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Improved activity and thermo-stability of the horse radish peroxidase with graphene quantum dots and its application in fluorometric detection of hydrogen peroxide

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ABSTRACT

Graphene quantum dots (GQDs) have received extensive concern in many fields such as optical probe, bioimaging and biosensor. However, few reports refer on the influence of GQDs on enzyme performance. The paper reports two kinds of graphene quantum dots (termed as GO-GQDs and N,S-GQDs) that were prepared by cutting of graphene oxide and pyrolysis of citric acid and L-cysteine, and their use for the horse radish peroxidase (HRP) modification. The study reveals that GO-GQDs and N,S-GQDs exhibit an opposite effect on the HRP performance. Only HRP modified with GO-GQDs offers an enhanced activity (more than 1.9 times of pristine enzyme) and thermo-stability. This is because GO-GQDs offers a larger conjugate rigid plane and fewer hydrophilic groups compared to N,S-GQDs. The characteristics make GO-GQDs can induce a proper conformational change in the HRP for the catalytic performance, improving the enzyme activity and thermo-stability. The HRP modified with green luminescent GO-GQDs was also employed as a biocatalyst for sensing of H_2O_2 by a fluorometric sensor. The colorless tetramethylbenzidine (TMB) is oxidized into blue oxidized TMB in the presence of H_2O_2 by the assistance of HRP/GO-GQDs, leading to an obvious fluorescence quenching. The fluorescence intensity linearly decreases with the increase of H_2O_2 concentration in the range from 2×10^{-9} to 2×10^{-4} M with the detection limit of 6.8×10^{-10} M. The analytical method provides the advantage of sensitivity, stability and accuracy compared with present H_2O_2 sensors based on the pristine HRP. It has been successfully applied in the determination of H_2O_2 in real water samples. The study also opens a new avenue for modification of enzyme activity and stability that offers great promise in applications such as biological catalysis, biosensing and enzyme engineering.

Keywords: graphene quantum dots; horse radish peroxidase; hydrogen peroxide; colorimetric method

1. Introduction

Enzyme is large biological molecule that regulate chemical reaction in numerous biological processes, including signal transduction, gene expression, immune response, metastasis and metabolism [1-3]. Enzyme is also used in pharmaceutical and medical fields, food and environmental industry, biofuel area, life science studies as well as biosensors [4-6]. The regulation of enzyme activity and stability is very important and has always attracted great attention [7,8]. To date, various enzyme regulators, ranging from proteins, peptides and synthetic organic molecules, have been discovered [9-11]. Recently, several reports referred to graphene and graphene oxide (GO)' applications in the enzyme immobilization and regulation [12,13]. Owing to unique electronic, thermal, mechanical and chemical properties, graphene and GO are considered as promising alternatives for enzyme regulators to modulate enzyme activity and stability [14-16].

Great effort has been attempted to modulate enzyme activity and stability using graphene and GO in the recent years [17]. Zhang *et al*. reported GO as a modulator for horse radish peroxidase (HRP) and lysozyme simply by incubating them with GO [18]. The investigation shows that the electrostatic interaction between negatively charged GO and enzymes lead to an improved thermal stability and a wide active pH range for phenolic compounds removal with high efficiency. Guo *et al.* used graphene sheets as the substrate to immobilize HRP and oxalate oxidase [19]. In contrast to GO, graphene exhibits a higher enzyme loading, implying that hydrophobic interaction is the main driving force and a higher activity, indicating that graphene with less surface functional groups bring less perturbation to enzyme structure. However, 2D graphene is not suitable for subsequent enzyme performance in practical applications due to its easy aggregation and poor dispersion in common solvents [20]. Therefore, graphene and GO mostly reported as an enzymatic inhibitors due to the synergic effect of different interactions [21-23]. To meet the need of practical applications, the chemical modification of graphene and GO is an effective approach to largely improve the solubility and processability in most of the solvents [24,25]. One the one hand, graphene has a very poor solubility. The characteristic makes its modification extremely hard. On the other hand, graphene or GO sheets are predominantly micro meter-sized from several hundred nanometers to tens of micrometers. Such large sheets may fully coat on the enzyme surface. This may seriously limit the conformational change of enzyme for the catalytic performance and the entrance of substrate into the active center of the enzyme, leading to an obvious reduce of the enzyme activity. However, these problems can be effectively overcome by reducing size of

graphene sheets and number of hydrophilic groups in GO. Graphene quantum dots (GQDs), one type of 0D graphene sheets with lateral size less than 10 nm, have attracted researchers because of robust chemical inertness, low cytotoxicity, excellent biocompatibility, high photostability and ease of preparation [26-28]. Particularly, small size and controllable polarity make it more suitable for enzyme modification. However, only few reports refer on the influence of GQDs on enzyme performance.

The study reports two kinds of graphene quantum dots (termed as GO-GQDs and N,S-GQDs) that are prepared by cutting of graphene oxide and pyrolysis of citric acid and L-cysteine, and their use for the HRP modification. The result demonstrates that GO-GQDs and N,S-GQDs give an opposite effect on HRP performance. Only use of the GO-GQDs leads to an enhanced activity and stability. The HRP/GO-GQDs was applied as a biocatalyst in the fluorometric detection of H_2O_2. The analytical method provides the advantage of sensitivity, stability and accuracy compared to present H_2O_2 sensors.

2. Materials and methods

2.1. Materials and reagents

Tetramethylbenzidine (TMB), N, N-dimethylformamide (DMF), Horseradish peroxidase (HRP, 250 U·mg^{-1}) were purchased from Sigma-Aldrich Chemical Company (Mainland, China). Natural flake graphite was purchased from Qingdao Henlide Graphite Company (Qingdao, China) with an average particle size of 20 μm. Hydrogen peroxide (H_2O_2, 30%), citric acid, L-cysteine and all other reagents employed were purchased from Shanghai Chemical Company (Shanghai, China) with the highest quality. GO was prepared from natural graphite powder by a modified Hummers method [29]. Sodium acetate buffer (pH 4.0, 0.2 M,) was prepared in the laboratory. Ultrapure water (18.2 MΩ cm) purified from Milli-Q purification system was used throughout the experiment.

2.2. Apparatus

Transmission electron microscope (TEM) images were obtained by using JEOL-2100F transmission electron microscope equipped with INCA X-ray energy dispersive spectrometer at 200 keV. The sample was prepared by dropping the GQDs suspension on 300 mesh copper TEM grids covered with thin amorphous carbon films. UV-visible spectra were recorded on the TU-1901 spectrometer. Infrared spectrum (IR) was recorded on the Nicolet FT-IR 6700 spectrometer. Circular dichroism (CD) measurements were carried out on the MOS-450 circular dichroism spectrometer, X-ray photoelectron spectroscopy (XPS) measurements were performed using a PHI 5700 ESCA spectrometer with monochromated Al KR radiation (*hv*=1486.6 eV). Fluorescence spectra

were recorded on a Cary Esclipse fluorescence spectrophotometer (Agilent, Japan) with an excitation wavelength of 380 nm.

