

RESEARCH ARTICLE

Immobilization of formate dehydrogenase on polyethylenimine-grafted graphene oxide with kinetics and stability study

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Abstract

Graphene oxide-based nanomaterials are promising for enzyme immobilization due to the possibilities of functionalizing surface. Polyethylenimine-grafted graphene oxide was constructed as a novel scaffold for immobilization of formate dehydrogenase. Compared with free formate dehydrogenase and graphene oxide adsorbed formate dehydrogenase, thermostability, storage stability, and reusability of polyethylenimine-grafted graphene oxide-formate dehydrogenase were enhanced. Typically, polyethylenimine-grafted graphene oxide-formate dehydrogenase remained 47.4% activity after eight times' repeat reaction. The immobilized capacity of the polyethylenimine-grafted graphene oxide was 2.4-folds of that of graphene oxide. Morphological and functional analysis of polyethylenimine-grafted graphene oxide-formate dehydrogenase was performed and the assembling mechanism based on multi-level interactions was studied. Consequently, this practical and facile strategy will likely find applications in biosynthesis, biosensing, and biomedical engineering.

KEYWORDS

assembly, formate dehydrogenase, graphene oxide, immobilization, polyethylenimine

1 | INTRODUCTION

Polyethylenimine (PEI) has been reported to be a biocompatible for enzyme immobilization for its predominant characteristic of high intensity of positive charge, which prevents the dissociation of enzyme multimers by strengthening the interaction of subunits [1–3]. PEI-CLEAs (cross-linked enzyme aggregates) have been applied to hydrolyze the fish oil and enrich polyunsaturated fatty acids efficiently with organic

solvent tolerance [4]. PEI-metal complexes acted as coating reagents to encapsulate oxidoreductases was also proved to be an efficient way [5,6].

Graphene oxide is an ideal material for the immobilization of enzymes on nanostructured materials due to its functional oxygen-containing groups, which makes it feasible to immobilize various enzymes. The unique structure, biocompatibility, and the possibilities of functionalizing the surface endow graphene oxide-based nanomaterials extensively applied in

Abbreviations: FDH, formate dehydrogenase; GO, graphene oxide; GO-FDH, graphene oxide adsorbed formate dehydrogenase; GO-PEI, polyethylenimine-grafted graphene oxide; PEI, polyethylenimine; SEM, scanning electron microscopy.

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enzymes immobilization [7]. Laccase was immobilized on reduced graphene oxide (rGO)/polymer or onto fGO (functionalized graphene oxide with terminal amine groups) as stable and powerful nanobiocatalysts and exhibited promising biocatalytic behavior [8,9].

Furthermore, the mechanism study of immobilization based on graphene oxide-based nanomaterials attracted great attentions. The improved trypsin activity and stability indicated the PEGylated GO-induced acceleration is substrate-dependent [10]. In our previous study, formate dehydrogenase (FDH) was assembled with graphene oxide-nickel composites (GO-Ni) with enhanced stability, the multiple interactions such as electrostatic forces and coordination bond revealed the immobilization mechanism [11].

The electrostatic interaction between the graphene oxide sheets lead to stacking in aqueous solution. To overcome this problem, one of the most efficient methods is to modify graphene oxide by the polyelectrolytes with opposite charge. Polyethylenimine is regarded as one of the ideal cationic polyelectrolytes to functionalize graphene oxide and form hybrid materials to promote the potential application of GO [12,13]. In this work, polyethylenimine-grafted graphene oxide (GO-PEI) was constructed as a novel scaffold for the immobilization of formate dehydrogenase.

2 | MATERIALS AND METHODS

2.1 | Chemicals

Graphite, NAD^+ , and polyethylenimine (branched PEI, 25 KDa) were purchased from Sigma Chemical Company (Tianjin, China). Kanamycin and IPTG were purchased from TransGen Biotech (Shanghai, China). The LB media was purchased from Sangon Biotech (Shanghai). All other chemicals were analytical grade and purchased from Sinopharm Chemical Reagent (Shanghai, China).

