7b, the degree of turn-on emission intensity reduced with the increase of UI concentration, which suggested that the rate of UA hydrolysis was reduced. And compare inhibitor concentration in Fig. 7a to Fig. 7b, a smaller OP concentration could turn-on the fluorescence obviously. IC_{50} is defined as the concentration of the inhibitor required to achieve 50% of inhibition efficiency which always be used to value the inhibition effect.[45] The IC_{50} of OP was estimated to be 35 μM. The above results suggest that the proposed system can be utilized for the screening of potential uricase inhibitors.

4. Conclusions

In this paper, we developed a simple and sensitive biosensor for H_2O_2 and UA based on GQDs and uricase. UA is oxidized by uricase, and the H_2O_2 formed is used to oxidize phenol via catalysis by HRP. The oxidation product of benzoquinone acts as an efficient quencher for GQDs, and the quenching degree was proportional to UA concentration. The biosensor was then utilized to the UA detection in human serum samples with satisfactory accuracy and reproducibility. This enzyme HRP/oxidase assays process also could be use in other target detection with H_2O_2 generated. It also provides a platform for further development of enzymatic biosensors based on GQDs.

Acknowledgements

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Conflicts of Interest

There are no conflicts to declare.

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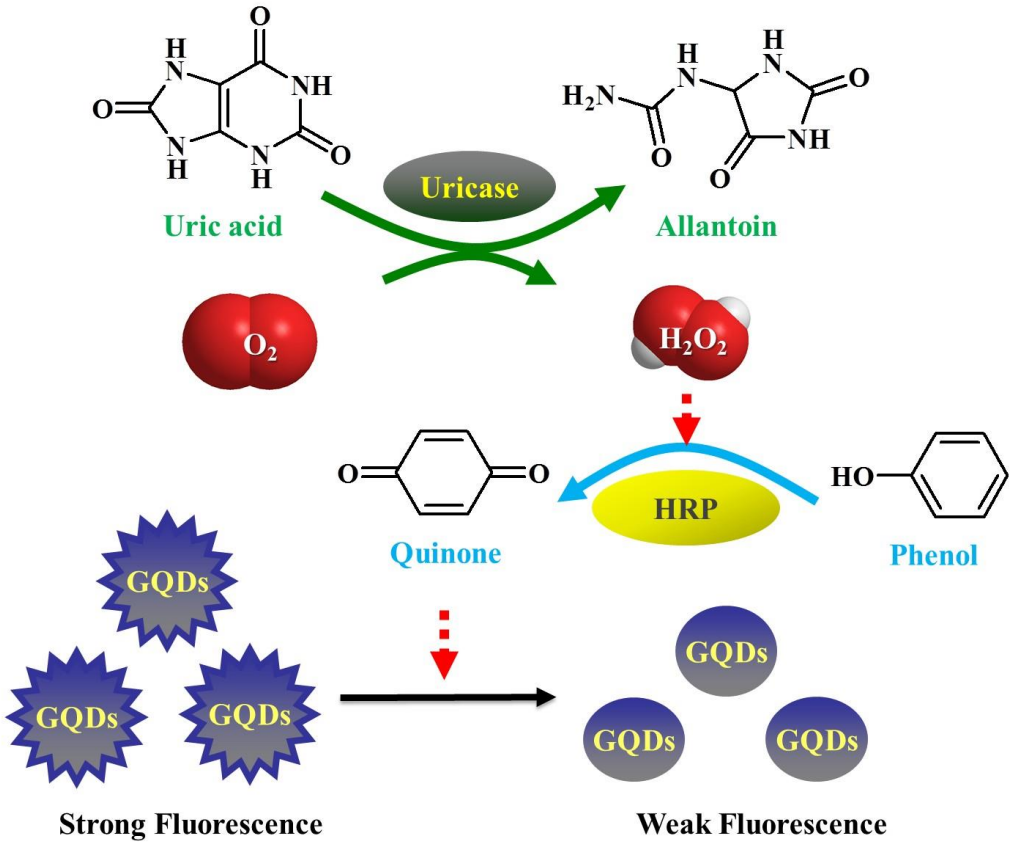
Table 1 Comparison with other analytical methods for uric acid.