2.3. GQDs synthesis

GO-GQDs was prepared from GO by one-step solvothermal method [30]. In a typical procedure, all glassware used in the experiments were cleaned in fresh aquaregia ($HCl:HNO_3$=3:1) and then rinsed thoroughly in ultrapure water. GO of 3 g was dispersed in a 100 ml DMF. After sonicated for 30 min, the dispersion was transferred into a 250 ml of Teflon autoclave and heated at 200℃for 8 h. The resulting brown suspension was filtrated by using a 220 nm microporous membrane to remove black precipitates, dialyzed to remove large graphene sheets, evaporated to remove solvent by using a rotary evaporator, and finally dried at 80℃to obtain solid GO-GQDs.

N,S-GQDs was prepared by pyrolysis of citric acid and L-cysteine [31]. Briefly, 4 g of citric acid and 2 g of L-cysteine were dissolved in 10 ml of ultrapure water. Followed by evaporating at 70℃until all solvent was completely removed from the system. The mixture was heated at 200℃for 3 h and the resulted black solid was dissolved by dropwise addition of 1 M NaOH until pH of the solution was neutral. The solution was subsequently purified by dialyzing and a powdery product was obtained by lyophilisation.

2.4. Enzymatic activity measurements and H_2O_2 detection

To investigate the effect of GQDs on HRP activity, the catalytic oxidation of TMB in the acetate buffer and H_2O_2 by HRP in the presence or the absence of GQDs was test. In a typical procedure, the HRP solution (100µl, 0.1 µg ml^{-1}) was mixed with 200 µl of GO-GQDs (500 µg ml^{-1}). After 12 min incubation, the mixture was added into the mixed solution of 200 µl of 5 mM TMB, 200 µl of 1 mM H_2O_2 and 4 ml 0.2 M acetate buffer. The absorbance change at 652 nm was measured by UV-Vis spectroscopy and repeated three times in all experiments.

For the determination of H_2O_2, 100 µl of HRP (0.1 µg ml^{-1}) was mixed with 200 µl of GO-GQDs (875 µg ml^{-1}). After 12 min incubation, the mixture and 200 µl of TMB (5 mM) were added into 4.5 ml of the acetate buffer solution (pH 4.0) containing H_2O_2 of known concentration or real water sample. The mixed solution was incubated for 30 min and subjected to fluorescence measurements on the fluorescence spectrophotometer with an excitation wavelength of 380 nm.

3. Results and discussion

3.1. Effect of GQDs on the HRP activity

The oxidation of TMB with H_2O_2 by HRP was used as a model reaction to investigate on the effect of GQDs on HRP activity (shown in Fig. 1). During the oxidation, colorless TMB will change into blue oxidized product. When the reaction time was fixed at 30 min, the absorbance at 652 nm can well present the reaction rate. Fig. 2 presents absorption spectra of the solution that was incubated for 30 min in the presence or absence of HRP.

<The preferred position for Fig. 1>

It can be seen that absorbance is very small in the absence of enzyme, showing a quite slow reaction process. The fact verifies that TMB is difficult to be oxidized by H_2O_2 in the absence of catalyst. However, the absorbance will rapidly increase in the presence of HRP, indicating that HRP offers the catalytic activity towards the oxidation of TMB. In addition, Fig. 2 also shows that the HRP/GO-GQDs as a biocatalyst for the reaction brings an obvious absorbance increase at 652 nm, indicating an enhanced enzyme activity. The result confirms that the introduction of GO-GQDs improves the HRP activity. However, HRP/N,S-GQDs gives a lower activity compared to pristine HRP. The above results demonstrate that GO-GQDs and N,S-GQDs exhibit an opposite effect on the HRP performance, which could be attributed to their differences in the structure and properties.

<The preferred position for Fig. 2>

Kinetic behaviour of the reaction was researched by the initial rate method. The change in the absorbance at 652 nm with different incubation time was monitored on the spectrometer, in which the final concentration of enzyme and H_2O_2 was fixed at 2 ng ml^{-1} and 0.5 mM, respectively. Based on the each absorbance value, we calculate the corresponding reaction rate. The absorbance response plateau was observed when the TMB concentration is more than 20 mM, showing the characteristics of Michaelis–Menten kinetic mechanism. The apparent Michaelis–Menten constants (K_m) can be calculated according to the Lineweaver–Burk equation (1):

$$\frac{1}{V} = \frac{K_m}{V_{max}} \times \frac{1}{C} + \frac{1}{V_{max}} \qquad (1)$$

Here, V is initial reaction rate, C is TMB concentration, K_m is Michiaelis-Menten constant, and V_{max} is maximal reaction rate. The relationship curves of TMB concentration with the reaction rate was shown in Fig. 3. K_M can be obtained by calculating the slope of the curve in Fig. 3. The $K_m s$ of HRP/GO-GQDs and pristine HRP are found to be 7.53 mM and 14.61 mM, implying that the activity of HRP/GO-GQDs is 1.9-fold higher than that of

pristine HRP. However, K_m of the HRP/N,S-GQDs is about 24.68 mM. The value is more than 1.68-fold compared with that of pristine HRP. The result shows that the introduction of N,S-GQDs will bring an obvious decreased enzyme activity (more than 1.68-time).

<The preferred position for Fig. 3>

To understand the effect of GQDs on the enzyme activity, the CD spectra of HRP before and after modified with GQDs were measured. The ratio of each secondary structure in the HRP was calculated by using the SELCON3 analytical program. As shown in Fig. 4, the introduction of GO-GQDs leads to an obvious increase of α-helix and decrease of β-sheet and random coil, which could improve the enzyme activity. On the contrary, the addition of N,S-GQDs leads to a reduce of α-helix and β-turn, and increase of β-sheet and random content, indicating a prominent increase of the unordered enzyme structure. The unordered structure is no favor for catalytic oxidization of TMB and leads to a decreased enzyme activity.

<The preferred position for Fig. 4>

3.2. Effect of GQDs on the thermo-stability

Enzyme stability could largely influence on the reproducibility and reliability of the biosensor, particularly the resistance to high temperatures. Herein, the thermo-stability of the pristine HRP and the HRP modified by different GQDs was evaluated by measuring their residual activities after incubation at 60℃with different time intervals. Fig. 5 presents the residual activities of the pristine HRP and the HRPs modified by N,S-GQDs and GO-GQDs with different incubation time. In general, the activity for each enzyme will slowly decrease, rapidly drop and keep almost constant with increasing incubation time. However, the change exists obvious difference for different enzyme. The activity of HRP/GO-GQDs can remain almost unchanged within 2 h and its activity keeps about 45 % of the original activity after 12 h, indicating a relatively higher thermo-stability. The activity is remarkably higher than that of the pristine HRP (more than 22 %). This could be attributed that the introduction of GO-GQDs improves the structure stability under such a high temperature. However, the interaction between the N,S-GQDs and the HRP will partly destroy the structure of HRP for the HRP/N,S-GQDs and results in an obvious decrease in the thermo-stability. Therefore, the activity of HRP/N,S-GQD is about 16 % of the original activity and much lower than that of the pristine HRP and HRP/GO-GQDs.