2.2 | Strains and culture conditions

FDH gene was obtained from *Candida boidinii* (190–1284 nucleotide, GeneBank ID: AF004096.1), which was sequenced and synthesized by Sangon Biotech (Shanghai, China). FDH was expressed in a pET28a, BL21 (DE3) with an N-terminal His-tag and cultured in LB media with 50 $\mu\text{g}/\text{mL}$ kanamycin at 37°C until the value of OD_{600} reached 0.5~0.6, then IPTG was added to 1 mM and incubated for 24~36 h at 16°C, 200 rpm.

2.3 | Enzyme extraction and purification

The cells were centrifuged at 8000 rpm for 10 min, washed twice with PBS buffer and resuspended, and ultrasonicated for 35 times of 3 s working and 6 s cooling in an ice bath. Then the mixtures were centrifuged at 10 000 rpm for 15 min

PRACTICAL APPLICATION

In this study, polyethylenimine-grafted graphene oxide was applied for the immobilization of formate dehydrogenase with enhanced stability and reusability. Morphological characterization and kinetics of immobilized enzyme were performed to verify the mechanism insights of the enzyme stability improvements. Multi-level interactions between and enzyme and scaffold polyethylenimine-grafted graphene oxide was analyzed. A practical and simple strategy based on graphene oxide polymer as biocompatible supporter for enzyme immobilization was developed, which is potentially applied in the biosynthesis, biosensing, and biomedical engineering.

under 4°C to remove the debris of cells. After that, the crude extract was filtered through a membrane filter (0.22 μm) and loaded onto a 5 mL His-Trap HP affinity column. Ten column volumes of binding buffer (20 mM sodium phosphate, 0.5 M NaCl, 20 mM imidazole, pH 7.4) were used to wash the unbound protein. The column was eluted with elution buffer (20 mM sodium phosphate, 0.5 M NaCl, 0.5 M imidazole, pH 7.4) by one-step elution. The fractions with the desired activity were desalted and concentrated using a Macrosep Advance Centrifugal Device (cut-off 10 kDa, Pall, East Hills, NY, USA). Protein concentrations were determined with a modified Bradford protein assay kit (Sangon Biotech) using bovine serum albumin as a standard, as described by Bradford.

2.4 | Enzyme activity assay

FDH activity was measured according to the methods reported [11]. Concentration of NADH was detected at 340 nm using an Infinite 200 PRO microplate reader (TECAN). One unit (U) of enzyme activity was defined as the quantity of enzyme catalyzing the formation of 1 μmol NADH per min. Specific activity was recorded as U/mg protein. Parallel experiments were repeated four times.

To investigate the effect of pH on free enzyme and immobilized enzyme, enzyme activities were measured in the buffer with different pH range (potassium phosphate buffer, pH 6.0~7.5; Tris-HCl buffer, pH 7.5~10.0; Glycine-NaOH buffer, pH 10.0~11.0). Temperature effects on the activities of the enzyme were conducted at the temperature ranging from 30 to 70°C. Thermostability of enzymes was measured by calculating the residual enzymatic activity of free and immobilized FDH after incubating under pH 10 and 60°C and taking samples every 15 min. The activity was expressed as relative

forms (%) with the maximal value of enzyme activity at a certain pH or temperature as 100%.

2.5 | Determination of kinetic parameters

The kinetic parameters of FDH and the immobilized FDH were studied by assaying the enzyme activity with sodium formate at different concentrations (5–80 mM). Lineweaver–Burk method was applied for the calculation of K_m , V_{max} , and k_{cat} .

2.6 | Preparation of immobilized enzymes GO-PEI-FDH

Graphene oxide (GO) was prepared by the Modified Hummers method [14,15]. The carboxylation process is to modify hydrogen groups and epoxy groups on the surface of graphene oxide. Here, 2.5 g NaOH and 2.5 g sodium hypochlorite were added to 50 mL graphene oxide solution (1 mg/mL), and after ultrasonic procedure (100 W, 2 h), the mixture was dialyzed for 3~5 days to get the GO-COOH solution.

