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Immobilization of Glucose Oxidase on Polydopamine-Functionalized Graphene Oxide

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Abstract In this study, graphene oxide (GO) was modified with dopamine to create a matrix for enzyme immobilization. Dopamine can self-polymerize to get polydopamine (PDA) and coated on GO surface. At the same time, GO was reduced to get PDA/rGO biocomposite. The PDA/rGO may offer adherent surface for enzyme immobilization. Glucose oxidase (GOD), an oxidoreductase, was chosen as model enzyme and can be easily immobilized on PDA/rGO. Experimental results indicated that the thermal and pH stability as well as the storage stability and resistance toward the denaturing agents of GOD were significantly improved after immobilization. The Michaelis constant (K_m) of the immobilized GOD was very close to that of the free GOD. This study offers a versatile approach for deposition of biopolymer on GO and provides a way for enzyme immobilization. Hopefully, the immobilized GOD may be further applied for biosensor and biofuel cell applications.

Keywords Graphene oxide · Dopamine · Glucose oxidase · Self-polymerize · Immobilization

Introduction

In recent years, inspired by the strong adhesion of proteins in mussels and geckos to universal substrates, 3,4-dihydroxyphenethylamine (i.e., dopamine) was used to carry out surface modification for various inorganic and organic materials [1–8] due to self-polymerization of dopamine in aqueous solution and the ability to form uniform films under mild conditions as well as its excellent adhesive ability [3, 9]. The self-polymerization and adhesive properties of dopamine as well as the hydrophilicity of polydopamine (PDA) provide a convenient and effective method for the hydrophilic modification of substrates. More importantly, PDA layer contains a large amount of unsaturated active valents that can provide a versatile platform for

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further surface functionalization [10]. For example, PDA-modified materials were used to immobilize biomolecules such as bovine serum albumin (BSA) [11] and α-amylase [12] by the coupling between o-benzoquinone of PDA and amino group of protein onto carriers. These reports provide a facile way for enzyme immobilization on many types of materials.

Recently, owing to its exceptional structural, mechanical properties, and potential applications, graphene oxide (GO) has received widespread attention in various domains [13–17]. Lee et al. [18] reported a method for simultaneous reduction and surface functionalization of GO by a one-step mussel-inspired surface chemistry method based on the oxidative polymerization of norepinephrine. The poly(norepinephrine)-coated reduced GO (rGO) became a multipurpose platform for graphene nanocomposite materials such as polymerization of ε-caprolactone and deposition of AgNPs. Moreover, graphene/polymer composites are promising candidates for materials with good mechanical and conducting properties. Thus, facile incorporation of polymer(s) into graphene-based materials is of interest. Additionally, choosing a material with non-toxic, stable, and cost-effective characteristics is important for chemical reduction of GO. Dopamine, a derivative of norepinephrine, is a potential material for chemical reduction of GO under alkaline conditions via oxidative polymerization. PDA, which is rich in hydrophilic catechol and amino groups [4], also contributes to the high dispersivity and stability of GO and disrupts the aggregation of GO. Additionally, the functionalized surfaces are chemically active to various thiol- or amine-containing molecules, demonstrating a new platform of surface chemistry. However, to the best of our knowledge, the surface function of GO by the method of oxidative polymerization and adhesion of dopamine and its application for enzyme immobilization have not yet been reported.

Thus, in this study, a facile method was developed for immobilizing biomolecules on GO that was reduced and modified by dopamine. The method involved simple dip coating and self-polymerization of dopamine on GO to form PDA/rGO composite, followed by conjugation of biomolecules on PDA/rGO surface. Glucose oxidase (GOD) was chosen as a model enzyme to be immobilized on the PDA/rGO. The effect of different coating and immobilizing conditions, such as coating time, concentration of dopamine and GOD, and immobilizing time, was investigated in detail. The biocatalytic characteristics of the immobilized GOD were studied.

Material and Methods

Materials

Dopamine and tris(hydroxymethyl)aminomethene (Tris) were purchased from Alfa Aesar China Ltd. (China). GOD from *Aspergillus niger* (E.C.1.1.3.4) and HRP (E.C.1.11.1.7) were purchased from Sigma and stored at −20 °C before use. All the chemicals were analytical reagents and used without further purification.