<The preferred position for Fig. 5>

3.3. Fluorescence response of the reaction system to H_2O_2

Fig. 6 shows that fluorescence spectrum of the GO-GQDs gives an emission peak at 515 nm upon excitation at 380 nm. The fluorescent intensity has a slight decrease after the addition of HRP. No fluorescent change is observed after the addition of single H_2O_2 or TMB. However, the fluorescent intensity will greatly decrease after added the mixed H_2O_2 with TMB. The HRP modified with GO-GQDs offers an enhanced activity and its addition result that colorless TMB will be rapidly changed into blue oxidized TMB in the presence of H_2O_2, which accompanies the fluorescence quenching. The investigation further reveals that the degree of fluorescence quenching increases with the increase of H_2O_2.

<The preferred position for Fig. 6>

We suggest such a fluorescence quenching could be attributed to the formation of the oxidized TMB, a blue product with the maximum absorption at a wavelength of 652 nm. Fig. 7 shows the specific absorption of blue product overlaps with the emission spectrum of GO-GQDs. There is the possibility of fluorescence resonance energy transfer (FRET) between the GO-GQDs and the oxidized TMB, since the oxidized TMB could fully contact with the GO-GQDs in the solution and leads to the decrease of the fluorescence intensity of GO-GQDs [32]. In the absence of HRP, no oxidized TMB is produced and thus there is no change in the fluorescence intensity of the HRP/GO-GQDs. This further demonstrates the fluorescence quenching arises from formation of the oxidized TMB. In addition, full contact of the oxidized TMB with GO-GQDs also influences on the surface state of GO-GQDs, further resulting in the quenching fluorescence [33].

<The preferred position for Fig. 7>

3.4. Condition optimization for the detection of H_2O_2

The quenching effect is dependent on formation of the oxidized TMB, and more oxidized TMB results in the increase of the quenching degree. Therefore, the conditions for TMB oxidation were optimized by the colorimetric method. The effect of GO-GQDs concentration on the sensitivity was tested, in which the HRP/GO-GQDs was employed as a biocatalyst for the oxidation reaction. Fig. 1S is the relationship curve of the absorbance with the GO-GQDs concentration. The absorbance increases with increasing GO-GQDs concentration, verifying that a relatively high concentration of GO-GQDs leads to a higher the enzyme activity when the concentration is lower than 30 μg ml^{-1}. However, the increase in the absorbance tends to remain almost constant when the GO-GQDs concentration excesses 30 μg ml^{-1}. To obtain a high sensitivity, the

GO-GQDs of 35 μg ml^{-1} was used for the HRP modification in the following experiments. In addition, the incubation time also plays an important role in improving the HRP activity, thus affecting the sensitivity for the H_2O_2 detection. In the study, the effect of the incubation time between HRP and GO-GQDs on the enzyme activity was also examined by monitoring visible absorption spectra of the oxidized product. Fig. 2S shows the change of absorbance largely depends on the incubation time. The absorbance increased rapidly with increasing the incubation time and then tended to reach a maximum value within 10 min., verifying that the interaction between the HRP and the GO-GQDs can complete with 10 min and results in a high enzyme activity. However, the absorbance remains nearly no change with use of a longer incubation time. This indicates that the interactions between HRP and GO-GQDs achieve to the dynamic balance. Thus, the incubation time of 12 min was chosen for the detection of H_2O_2 based on time-saving consideration.

The pH of reaction medium is an essential factor for influence on the structure and activity of HRP. Fig. 3S shows absorbance of the solution at 652 nm in different pH with the HRP/GO-GQDs as biocatalyst. It can be seen that pH value of the reaction medium has a serious effect on the absorbance. When the pH was fixed at 3, the absorbance was small due to the inactivation of enzyme in a more acidic solution. In the case of pH 4.0 and 5.0, the maximum absorbance could be obtained, indicating that the oxidation of TMB occurs easily under these two acidic media. However, the absorbance rapidly decrease with the increasing pH from 5 to 8. The fact that enzyme activity is insensitive to pH indicates that electrostatic interaction may be the driving force for the modification of HRP. The isoelectric point (PI) of HRP is 7.2, the enzyme activity was dramatically dropped when the pH was above the enzyme PI. In addition to a weak interaction between HRP and GO-GQDs, it is also because TMB has a poor solubility and H_2O_2 can be easily decomposed in basic solution. To obtain a high sensitivity, the acetate buffer with pH 4.0 is taken as the optimal reaction solution.

3.5. Calibration, linearity and detection limit

The HRP/GO-GQDs was developed as a nanosensor for the fluorescent detection of H_2O_2. To evaluate the analytical performance for quantitative analysis of H_2O_2, various concentrations of H_2O_2 were added into the reaction system under the optimal conditions and the fluorescent spectra were recorded. The fluorescence response of the nanosensor for different concentration of H_2O_2 and the calibration plot of logarithmic H_2O_2 concentration vs. corresponding maximum fluorescence intensity was shown in Fig. 8. The fluorescent intensity at 515 nm linearly decreases with the increase of H_2O_2 concentration in the range of 2×10^{-4} to 2×10^{-9} M. The

linear equation was F = -8.3609logC + 344.27, with statistically significant correlation coefficient of 0.9958, where F is fluorescence intensity at 515 nm and C is H_2O_2 concentration (M). The detection limit was 6.8×10^{-10} M that was obtained from the signal-to-noise characteristics of these data (S/N=3). The H_2O_2 was repeatedly measured ten times in 2×10^{-6} M standard solution under the same conditions. A relative standard deviation of 2.5 % for the measurements was obtained, indicating a good precision. These analytical parameters are better than that of the reported H_2O_2 sensors based on HRP and the proposed method can be widely used for detection of trace H_2O_2 [34].

<The preferred position for Fig. 8>

3.6. Interference study

The interference from possible substances existed in natural water was investigated to evaluate selectivity of the sensor. Ca^{2+}, Mg^{2+}, Na^{+}, SO_4^{2-}, CO_3^{2-}, NO_3^{-} and Cl^{-} are main ions in natural water. The result shows that 100 mM of the each ion leads to far less than 5% of the fluorescence quenching. This is because the above ions do not react with HRP and GO-GQDs and therefore do not interfere with the determination. Fe^{3+}, Hg^{2+}, Ni^{2+} and Cu^{2+} exist in natural water at trace level. These ions can combine with GO-GQDs to produce a complex, which greatly affects the surface states of GO-GQDs due to the surface modification. The adsorption of metal ions also lead to the fluorescence quenching [35]. For example, the existence of 0.1 mM Fe^{3+} causes more than 30 % of the fluorescence quenching. To eliminate the interference of these ions with detection of H_2O_2, EDTA was added into water sample prior to the addition of biocatalyst. The result indicates that Fe^{3+}, Hg^{2+}, Ni^{2+} and Cu^{2+} will rapidly combine with EDTA to form stable complex, which cannot react with HRP/GO-GQDs and cause the fluorescence quenching. The 1 mM of Fe^{3+}, Hg^{2+}, Ni^{2+} or Cu^{2+} do not interfere with the fluorescent detection of H_2O_2 in the presence of 2.5 mM EDTA.