Ten milliliters of PEI solution (10 mg/mL, pH 8) was added to 10 mL GO-COOH solution (1 mg/mL) mentioned above and dispersed by ultrasonic method for 5 min. Fifty milligram 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride was then added to the mixture and stirred for 30 min at room temperature, extra 150 mg ethylcarbodiimide hydrochloride was added to the mixture and reacted for 12 h. After the reaction, 2 g NaCl and 2 g urea were added to the mixture, and centrifuged for 15 min at 10 000 rpm to remove the precipitate. The supernatant was collected and used 100 KDa Microsep to ultrafilter for over four times for removing the unbound PEI and getting the stable polyethyleneimine grafted graphene oxide solution (GO-PEI).

FDH was immobilized on the GO and GO-PEI, respectively to investigate the activities of the enzymes before and after immobilization [11]. FDH solution was dispersed in 1 mg/mL GO-PEI solution mentioned above. The mixture was incubated at 4°C for 4 h with occasional shaking, and then centrifuged at 8000 rpm for 15 min. The supernatant was collected to determine the enzyme loading capacity. The precipitate was centrifuged and rinsed three times with the Tris-HCl buffer (pH 10) to remove unabsorbed enzymes. Amount of immobilized enzyme was evaluated by the difference of enzyme concentration before and after immobilization in solution.

2.7 | Morphology characterization

Morphology of nanoparticles was observed by scanning electron microscopy (SEM, Zeiss Sigma). For SEM, 10 μ L of the immobilized enzyme was dropped on a silicon chip and allowed to evaporate and dry overnight at room temperature,

the sample was then coated with platinum (2-nm thickness) using a JEOL JFC 1600 (JEOL, Tokyo, Japan) with an electric current of 10 mA for 30 s before imaging with a Zeiss Sigma SEM (Carl-Zeiss AG, Germany). Circular dichroism spectrum (CD) of FDH, GO adsorbed FDH (GO-FDH), and GO-PEI-FDH were determined by a Jasco J-810 CD spectropolarimeter (Biologic, Japan) within the range of 190–400 nm with an interval of 1 nm.

2.8 | Storage stability and reusability

Free FDH and immobilized enzyme GO-FDH, GO-PEI-FDH were stored at 4°C, the storage stabilities were studied and compared by measuring the residual activities every 2 days within 20 days. The reusability of immobilized enzyme GO-FDH and GO-PEI-FDH were explored by repeating the enzymatic reaction for eight times. After finishing each cycle of reaction, the immobilized enzyme was washed by Tris-HCl buffer (pH 10) and re-collected by centrifugation at 10 000 rpm for 15 min (4°C). The first cycle of enzyme activity was set as 100%, and the remaining activity was counted for percent form (%) compared with that of the first reaction.

3 | RESULTS AND DISCUSSION

3.1 | Loading capacity of GO-PEI

To investigate the enzyme loading capacity of GO-PEI, purified FDH (0–0.8 mg/mL) was added to GO and GO-PEI solution, respectively. The immobilization capacity of GO-PEI (0.566 mg/mg), which was enhanced by 2.4 folds compared with GO (0.231 mg/mg) under pH 10 (Figure 1). Increasing of loading capacity could be explained by cationic properties of PEI, which prevent graphene oxide from stacking and increase the binding surface.

3.2 | Recovery yield of enzyme activity

Recovery of enzyme activity is a key parameter for enzyme immobilization. Activity recovery of immobilized enzyme GO-FDH and GO-PEI-FDH were 73.3 and 78.9%, respectively (Figure 2). Conjugating graphene oxide surface with branched polyethyleneimine would relieve the electrostatic and hydrophobic interactions which may change the conformation of proteins and disrupt the biological activity [16]. Therefore, GO-PEI as hybrid material not only provided abundant binding sites for further increasing the loading capacity of enzyme, but also acted as a soft biocompatible scaffold to enhance the enzyme stability.