Materials	Limit of detection (μM)	Liner range (μM)	Reference
Pt NPs	4.2	0-8000	36
Tb-dtpa-bdap	5.8	10-50	37
Au-SiO_2	2.58	10-500	38
Ag/Au NRs	0.065	0.1-1	39
Cu(II)/Cu_2O/N-GQDs	0.041	0.16-4	40
CDs-MnO_2	0.8	2.5-30	41
Fe_3O_4 NPs	20	50-15000	42
CoOOH nanoflakes	1	10-1000	43
GQDs	0.06	0.2-14	This work

Table 2 UA determination in dilute serum (1%) samples (n = 3).

Sample	Added (μM)	Measured by our method (μM)	Measured by an UA meter (μM)	Recovery (%)	RSD (%)
1	0	1.61	1.59	-	2.6
	2.00	3.66	-	102.5	3.3
	5.00	6.67	-	101.2	2.7
2	0	1.47	1.50	-	3.9
	2.00	3.44	-	98.5	2.8
	5.00	6.46	-	99.8	1.5



Scheme 1 Schematic representation of the sensing strategy.

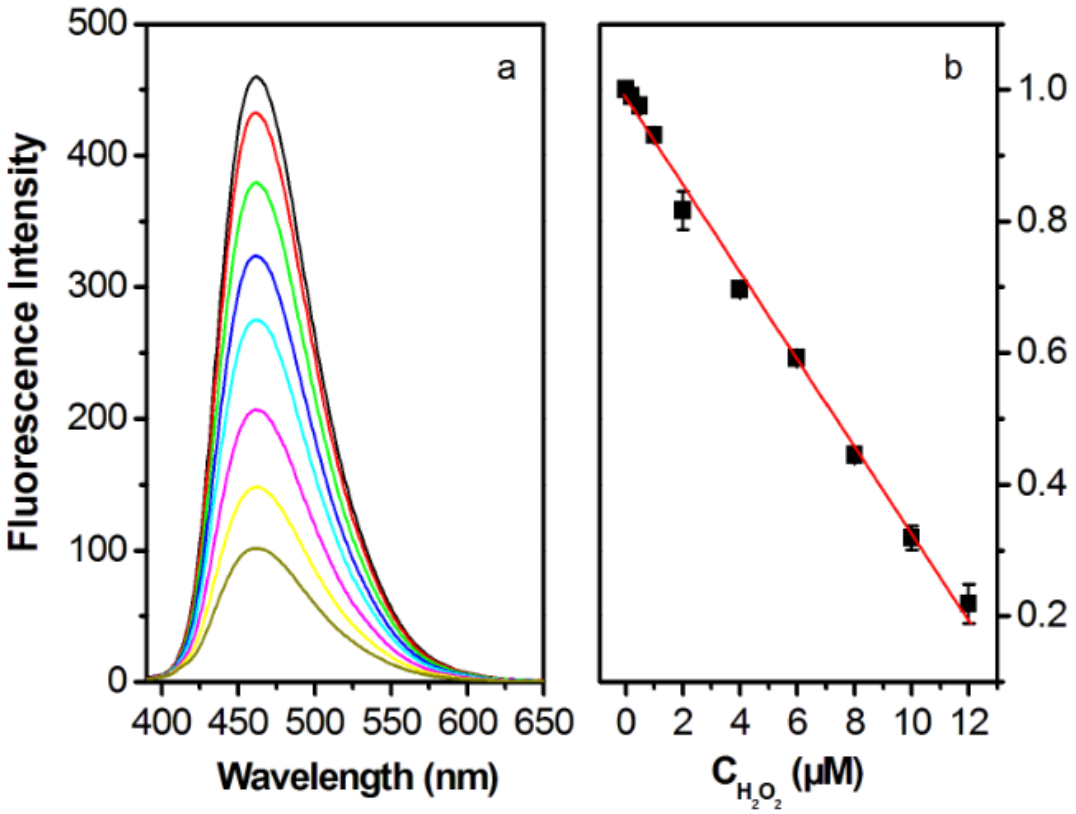


Fig. 1 The right figure shows PL spectra of GQDs in different concentrations of H_2O_2. a–j represents the concentrations of H_2O_2 of 0, 0.2, 0.5, 1.0, 2.0, 4.0, 6.0, 8.0, 10.0, and 12.0 μmol/L, respectively. The right figure shows the linear relationship between I_0/I and concentration of H_2O_2. The excitation wavelength was 370 nm.