3.7. Sample analysis

The feasibility of the newly proposed method for possible applications was investigated by analyzing H_2O_2 in real water samples. All water samples including lake water, river water, rain water, ground water and tap water, were collected from the campus at jiangnan University. The recovery experiments with different amounts of H_2O_2 were test by measuring the fluorescence response to each spike sample. The concentration of H_2O_2 in each water sample was determined from the calibration curve and the value was used to calculate the

concentration in the original sample. The results of detection of H_2O_2 in real water samples are given in Table 1. The recovery of H_2O_2 for real samples analysis is in the range from 98 to 106%, indicating the proposed method was satisfactory for H_2O_2 analysis with a good accuracy and precision.

<The preferred position for Table 1>

3.8. Mechanism for the effect of GQDs on the HRP performance

To understand the mechanism for the effect of GQDs on the HRP performance, the structure characteristics of two kinds of GQDs were investigated using IR, XPS and TEM technologies. The results were shown in Fig. 9. For the GO-GQDs, only several weak IR absorption bands can be observed on the IR spectrum. Their intensity is obviously weaker than that of GO, verifying that the most of oxygen-containing groups have been removed during the synthesis of GO-GQDs [36]. The very weak absorption of the most characteristic bands, including –OH (3420 cm^{-1}), epoxy (1045 cm^{-1}), C=O (1700 cm^{-1}) and –$CON(CH_3)_2$ (1130 and 1640 cm^{-1}), imply the small amount of these groups on the GO-GQDs. There are a high peak of graphitic carbon (284.5 eV) and weak peak of oxygenated carbon and nitrous carbon on the C_{1s} XPS spectrum of GO-GQD. The result further confirms that the GO-GQDs contain a few oxygen-containing groups. There are two peaks of both N-C bond of methylamine (399.5 eV) and amide-N (401.2 eV) on the N_{1s} XPS spectrum of GO-GQDs in Fig. 4S, indicating the existence of amide attached on the GO-GQDs. On the other hand, the TEM analysis in Fig. 10A and B reveals that the GO-GQDs is composed of a relatively big graphene sheets with an average diameters of 4.2 nm and the topographic heights of 1.0 nm (shown in Fig. 5SA). The above results demonstrate that the GO-GQDs is composed of relatively big graphene sheets with few hydrophilic groups. Fig. 9A indicates that the N,S-GQDs offers much stronger absorption at 3420 cm^{-1} of -OH stretching vibration, 1701 cm^{-1} of -C=O, 1600 and 1400 cm^{-1} of -COOH, indicating that the N,S-GQDs contains the rich of oxygen-containing groups. There is a high proportion of peak at high binding energy on the C_{1s} XPS spectrum, further confirming the existence of rich oxygen-containing groups. There are pyridinic type (399.9 eV) and pyrrolic type (400.6 eV) N atoms on the N_{1s} XPS spectra, as well as C-S-C units on S_{2p} XPS spectra in Fig. 6S. In addition, the TEM analysis in Fig. 10C verifies that the size of graphene sheets in the N,S-GQDs is quite uniform with an average size of 2.3 nm and the topographic height of 0.9 nm (shown in Fig. 5SB). The above results confirm that the N,S-GQDs is composed of small graphene sheets with rich of hydrophilic groups.

<The preferred position for Fig. 9>

The interaction between enzyme and GQDs is critical factor for influencing on enzyme activity and stability. The interaction strongly depends on the hydrophilic groups and structure of GQDs [37]. Thus, an opposite effect of GO-GQDs and N,S-GQDs on the enzyme performance could be attributed to their difference in the surface groups and structure. Based on the results of IR, XPS and TEM analysis, we know that the GO-GQDs is composed of big graphene sheets with few hydrophilic groups. The characteristic makes it can combine with the enzyme by a weak electrostatic attraction. The combination increases stability of HRP structure, leading to an improved thermo-stability. The combination brings an increase of the ordered structures of HRP, which were confirmed by the result of CD analysis. This will lead to an enhanced enzyme performance. In addition, GO-GQDs also possesses a high conjugated system inherited from the chemical reduction of GO, which will promote the electron transfer [38]. The characteristic make GO-GQDs has a high affinity for TMB, which increases local concentration of the substrate and be propitious to the catalytic activity of HRP in the TMB-H_2O_2 system [39]. Unlike GO-GQDs, N,S-GQDs is composed of small graphene sheets with the rich of hydrophilic groups. The rich of hydrophilic groups bring a strong interaction between the N,S-GQDs and the HRP due to strong electrostatic attraction between the enzyme and the N,S-GQDs. Such a strong interaction induces a big conformation change in the enzyme, leading to an obvious reduce of the enzyme activity. On the other hand, small graphene sheets make it can fully contact with HRP, resulting in a large perturbation of the enzyme molecules and the decrease of enzyme activity and stability.

<The preferred position for Fig. 10>

Conclusions

We demonstrated an improved activity and thermo-stability of horse radish peroxidase with GO-GQDs and its application in the fluorometric detection of hydrogen peroxide. The study shows that the effect of graphene quantum dots on the enzyme performance seriously depends its hydrophilic groups and sheet size. Only graphene quantum dot with little hydrophilic groups and large sheets can improve the enzyme activity and thermo-stability. Graphene quantum dot with rich of hydrophilic groups and small sheet will inhibit the enzyme performance. More importantly, the study provides a promising approach for adjusting enzyme performance for many potential applications in biosensing, biocatalysis and enzyme engineering fields.

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Figure Captions

Fig. 1 The oxidizing reaction of TMB with H_2O_2 by catalysis of HRP.

Fig. 2 Absorption of the solution which was incubated for 30 min in the absence (a) and the presence of bare HRP (c) and HRP modified with N,S-GQDs (b) or GO-GQDs (d).

Fig. 3 Line weaver-Burk plot of TMB oxidation catalyzed by HRP (b) and in presence of GO-GQDs (a) and N,S-GQDs (c)

Fig. 4 The ratio of secondary structures in HRP and HRP modified by GQDs.

Fig. 5 Residual activity of HRP in the absence and presence of GQDs with different incubation time at 60℃.

Fig. 6 Fluorescence spectra of GO-GQDs (a), HRP/GO-GQDs (b) and HRP/GO-GQDs after the addition of single H_2O_2 (c), TMB (d) and the mixture of H_2O_2 and TMB (e).

Fig. 7 Absorption spectrum of oxidized TMB and fluorescence spectrum of GO-GQDs. Inserts show the photographs of GO-GQDs aqueous solution taken under visible light (left) and under 365 nm UV light (right).