Turnover number (k_{cat}) and enzyme efficiency (k_{cat}/K_m) of GO-FDH and GO-PEI-FDH were further compared. K_m and

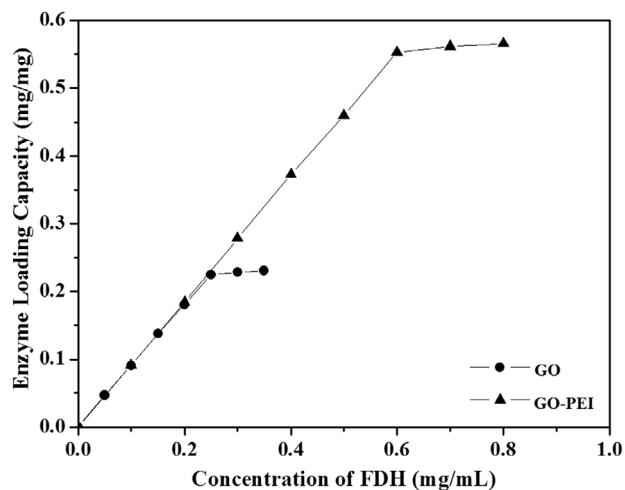


FIGURE 1 Effect of FDH concentration on the loading capacity of carriers. The concentration of graphene oxide was 1 mg/mL, the immobilization was conducted at pH 10

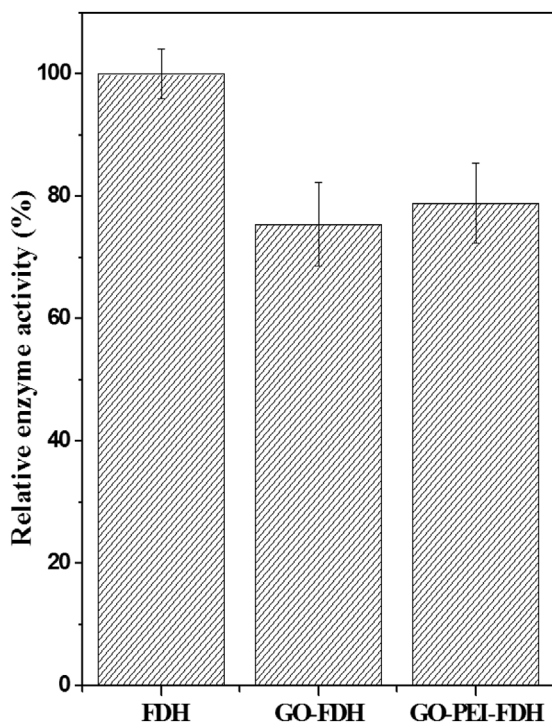


FIGURE 2 The relative activity of free FDH, GO-FDH and GO-PEI-FDH. Reaction conditions: sodium formate, 162 mM; NAD^+ , 1.62 mM; pH, 10; temperature, 30°C. The relative activity is expressed as a percentage of the original activity of free FDH

k_{cat} values were calculated by the Lineweaver–Burk method, which were listed in Table 1. The similar K_m values of the GO-FDH, GO-PEI-FDH, and free FDH indicated the similar affinity of the substrate. However, k_{cat} and k_{cat}/K_m of GO-FDH and GO-PEI-FDH were lower than those of free FDH, suggesting the decreasing catalytic efficiency of immobilized enzymes.

