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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 offer a larger conjugate rigid plane and fewer hydrophilic groups compared to N,S-GQDs. The characteristics can make GO-GQDs 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 $2 \times 10-9$ to $2 \times 10-4$ M with the detection limit of $6.8 \times 10-10$ 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.

1. Introduction

Enzyme is a large biological molecule that regulates 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 leads to an improved thermal stability and a wide active pH range for phenolic compound 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 brings 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 were mostly reported as 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]. On the one hand, graphene has a very poor solubility. The

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characteristic makes its modification extremely hard. On the other hand, graphene or GO sheets are predominantly micrometer-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 reduction 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 the 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), and horse radish peroxidase (HRP, 250 $U \cdot mg^{-1}$) 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 GQD 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 ($h\nu = 1486.6$ eV). Fluorescence spectra were recorded on a Cary Esclipse fluorescence spectrophotometer (Agilent, Japan) with an excitation wavelength of 380 nm.

2.3. GQD synthesis

GO-GQDs were 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 being sonicated for 30 min, the dispersion was transferred into a 250 ml of Teflon autoclave and heated at 200 °C 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 °C to obtain solid GO-GQDs.

N,S-GQDs were 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 °C until all solvent was completely removed from the system. The mixture was heated at 200 °C for 3 h and the resulting 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 tested. In a typical procedure, the HRP solution (100 μl, 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.

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

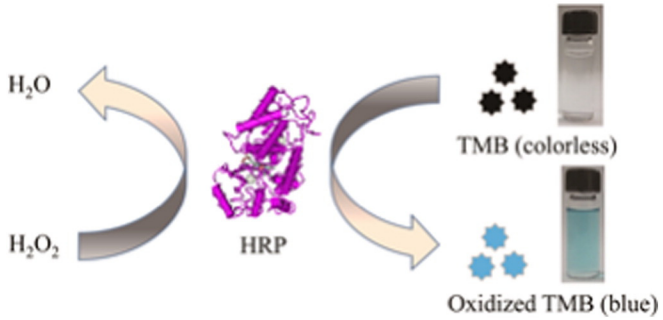

Fig. 1. The oxidizing reaction of TMB with H_2O_2 by catalysis of HRP.