Immobilization of GOD on PDA/rGO

The immobilization of GOD on PDA/rGO by a two-step process was demonstrated in Fig. 1. In the first step, the GO nanosheets synthesized from graphite powder according to a modified Hummer's method [19] were dispersed in distilled water, following ultrasonication treatment for 1 h to obtain the homogeneous GO dispersion. The GO dispersion was then mixed with Tris–HCl solution (pH 8.5, 0.1 M) containing dopamine (between 0.1 and 5.0 g/L) under agitation at 25 °C for a certain time to create an adherent PDA coating on the surface of GO

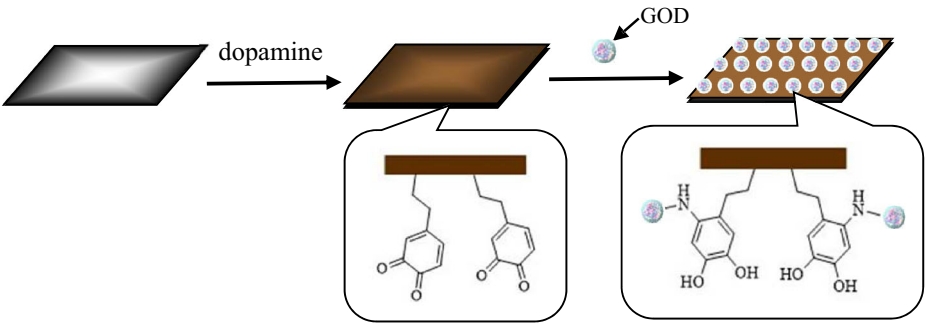

Fig. 1 The preparation process of GOD-PDA/rGO

nanosheets. The obtained PDA/rGO was rinsed several times with distilled water until the washing liquid became colorless.

In the second step, the GOD solution was added into the PDA/rGO dispersion (0.1 M phosphate buffer solution (PBS)). The immobilization process was carried out at 25 °C in a rotary shaker for a certain time. After centrifugation, the immobilized GOD denoted as GOD-PDA/rGO was collected and rinsed thoroughly with PBS to remove unbound GOD.

Assays of Enzyme Activity

The activities of the free GOD and GOD-PDA/rGO were determined using a conventional colorimetric method by measuring the amount of H_2O_2 produced as a result of catalytic oxidation of β-D-glucose by GOD [20]. Typically, to measure the activities of the free GOD and GOD-PDA/rGO, 1.5 mL of buffer solution containing HRP (0.25 mg), 4-aminoantipyrine (0.25 mg), and phenol (0.003 mg) was mixed with 1.5 mL of 13 % glucose solution under vigorous stirring at 25 °C for a certain time. The reaction mixture was filtered and added to a quartz cuvette along with the sample to perform the assay. Absorbance measurements were made using a Thermo 300 UV-visible single-beam absorption spectrometer. The activity at each point was calculated through the time-dependent increase of absorbance at 500 nm. The enzyme unit at each point was defined as the amount of enzyme catalyzing the oxidation of glucose per second at 25 °C. The relative activity was defined as the ratio of the residual activity to the initial activity of each sample.

Characterization

The powder X-ray diffraction (XRD) measurements of the samples were carried out on a D8 Advance diffractometer (Bruker AXS Ltd., Germany) by using Cu Kα radiation with scattering angles (2θ) of 5°–50°. Transmission electron microscopy (TEM) images were recorded on a JEM 2100 TEM system (200 kV, JEOL, Japan). The powdered samples were dispersed in absolute ethanol and mixed uniformly by ultrasonication. A copper grid was used and dipped into the dispersion of specimens. Then, the grid is dried (by heater) in vacuum. Fourier transform infrared (FT-IR) spectra were obtained by using a Brukervector 22 FT-IR spectroscopy (Bruker Optical Instrument Co., Germany) under ambient conditions. The test samples were prepared by dispersing the as-prepared products in potassium bromide (KBr) uniformly and pressing these powders into pellets. The spectra were obtained in the wave number range from 4,000 to 400 cm^{-1}.