TABLE 1 Enzyme kinetic parameters

Samples	V_{max} ($\mu\text{M}/\text{min}/\text{mL}$)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\text{s}^{-1} \text{mM}^{-1}$)
FDH	0.0132	5.2770	3.4693	0.6574
GO-FDH	0.0129	5.6880	3.3820	0.5946
GO-PEI-FDH	0.0123	6.0714	3.2758	0.5395

3.3 | Effect of temperature and pH

The optimal reaction temperature and reaction pH were investigated. In the tested temperature range, the optimum temperature of FDH, GO-FDH, and GO-PEI-FDH was 60°C (Figure 3A). The scaffolds GO and GO-PEI did not change the optimal reaction temperature of FDH. Interestingly, GO-FDH and GO-PEI-FDH exhibited a higher relative enzyme activity than the free enzyme at temperatures between 40–70°C, indicating that thermostability was enhanced. The immobilized enzyme GO-FDH and GO-PEI-FDH kept the same optimal reaction pH of free FDH (Figure 3B). When pH is more than 10, positively charged PEI buffered pH change from the bulk solution, illustrating biocompatibility of GO-PEI. Moreover, PEI can prevent the dissociation of multimeric enzyme subunits, and maintain the activity of GO-PEI-FDH in an alkaline environment [5].

3.4 | Stability of immobilized enzymes

Thermostability of free FDH and immobilized FDH was studied under 60°C by measuring the residual activities every 30 min. GO-FDH and GO-PEI-FDH enzyme activity retained 18.2 and 24.8% of its initial activity, respectively, while free FDH only retained 2.78% of its initial activity (Figure 3C). Compared with FDH and GO-FDH, GO-PEI-FDH exhibited enhanced thermostability due to the self-assembled structure of the GO-PEI and FDH. Positively charged PEI with a high proportion of amine protonation interacts with FDH through electrostatic interactions and hydrogen bonds, and then maintain the structure of protein at high temperature [17].

To investigate the storage stability, the free enzyme and immobilized enzyme were both stored at 4°C and the enzyme activity was determined every two days (Figure 3D). After 20 days, GO-PEI-FDH retained 24.9% of its initial activity, and GO-FDH retained 22.8%, while free FDH only maintained 3.48%. The results indicated the scaffolds GO and GO-PEI greatly improved the storage stability of enzyme. During the long storage, GO-FDH tended to aggregate, however, as a cationic polymer, PEI can bind with negatively charged graphene oxide to promote uniform dispersion of graphene oxide and reduce the degree of aggregation, which lead to the better storability of GO-PEI-FDH over GO-FDH.