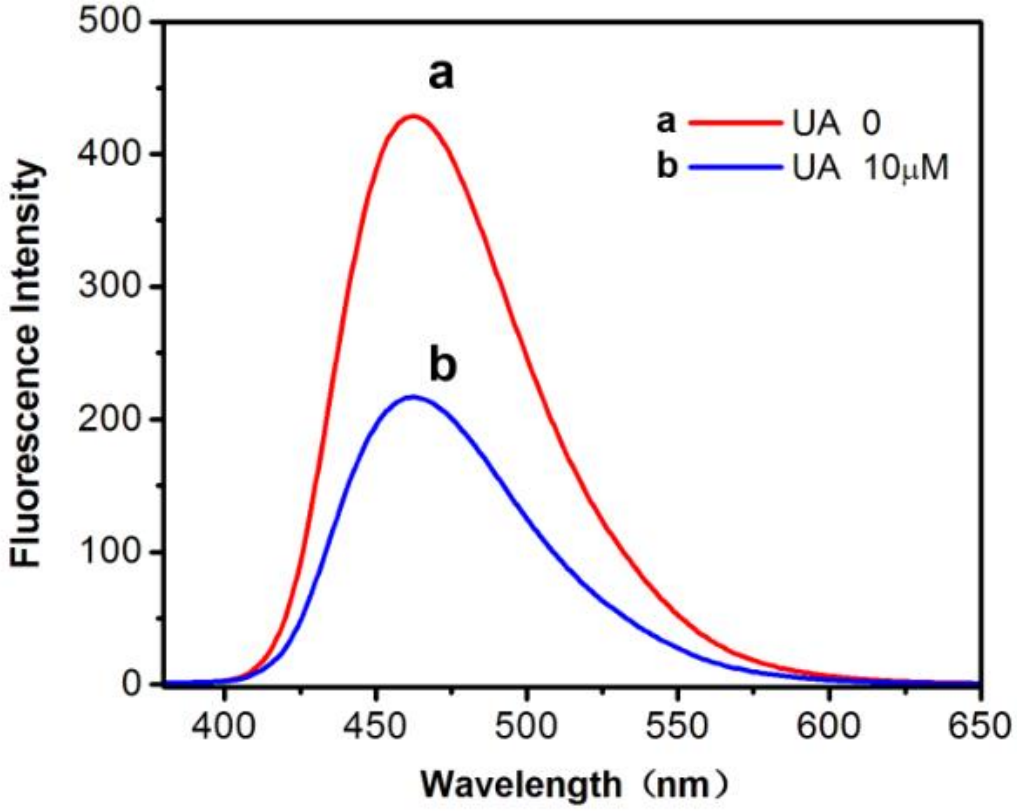


Fig. 2 The fluorescence spectra of GQDs-HRP-uricase-phenol system in the absence (a) and presence of UA (b). Concentration: 10% GQDs, 0.5 μg/mL HRP, 0.5 mM phenol, 10 μmol/L UA, and 0.5 mU/mL uricase.