Results and Discussion

Preparation of GOD-PDA/rGO

In the current work, dopamine was employed to perform surface modification of GO. As is well known, the isoelectric point (pI) of dopamine was 9.7, and the GO had net negative surface charges in the pH range from 4.0 to 11.0 [21, 22]. Under the condition of pH 8.5, the positively charged dopamine was easily attracted to the surface of the negatively charged GO by electrostatic interaction. Dopamine could self-polymerize at alkaline pHs [23] to form a stable adherent PDA coating onto the surface of GO. The PDA/rGO coating contained active quinone groups that could react with the amino groups of the GOD through Michael addition and/or Schiff base reaction and then resulted in immobilization of GOD onto the surfaces of the PDA/rGO [4, 10, 24–26].

The optimal conditions for GOD immobilization (preparation of GOD-PDA/rGO) were investigated. The enzymatic activity increased with the concentration of dopamine solution from 0.1 to 0.5 g/L (see Supporting Information, Fig. S1). Usually, the thickness and amount of the PDA coating layer increased with the increase of dopamine concentration [27]. The increased thickness and amount of the PDA coating layer containing abundant hydroxyl groups improved the hydrophilicity of the PDA/rGO and further improved loading of GOD that led to the increase of enzymatic activity. However, the enzymatic activity had only a slight increase when the concentration of dopamine solution increased from 0.5 to 5.0 g/L. For the adsorption time, up to 1 h with dopamine solutions at 0.5 g/L, initial slope and the enzymatic activity of the immobilized GOD raised fast, while for longer adsorption times, enzymatic activity did not change significantly. This was the typical adsorption behavior and agreement with the report of da Silva et al. [28].

GOD concentration had a dramatic effect on the activity recovery (AR, %) and adsorption efficiency (AE, %), which were strongly concentration-dependent. As shown in Fig. S2 (see Supporting Information), AR and AE increased with increase of the concentration of GOD, indicating that the increase of GOD concentration was in favor of GOD immobilization on PDA/rGO. But, after the saturation of enzyme, a further increase in the concentration of GOD did not result in a further increase of AR and AE. On the contrary, the AR and AE declined gradually when GOD concentration was higher than 0.15 mg/mL, showing characteristic bell-shaped curves.

The immobilization time may affect the probability of interactions between GOD and free space on PDA/rGO. For the time intervals tested, 1 h of immobilization time at optimal concentration of GOD solution (0.15 mg/mL) seemed to be the most appropriate time (see Supporting Information, Fig. S3). For shorter time intervals, the observed enzymatic activity was lower because there was not enough time for enzymes to firmly adsorb onto the carrier. While for the immobilization time above 1 h, enzymatic activity of GOD-PDA/rGO did not increase significantly.

Considering the above results, the optimal conditions for GOD immobilization on modified GO were as follows: initial dopamine concentration of 0.5 mg/mL, the adsorption time of dopamine on GO of 1 h, initial GOD concentration of 0.15 mg/mL, and the immobilization time of GOD on PDA/rGO of 1 h. These conditions were selected to perform the subsequent tests with GOD immobilized on modified GO.

In order to detect the variations of the layer structure of GO and rGO, XRD analysis was performed, and the results were shown in Fig. 2. There was a narrow and strong diffraction peak at 10.9° for GO, which was caused by intercalated water molecules and oxygen functionalities [29]. After the PDA reduction, the characteristic peak of GO at 10.9° totally disappeared and a new broad peak occurred at 23.8°. The result indicated that the interlayer

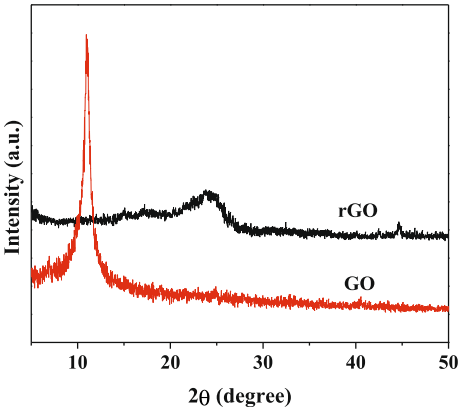

Fig. 2 XRD patterns of GO and rGO powders

distance of GO decreased after PDA reduction, from 0.81 nm (GO, 2θ=10.9°) to 0.37 nm (rGO, 2θ=23.8°), due to the elimination of oxygen-containing groups on GO [30]. The reduction of GO was due to the release of electrons during oxidative polymerization of dopamine [18], which suggested that the reduction of GO and oxidation polymerization of dopamine occurred simultaneously.