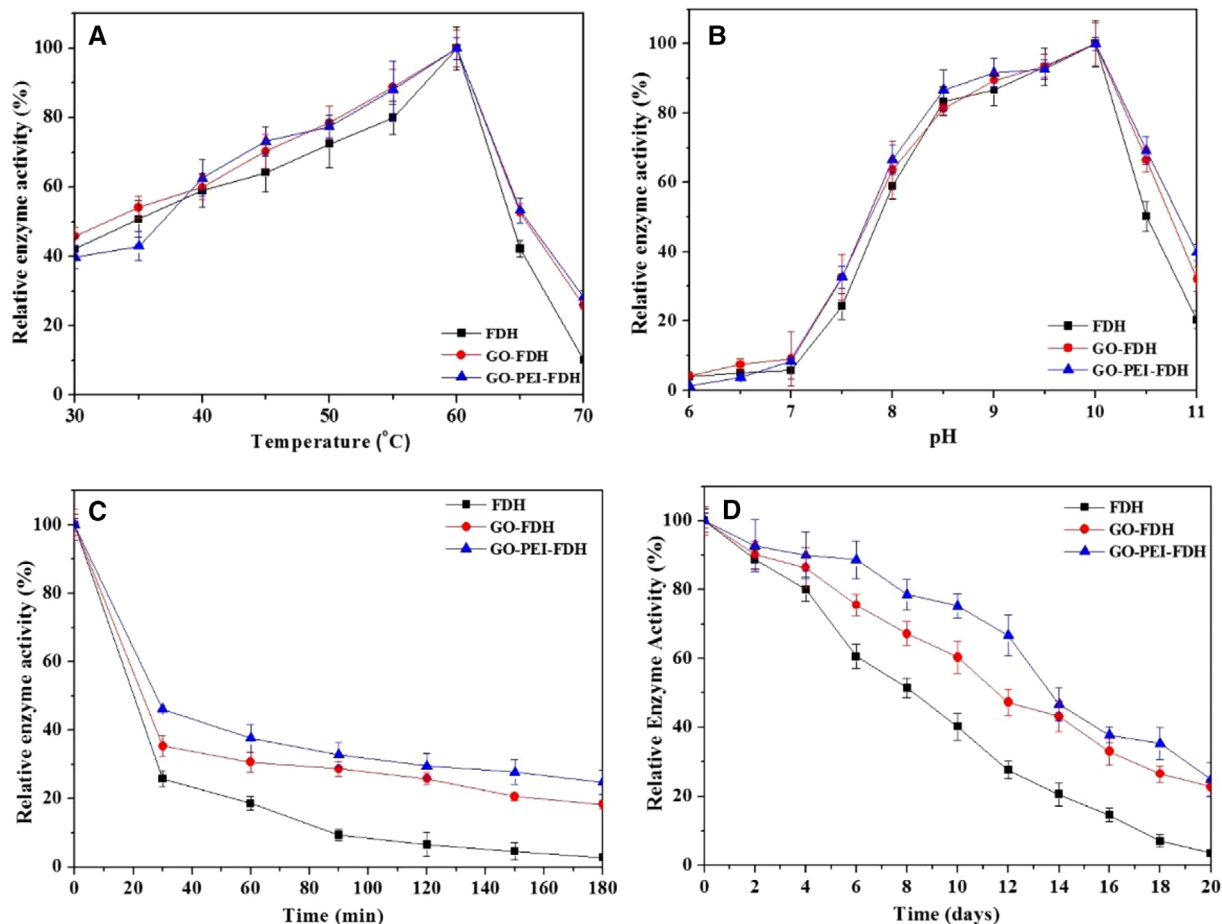


FIGURE 3 Enzyme properties of immobilized FDH and free FDH. (A) Optimal reaction temperature of immobilized FDH and free FDH. Reaction conditions: sodium formate, 162 mM; NAD^+ , 1.62 mM; pH, 10; temperature, 30°C. The activity was expressed as relative forms (%) with the maximal value of enzyme activity at 60°C as 100%. (B) Optimal reaction pH for immobilized FDH and free FDH. Reaction conditions: sodium formate, 162 mM; NAD^+ , 1.62 mM; temperature, 30°C. The activity was expressed as relative forms (%) with the maximal value of enzyme activity at pH 10 as 100%. (C) Thermostability of immobilized FDH and free FDH at 60°C. Reaction conditions: sodium formate, 162 mM; NAD^+ , 1.62 mM; pH, 10.0; temperature, 30°C. The activity was expressed as relative form (%) with the maximal value of enzyme activity at 0 min as 100%. (D) Storage stability of immobilized FDH and free FDH at 4°C. Reaction conditions: sodium formate, 162 mM; NAD^+ , 1.62 mM; pH, 10; temperature, 30°C. The activity was expressed as relative form (%) with the maximal value of enzyme activity at 0 days as 100%

3.5 | Reuse stability

The reusability of GO-FDH and GO-PEI-FDH nanoparticles were also exploited. The activity of GO-FDH and GO-PEI-FDH gradually decreased over eight cycles' reaction (Figure 4). GO-PEI-FDH retained 47.4% of its initial activity after cycling through eight reactions while GO-FDH kept 27.3%. Compared with GO-FDH, activity of GO-PEI-FDH dropped slowly, exhibiting the enhanced interactions between GO-PEI and FDH. Activity loss of GO-FDH was mainly caused by the desorption of FDH. For GO, the negative charged surface lead to the adsorption of enzyme by electrostatic interaction. Compared with GO-FDH, interaction strength of FDH with GO-PEI based on electrostatic interaction and hydrogen bonds was much stronger. PEI not only offer bind sites with the functional groups of graphene oxide, but also react with the functional amino acid residues

(histidine, aspartic acid, arginine, etc.) of FDH. The electrostatic interaction allows FDH to bind tightly to GO-PEI. In addition, van der Waals forces, hydrogen bonds were also present as auxiliary binding interaction between GO, PEI, and FDH.