Fig. 8 Fluorescence response of HRP/GO-GQDs in 0.0, 1×10^{-9}, 5×10^{-9}, 1×10^{-8}, 5×10^{-8}, 1×10^{-7}, 5×10^{-7}, 1×10^{-6}, 5×10^{-6}, 1×10^{-5}, 5×10^{-5}, 1×10^{-4}, 5×10^{-4}, 1×10^{-3}, 5×10^{-3} and 1×10^{-2} M of H_2O_2 (from top to bottom) (A) and the calibration plot of concentration of logarithmic H_2O_2 vs. Fluorescence intensity at 515 nm (B).

Fig. 9 FTIR of GO and GQDs (A), XPS of GQDs (B) and high-resolution C_{1s} of GO-GQDs (C) and N,S-GQDs (D).

Fig. 10 TEM images of GO-GQDs (A) and N,S-GQDs (C) and their size distributions.

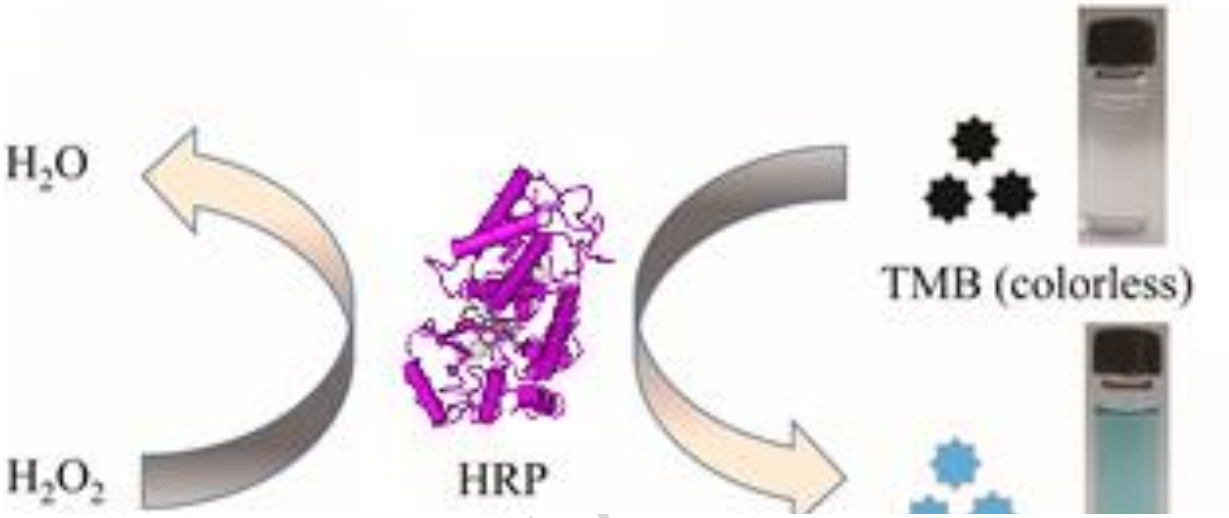

Figure 1

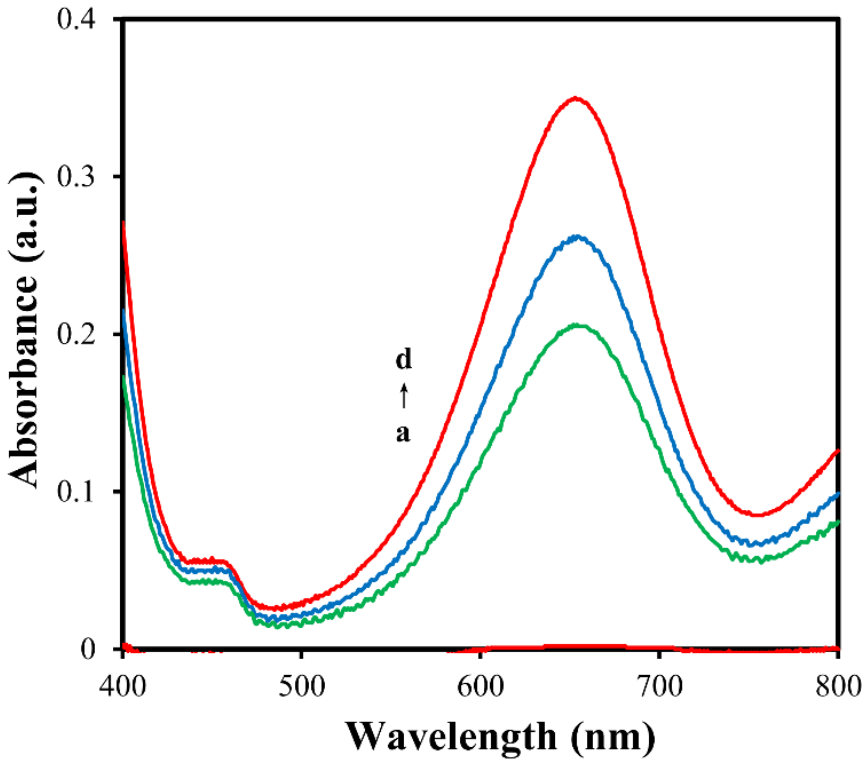

Figure 2