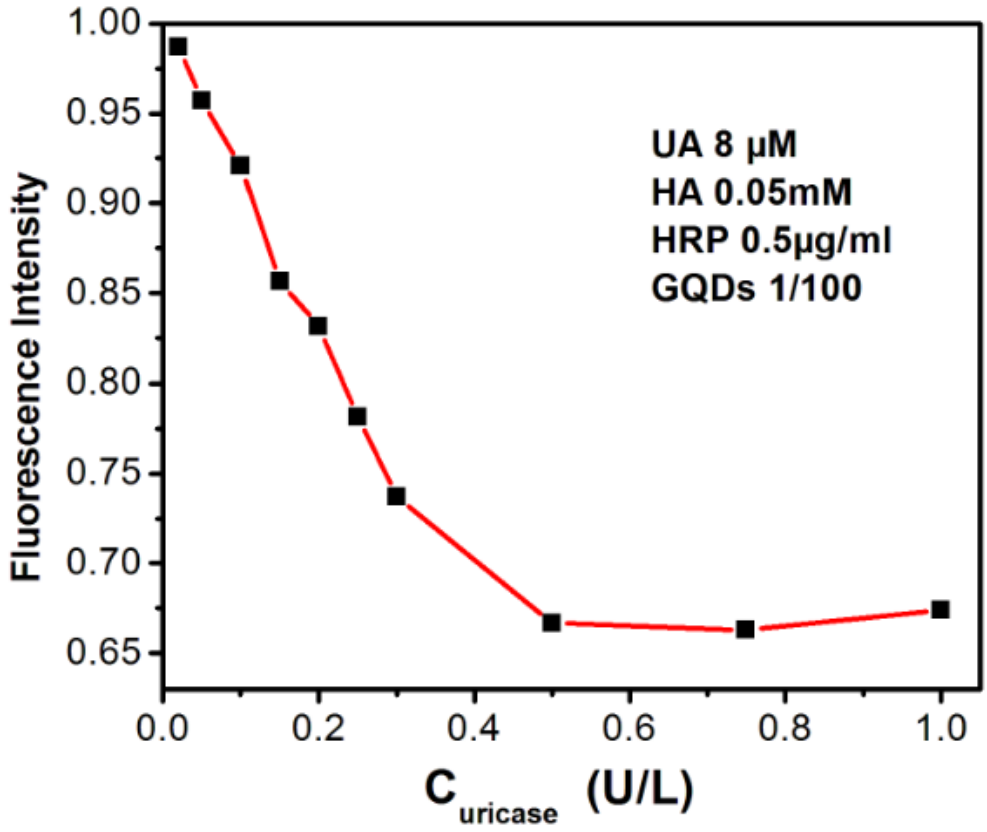


Fig. 3 The normalized PL intensity of UA biosensor with different respective enzyme concentration.

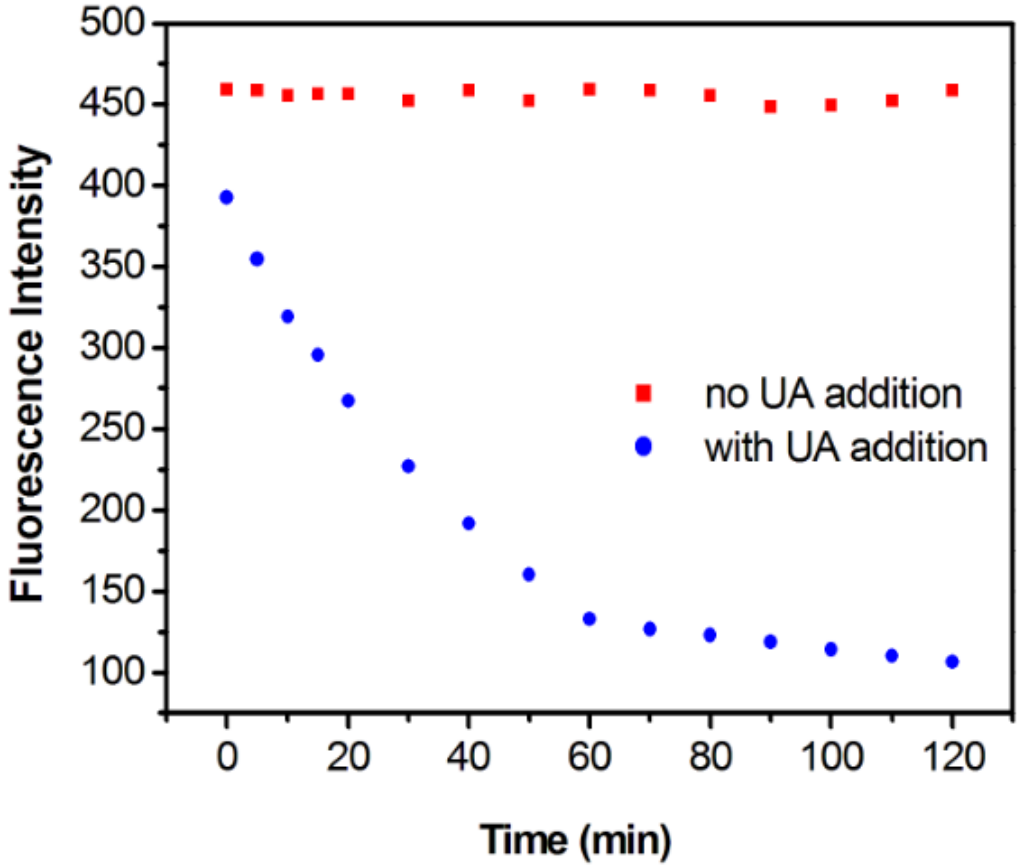


Fig. 4 The normalized PL intensity of UA biosensor with reaction time.