3.6 | Morphological analysis of GO-FDH and GO-PEI-FDH

Morphology of the immobilized FDH was measured by SEM. PEI-grafted graphene oxide exhibited amorphous structure (Figure 5B), which was quite different from the layer structure of graphene oxide. GO-PEI-FDH formed uniform particles (Figure 5C), showing rectangular and planar structure about 0.5–1 μm . Mechanism insight of assembly FDH with GO-PEI was investigated based on the analysis of multi-level interactions comprised of the electrostatic interaction, hydrophobic

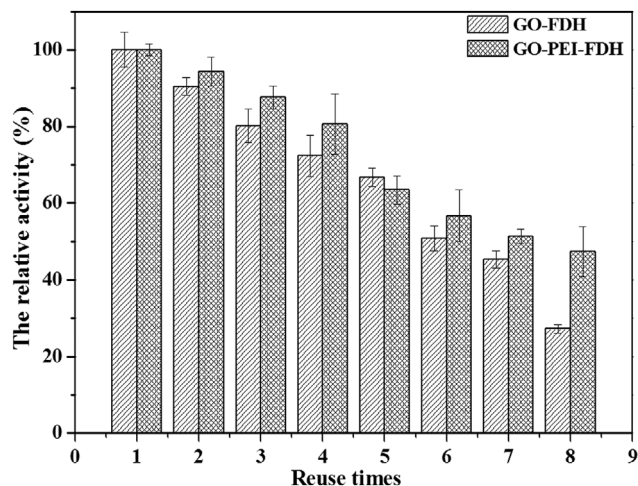


FIGURE 4 The recycling stability of GO-FDH and GO-PEI-FDH. Reaction conditions: sodium formate, 162 mM; NAD^+ , 1.62 mM; pH, 10; temperature, 30°C

interaction, and hydrogen bonds. In addition, GO-PEI-FDH achieved good dispersion than GO-FDH in solution, which can be explained by the electrostatic repulsion between PEI and PEI. The dispersion of the immobilization enzyme was beneficial for the retainment of enzyme activity due to its lower steric hindrance to the active sites. Moreover, multiple interactions of GO, PEI, and FDH ensured assembly balance during the immobilization process, thus formed the layer-by-layer sandwich structure.

GO is an oxygen-rich derivative of graphite, which was decorated with hydroxyl, epoxy, and carboxyl groups. These oxygen-containing groups were distributed randomly on the basal planes and edges of the GO sheets and provided a negative charged surface [18,19]. The enzyme loaded on GO is governed by the electrostatic interaction. Furthermore, due to their polarity, weak interactions with protein such as hydrogen bonds were formed (Figure 5D). The unmodified areas of the surface maintained hydrophobicity and free π -electrons,

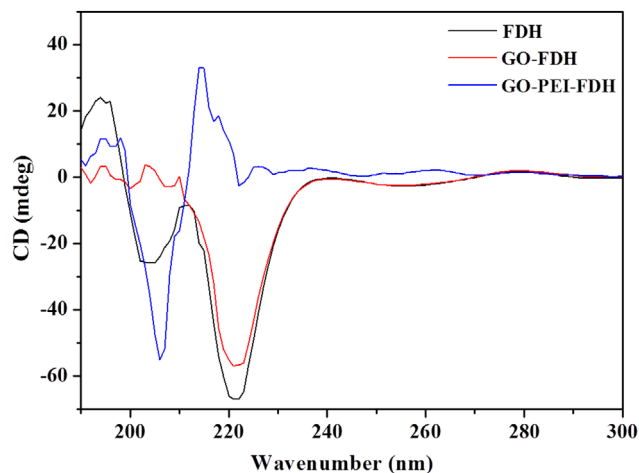


FIGURE 6 Circular dichroism spectrum of FDH, GO-FDH, and GO-PEI-FDH. Samples were dissolved in PBS solution; detection temperature was 25°C

making π - π interactions feasible, therefore multi-level interaction of GO with enzyme may affect conformational alteration and catalytic activity of FDH. Physicochemical property of nanomaterials influences the interactions with biomolecules and subsequently affects the immobilization as well as the conformation and biological function of conjugated enzyme.