The morphology of GO, PDA/rGO, and GOD-PDA/rGO was observed by TEM. The typical TEM image of GO was displayed in Fig. 3a. It could be seen that GO exhibited the sheet-like structure with some folds and small wrinkles. Figure 3b was the TEM image of PDA/rGO. A layered structure with many wrinkled and folded regions could be clearly observed, which was similar to the image of GO, indicating that dopamine produced conformal coatings on surfaces of GO sheets by its self-polymerization. A lot of the dark dots on PDA/rGO sheet could be observed from Fig. 3c, indicating that GOD was distributed on the surface of PDA/rGO sheet.

FT-IR spectra of GO, PDA/rGO, and GOD-PDA/rGO were shown in Fig. 4. The adsorption peaks of GO at 2,925, 2,852, and 1,626 cm^{-1} were contributed to the asymmetric stretching of C–H bond, symmetric stretching of C–H bond, and C=C bond, respectively [31]. A lot of oxygen groups such as −OH (3,422 cm^{-1}), carboxyl C=O (1,730 cm^{-1}) and C–OH (1,405 cm^{-1}), and epoxy C-O-C (1,058 cm^{-1}) were clearly observable, indicating the presence of oxygen-containing functional groups in GO [32]. Due to its abundance of oxygen-containing groups, GO was hydrophilic and could be excellently dispersed in water to form the homogeneous GO dispersion. After GO was functionalized with PDA, the spectrum of PDA/

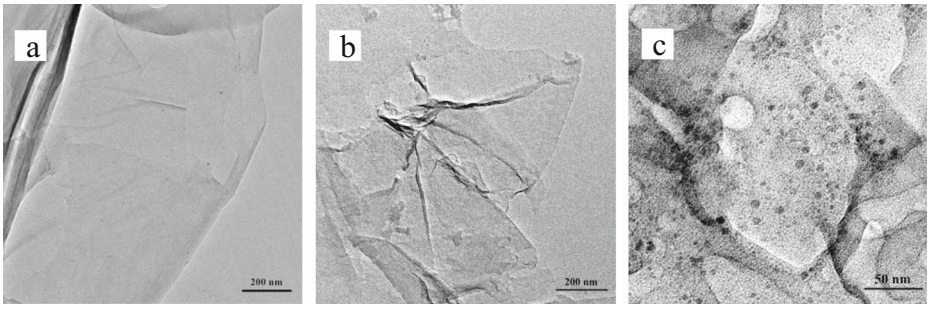

Fig. 3 TEM images of GO (**a**), PDA/rGO (**b**), and GOD-PDA/rGO (**c**)

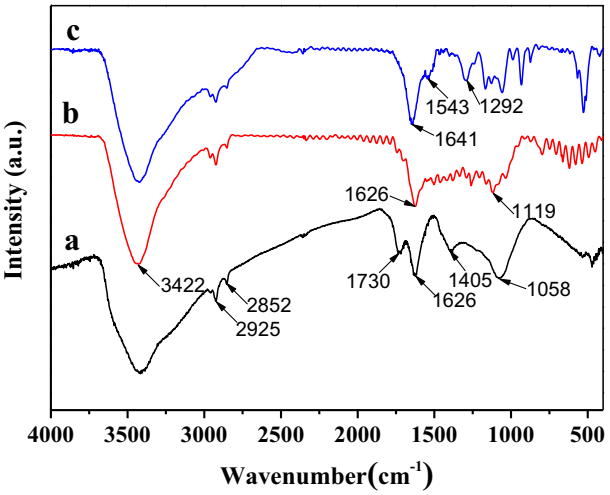

Fig. 4 FT-IR spectra of GO (**a**), PDA/rGO (**b**), and GOD-PDA/rGO (**c**)