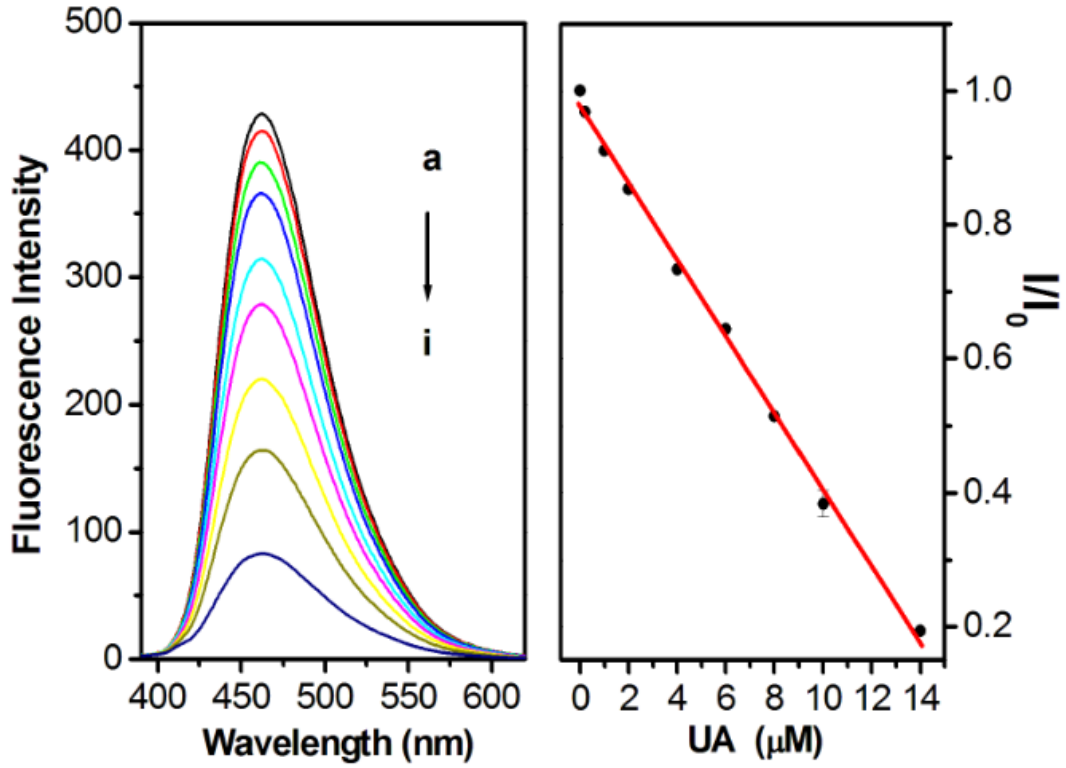


Fig. 5 (a) Effect of UA concentration on the PL intensity of 5.0 μmol/L GQDs in the presence of 0.5 U/L uricase, 0.5 μg/mL HRP, and 50 μmol/L phenol; (b) The linear relationship between the fluorescence intensity ratio of I/I_0 and the concentration of UA. (a–i) represents the concentration of UA of 0, 0.2, 1.0, 2.0, 4.0, 6.0, 8.0, 10.0, and 14.0 μmol/L, respectively.