3.7 | Structural analysis and mechanism study

To further explain the alteration of the catalytic behavior of immobilized enzymes and immobilization mechanism, the change of enzymes' secondary structure after immobilization was studied by circular dichroism (CD). GO-PEI-FDH had a significant negative absorption peak at 205 nm and a significant positive absorption at 218 nm, while the negative absorption peak at 222 nm and the positive absorption peak at 193 nm were relatively weak (Figure 6), indicating

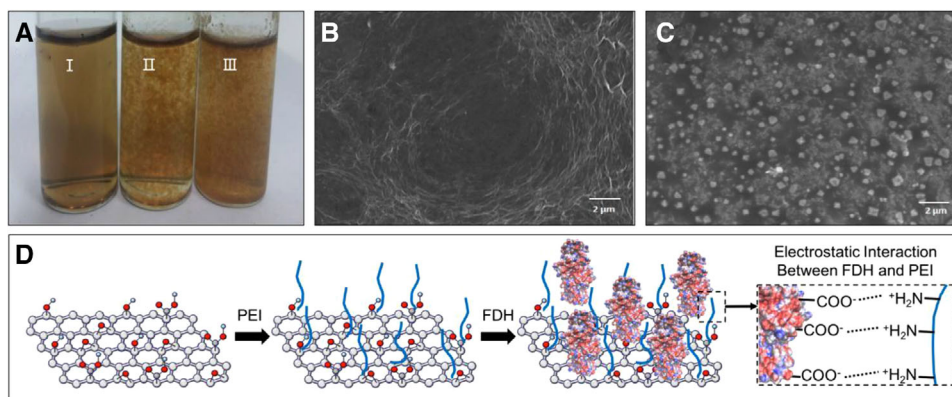


FIGURE 5 Morphology and immobilization mechanism. (A) The photographs of GO (I), GO-FDH(II), and GO-PEI-FDH(III) solution; (B) SEM image of scaffold GO-PEI; (C) SEM image of immobilized enzyme GO-PEI-FDH; (D) speculative mechanism of the immobilization process of GO-PEI-FDH

significantly reducing of the α -helical structure of FDH during the immobilization process [20,21]. The obvious change of the secondary structure of GO-PEI-FDH may be due to the interaction between GO-PEI and FDH.

GO-based nanomaterials have been applied as a solid substrate for enzyme immobilization through non-covalent adsorption and covalent binding. Activity of immobilized enzymes is affected by a combination of the GO chemistry, protein intrinsic property, and the immobilization procedure [22,23]. The conformation change and activity variations of immobilized enzymes based on GO have been investigated. The interaction with GO induced a structural transformation from α -helix to β -sheet, even to the unfolding of the glucose oxidase, which resulted in the main loss of enzyme activity [24]. Similar phenomenon was found in bilirubin oxidase, horseradish peroxidase, catalase, cytochrome c, and esterase [25–29]. The relation between the alteration of catalytic properties and structure transformation merits further investigation.

4 | CONCLUDING REMARKS

PEI drafted graphene oxide was applied for the immobilization of formate dehydrogenase. The stability and reusability of the immobilized enzyme have been compared with free FDH. The enzyme loading capacity of GO-PEI was 2.4-fold of that of GO. GO-PEI-FDH exhibited better stability and reusability. Morphological characterization and inactivation kinetics of GO-PEI-FDH were performed to propose the mechanism of stability enhancement. Multi-level interaction analysis of FDH and GO-PEI indicated that the electrostatic force played a significant role in the assembling process. Therefore, it is a practical and simple strategy and was developed based on GO-PEI as biocompatible supporter for enzyme immobilization, which is potentially applied in various biotechnological applications.

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CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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