rGO at 3,422, 2,925, 2,852, and 1,626 cm^{-1} was consistent with that of GO. But, adsorption peaks of carboxyl C = O (1,730 cm^{-1}) and C–OH (1,405 cm^{-1}), epoxy C-O-C (1,058 cm^{-1}) disappeared, indicating that GO was reduced [18, 32]. The result was in agreement with the XRD of rGO. Nevertheless, compared with GO, PDA/rGO showed broad absorption bands in the range of 1,500–1,000 cm^{-1}, such as at 1,379 cm^{-1} (CH_2 bending vibration), 1,333 cm^{-1} (C-O-H asymmetric bending vibration), 1,205 cm^{-1} (C–O asymmetric vibration), and 1,035 cm^{-1} (C–N stretching vibration) [33]. This phenomenon demonstrated that the PDA layer was successfully introduced onto the GO surface, and GO was reduced and functionalized simultaneously. In the spectrum of GOD-PDA/rGO, the bands centered at about 1,641 and 1,543 cm^{-1} were assigned to amide I (CO stretching of peptide) and amide II (N–H bending and C–N stretching) [16], respectively, which indicated that GOD was successfully immobilized on PDA/rGO. This result was agreed with the previous reports [11, 34].

Properties of free GOD and GOD-PDA/rGO

Effects of pH and Temperature on the Activity

Figure 5a displayed the effect of pH on the relative activities of free GOD and GOD-PDA/rGO over the range 3.5–8.0 for 2 h at 25 °C. The optimal pH for the free GOD and GOD-PDA/rGO was 5.5 and 4.5, respectively. The optimal pH of the GOD-PDA/rGO shifted toward slightly acidic pH, relative to the free GOD, agreeing with the previous report of α-amylase covalently immobilized on PDA-coated magnetic-chitin particles [12]. This shift of optimal pH for the GOD-PDA/rGO might be attributed to the amino or imino groups of PDA surface near GOD active site which could decrease the concentration of H^+ [12]. Figure 5a also showed that the GOD-PDA/rGO exhibited promising activity in the pH range from 3.5 to 5.5, which was a broader range than its free counterpart, indicating that PDA/rGO could maintain the enzyme activity in a wider pH range, and the pH stability of GOD was enhanced after the immobilization on PDA/rGO.

For determining the effect of temperature on the relative activities of GOD in free and immobilized forms, enzymes were incubated in PBS at various temperatures (20–70 °C) for

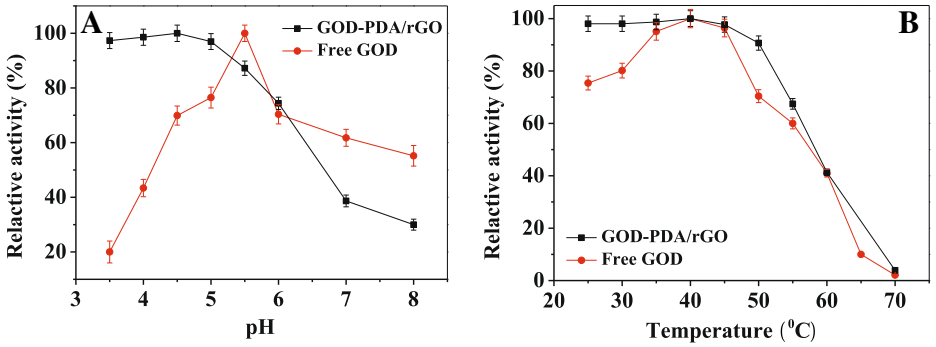

Fig. 5 **a** The effect of pH on the activity of free GOD and GOD-PDA/rGO in 0.1 M PBS; **b** the effect of temperature on the activity of free GOD (pH 5.5) and GOD-PDA/rGO (pH 4.5)

4 h, and then, the enzymatic activity was measured (as shown in Fig. 5b). Both the free GOD and GOD-PDA/rGO exhibited maximal activity at 40 °C and negligible activity above 65 °C. However, the GOD-PDA/rGO had a broader working temperature range than that of the free GOD at the temperature of 25–70 °C, especially in the temperature range 25–55 °C, which could be due to the multipoint interactions between GOD and PDA-coated GO surface [12]. The immobilization of GOD stabilized the rigidity of the enzyme and inhibited the conformational changes in enzyme molecules [12, 35] and then decreased denaturation of enzyme and improved temperature stability of enzyme. Additionally, the biocompatibility of PDA/rGO could also play an important role for the stabilization of GOD conformation. This result was consistent with those reported in the previous literatures [12, 36].