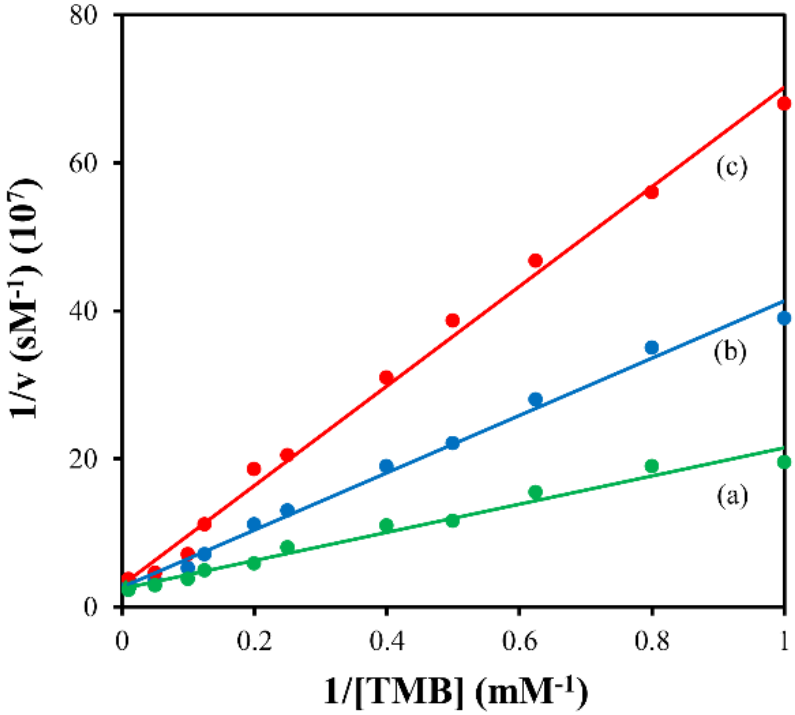

Figure 3

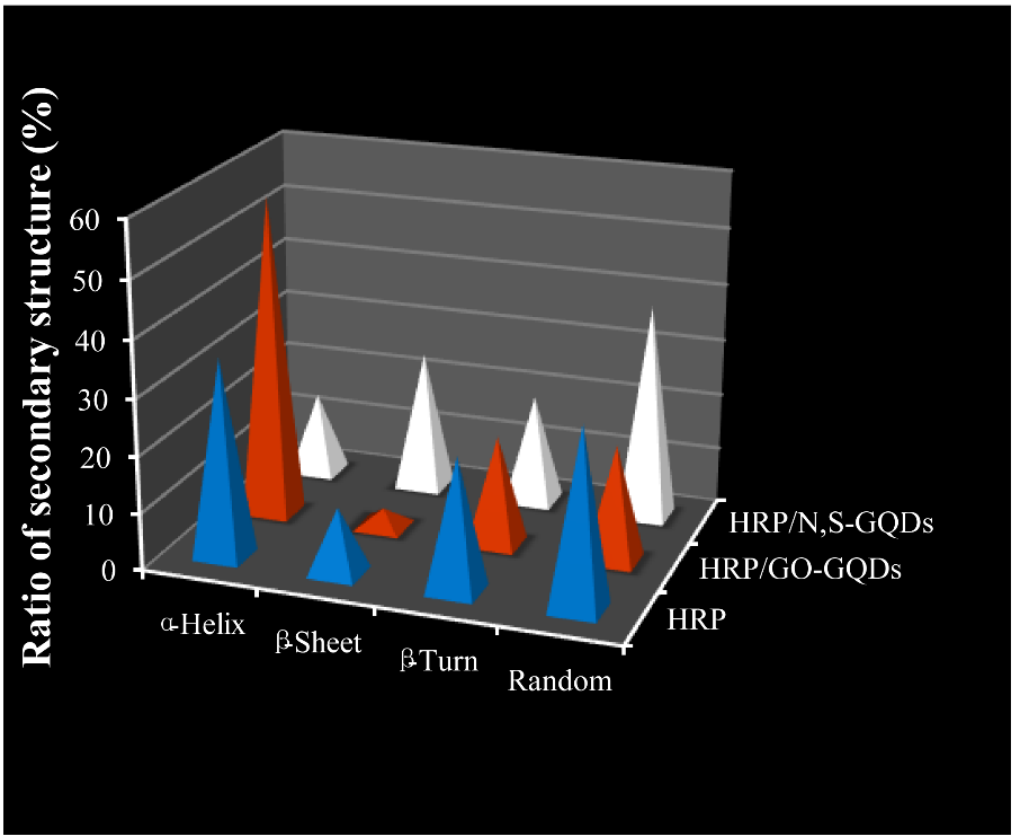

Figure 4

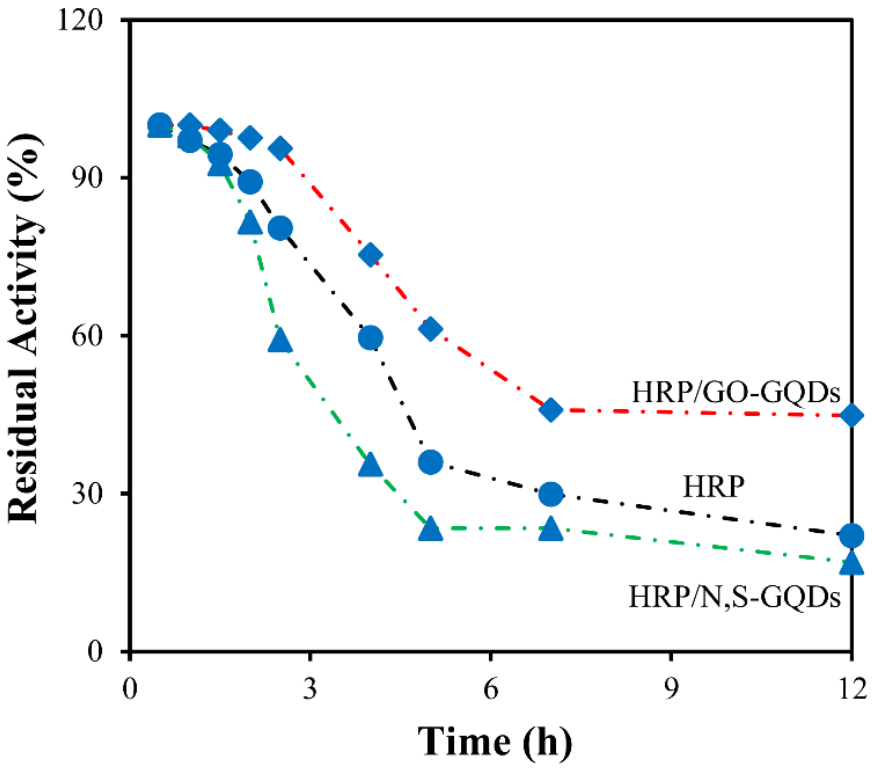

Figure 5

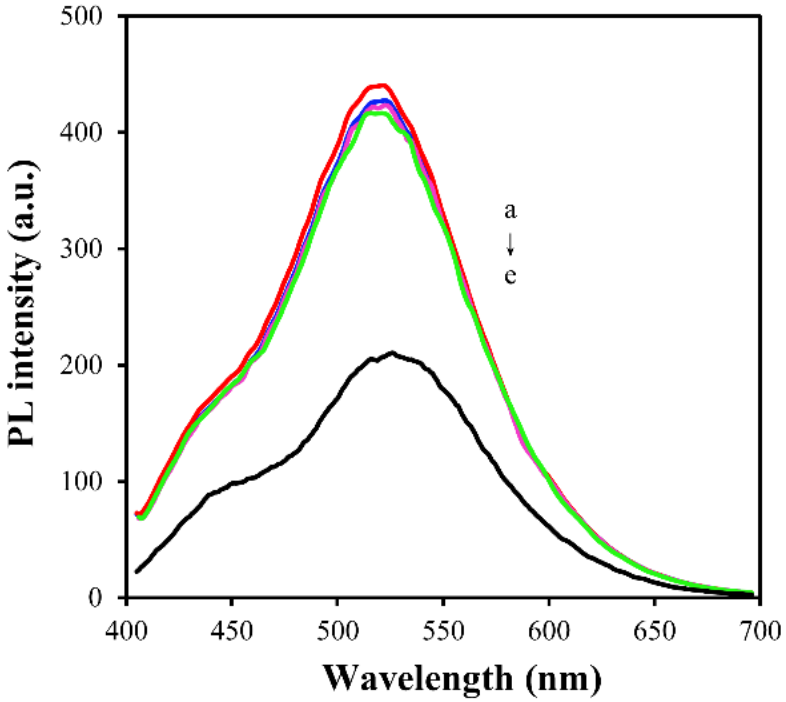

Figure 6

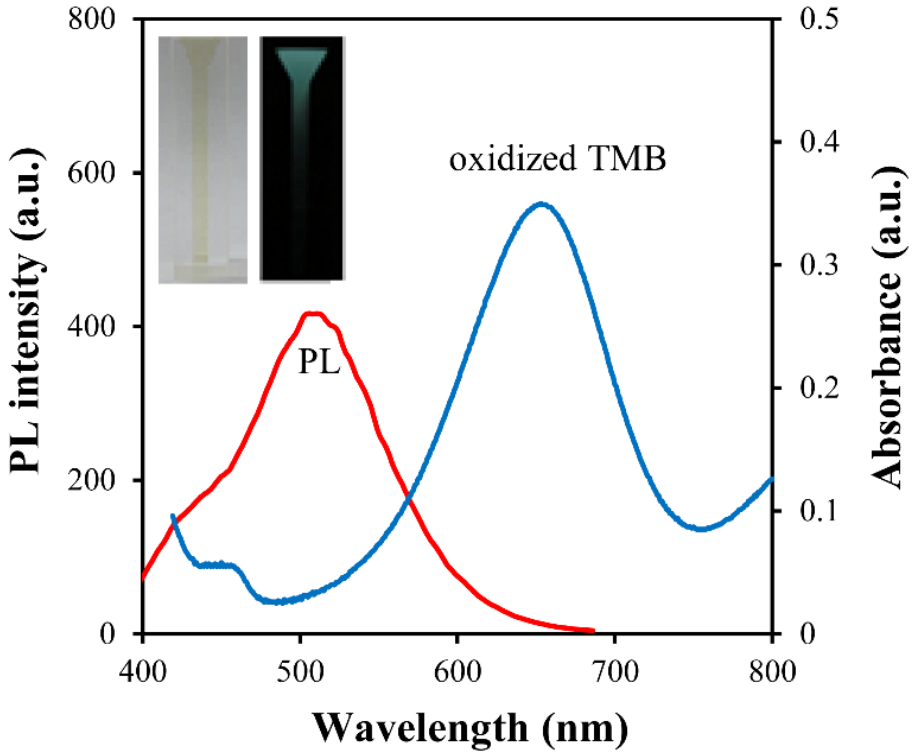

Figure 7

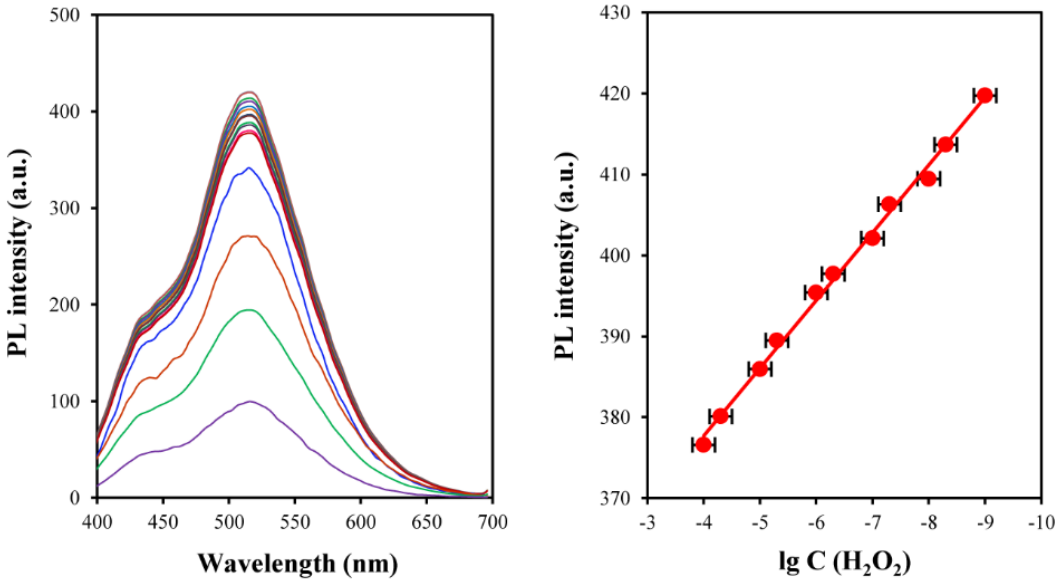

Figure 8

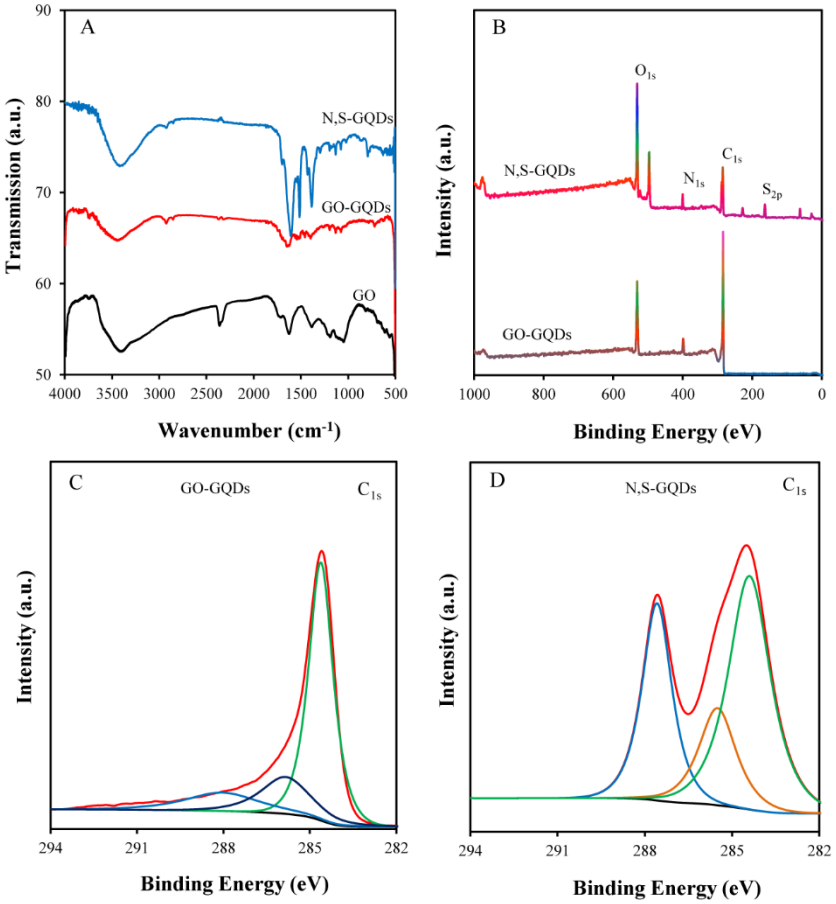

Figure 9

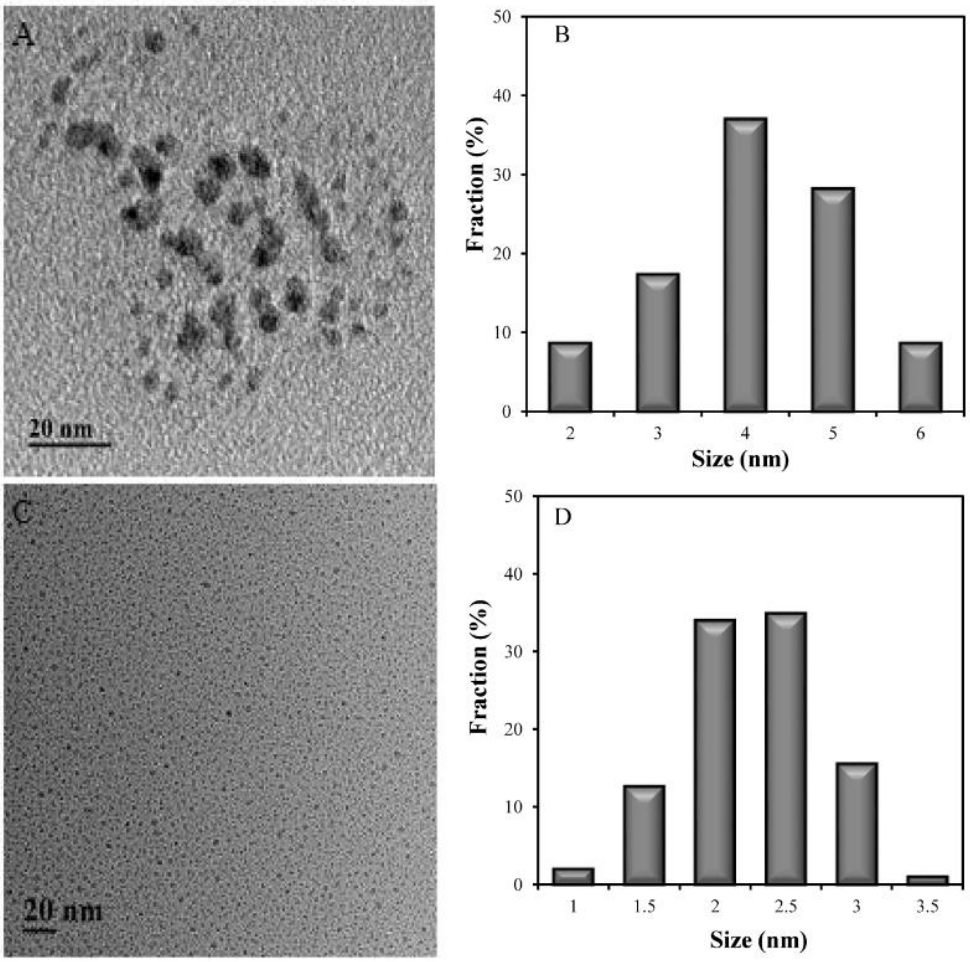

Figure 10

Table. 1

The results for the determination of H_2O_2 in real water samples (n=5)

Samples	Added (μM)	Found (μM)	Recovery (%)
Lake water	0.0	0.65	
	0.5	1.14	98.0
River water	0.0	0.57	
	1.0	1.58	101.0
Rain water	0.0	0.78	
	1.5	2.27	99.3
Ground water	0.0	0.83	
	1.5	2.42	106.0