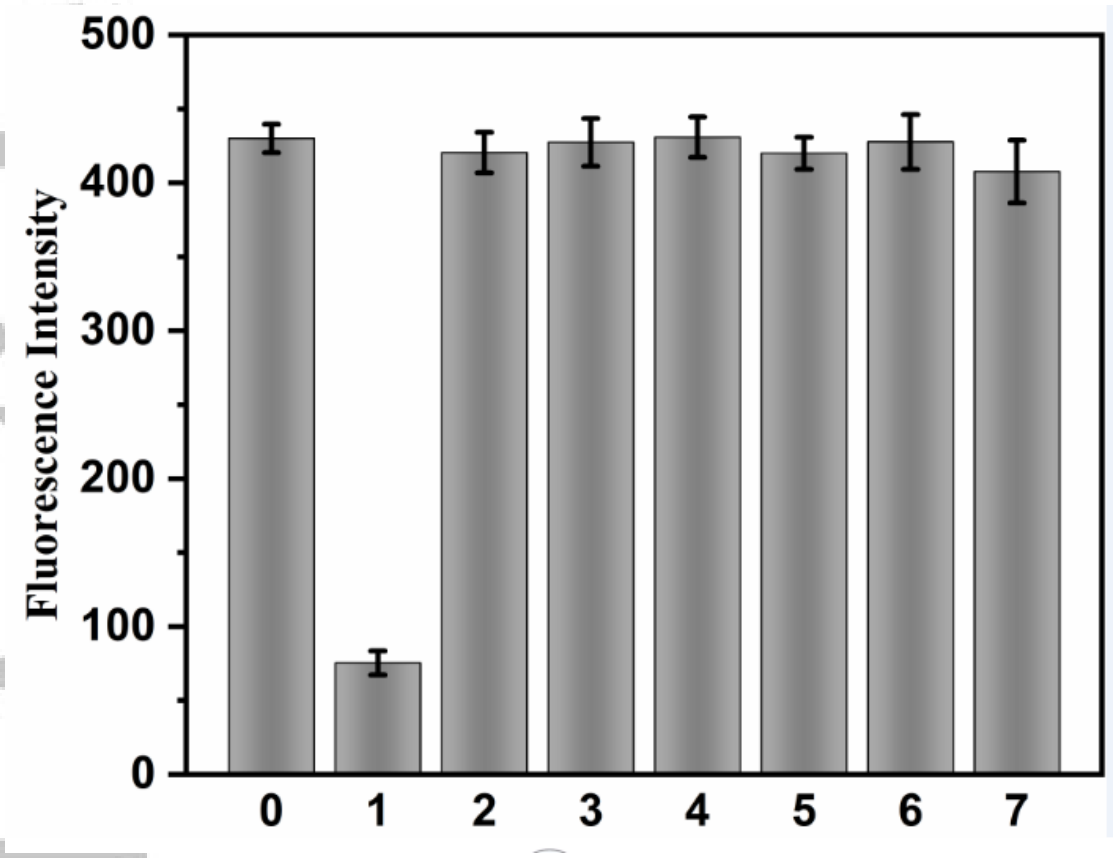


Fig. 6 Selectivity study. 0 - 7 represents the blank sample (no addition), 14 μM UA, 50 μM human serum albumin, 50 μM alkaline phosphatase, 50 μM Glucose, 10 mM Ca^{2+}, 10 mM Na^{+}, and 10 mM K^{+}.

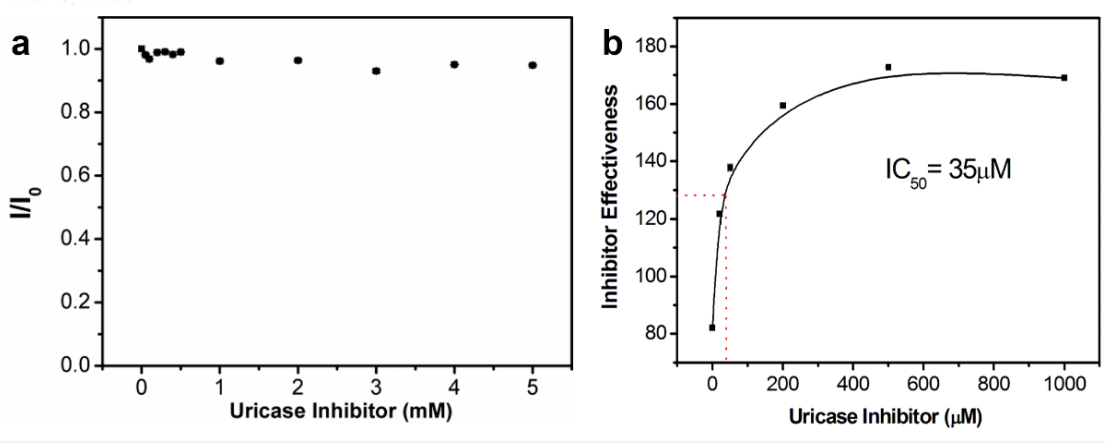


Fig. 7 (a) Effect of the inhibitor concentration on the PL intensity of GQDs. The inhibitor OP concentration is 0, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 1.0, 2.0, 3.0, 4.0, and 5.0 mM (b) Effect of the inhibitor concentration on the PL intensity of GQDs in the presence of 0.5 U/L uricase, 0.5 μg/mL HRP, and 50 μmol/L phenol. The inhibitor OP concentration is 0, 0.02, 0.05, 0.2, 0.5, and 1.0 mM.