Stability of free GOD and GOD-PDA/rGO

The immobilization of the enzyme often limited its freedom to undergo drastic conformational changes, thus resulting in increased stability [37]. The thermal stabilities of the free GOD and GOD-PDA/rGO were also studied and compared at 40 and 60 °C (as shown in Fig. 6a). The results showed that the enzyme activity decreased with the increase of incubation time. However, the decrease in the activity of the free GOD was much more severe than that of the GOD-PDA/rGO. The GOD-PDA/rGO still retained 65.44 % of its initial activity after 180 min of incubation at 60 °C, whereas only 44.82 % of its initial activity was maintained for the free GOD at the same conditions. These results showed that the immobilization of GOD on PDA/rGO could improve the stability of GOD against heat denaturation. These could be explained as follows: The immobilization of enzyme on a carrier often inhibited the conformational changes of enzyme, decreased denaturation of enzyme, and then improved the enzyme thermostability [35]. The improved enzyme stability in the form of GOD-PDA/rGO could be also attributed to the structural stabilization of GOD because of its extra chemical bonds (covalent bonds) on the PDA/rGO surfaces and geometric confinement imposed by the PDA/rGO that provided an added resistance to protein structure denaturation [38].

The storage stability was also one of the most important features for the application of the biocatalyst. For testing the storage stability, the free GOD and GOD-PDA/rGO in 0.1 M PBS (pH 5.5 and 4.5, respectively) were stored for scheduled times, as shown in Fig. 6b. The GOD-PDA/rGO maintained nearly 83.94 % of its initial activity after stored for 30 days at 4 °C, while the free GOD dropped to the same level of its initial activity only on the seventh day, indicating that the GOD-PDA/rGO was able to retain its activity for a longer period of time