Tap water	0.0	2.93	
	2.0	5.02	104.5

Improved activity and thermo-stability of the horse radish peroxidase with graphene quantum dots and its application in fluorometric detection of hydrogen peroxide

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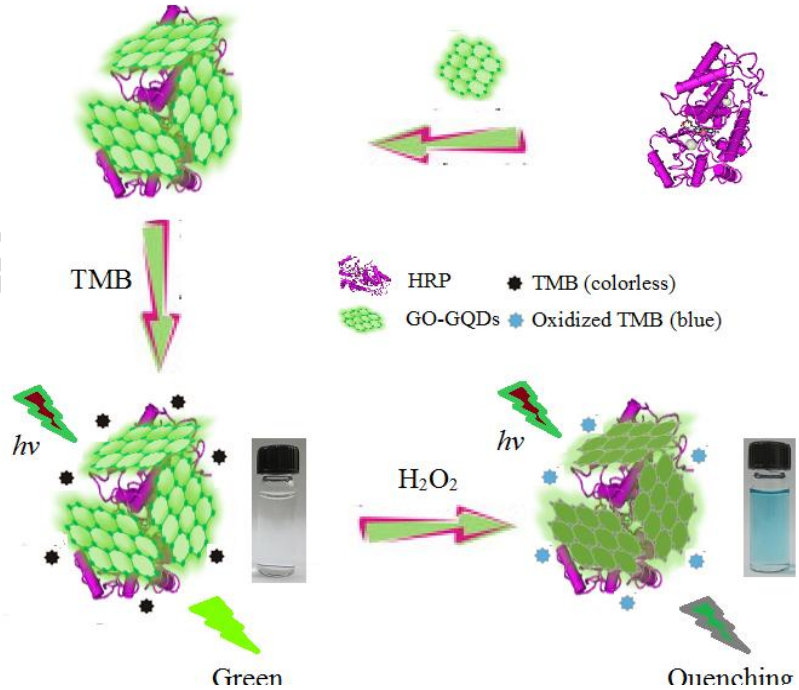
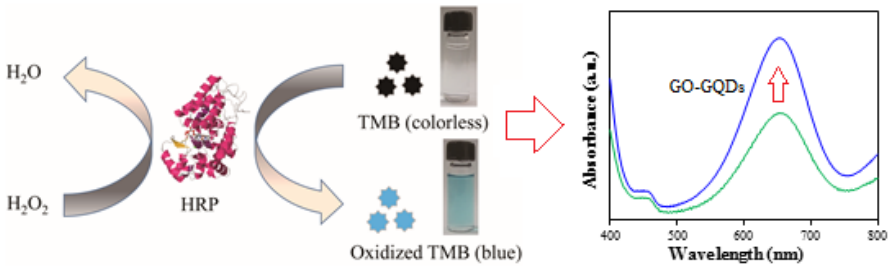
We reported an improved activity and thermo-stability of horse radish peroxidase (HRP) with the graphene quantum dot prepared by cutting of graphene oxide (GO-GQDs) and its application in fluorometric detection of hydrogen peroxide. The result demonstrates that the effect of graphene quantum dot on enzyme performance seriously depends its hydrophilic groups and sheet size. The study also provides a promising approach for adjusting enzyme performance for many potential applications in biosensing, biocatalysis and enzyme engineering fields.

Graphical abstract





Highlights:

- We first reported horse radish peroxidase modification with graphene quantum dots.
- N,S-doped graphene quantum dots greatly improve the enzyme activity and stability.
- The enzyme performance depends on hydrophilic groups and structure of graphene quantum dots.
- The proposed method provides the advantage of sensitivity, stability and accuracy.
- The study provides a promising approach for adjusting enzyme activity and stability.