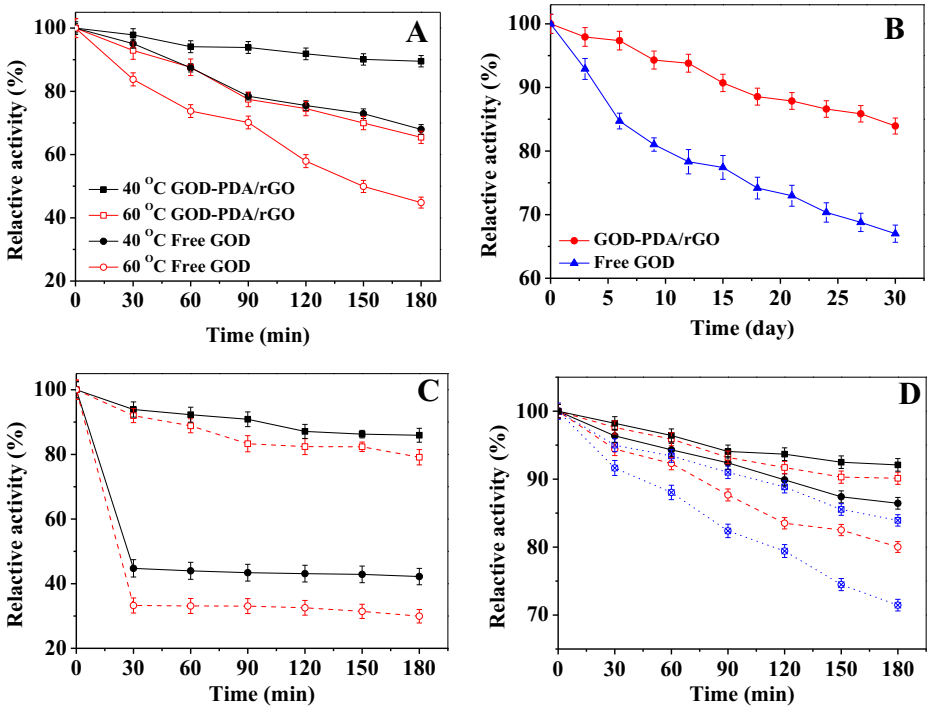

Fig. 6 **a** The thermal stability of free GOD (pH 5.5) and GOD-PDA/rGO (pH 4.5) pre-incubating at 40 and 60 °C for scheduled times; **b** the storage stability of free GOD and GOD-PDA/rGO at 4 °C for scheduled times; **c** the stability of free GOD (*black circle*) and GOD-PDA/rGO (*black square*) against 2 M (—) and 4 M GuHCl (—) at 25 °C for scheduled times; **d** the stability of free GOD (*black circle*) and GOD-PDA/rGO (*black square*) against 2 M (*black drawing light horizontal*), 4 M (*horizontal bar*), and 6 M (*bullet*) urea at 25 °C for scheduled times

than the free GOD. The result showed that the storage stability of GOD was remarkably improved after immobilization. Such a high stability might be ascribed to the good biocompatibility of the PDA/rGO and the effective immobilization of GOD. The enhanced storage stability of GOD arising from immobilization would be an advantage for its industrial application.

It is well known that guanidine hydrochloride (GuHCl) and urea are the most frequently used denaturants to study the protein stability. To evaluate the tolerance of the free GOD and GOD-PDA/rGO to GuHCl and urea, a series of reactions were carried out at different GuHCl and urea concentrations. As shown in Fig. 6c, the GOD-PDA/rGO retained 79.11 % of its initial activity at 4 M GuHCl for 180 min, whereas the activity of the free GOD dropped to 29.93 % under the same conditions, indicating that the free GOD was more sensitive to GuHCl than GOD-PDA/rGO. In addition, at 4 M urea, the free GOD and GOD-PDA/rGO dropped to 80.01and 90.12 % of their initial activities for 180 min, respectively, and the difference was more significant with increase of urea concentrations (as shown in Fig. 4d). These results indicated that the stabilities of GOD against GuHCl and urea were improved after immobilization, and PDA/rGO could protect and stabilize GOD against denaturation. The possible explanation of this stability could be that the active sites of GOD became less accessible for the GuHCl and urea after immobilization [39].

Determination of Kinetic Parameters

The apparent Michaelis-Menten kinetic parameter (K_m) and the maximum reaction velocity (V_{max}) of the free GOD and GOD-PDA/rGO were determined. The kinetic parameters were calculated by curve fitting the plot of reaction rate versus substrate concentrations (as shown in Supporting Information Table S1). The calculated K_m was 27.33 mM for the GOD-PDA/rGO and 22.55 mM for the free GOD, respectively. The results showed that the K_m of the GOD-PDA/rGO was close to that of the free GOD, indicating that the GOD-PDA/rGO possessed high biological affinity to glucose, and PDA/rGO provided a favorable microenvironment for enzyme-substrate reaction. The V_{max} of the GOD-PDA/rGO was also close to that of the free GOD, which might be mainly due to the decrease of the diffusional resistance because the hydrophilic groups of the PDA/rGO could be attributed to the migration of substrate from solution to the active sites of the immobilized GOD [35], and then improved concentration of substrate near the active sites of enzyme, resulting in the improvement of enzymatic efficiency.

Conclusions

In summary, a novel method for immobilizing GOD on GO was developed. GO was firstly modified and reduced by coating PDA layer under mild conditions, and then, the modified GO was successfully used for GOD immobilization. The results showed the GOD-PDA/rGO possessed enzymatic activity in a wider pH and temperature range. It was also noticed that the GOD-PDA/rGO had improved storage stability and durability for GuHCl and urea. The K_m values of the free GOD and GOD-PDA/rGO were similar, indicating that the GOD-PDA/rGO had high affinity for glucose. Hopefully, the method developed in this work can construct a platform for the immobilization of various enzymes.

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