Metal-Organic Framework Hybrid Materials of ZIF-8/RGO for Immobilization of D–amino Acid Dehydrogenase

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Abstract

Immobilization of D-amino acid dehydrogenase (DAADH) by the assembly of peptide linker was studied for biosynthesis of D-phenylalanine which is an unnatural amino acid. Hybrid material of ZIF-8 and reduced graphene oxide (RGO) were applied for the immobilization of DAADH from Ureibacillus thermosphaericus. Activity of DAADH/ZIF-8/RGO was enhanced by 1.65 folds than free enzyme. DAADH/ZIF-8/RGO remained 53.4% of its initial activity at 50 °C for 10 h. At the same time the free enzyme was inactivated. The result indicated that the immobilization greatly improved the thermostability of DAADH and the stability in hyperalkaline solution. Kinetic parameters indicated that DAADH/ZIF-8/RGO had greater affinity of phenylpyruvate as Vm /Km of DAADH/ZIF-8/RGO was 1.27-fold than free enzyme. After seven recycles, the activity of DAADH/ZIF-8/RGO remained 64.3%. Furthermore, one step separation and immobilization by ZIF-8/RGO/Ni-DAADH had 1.5-fold activity enhancement. Combination of peptide linker and MOF immobilization, thermostability of the dehydrogenase was significantly improved.

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Immobilization of D-amino acid dehydrogenase (DAADH) by the assembly of peptide linker was studied for biosynthesis of D-phenylalanine which is an unnatural amino acid. Hybrid material of ZIF-8 and reduced graphene oxide (RGO) were applied for the immobilization of DAADH from *Ureibacillus thermosphaericus* . Activity of DAADH/ZIF-8/RGO was enhanced by 1.65 folds than free enzyme. DAADH/ZIF-8/RGO remained 53.4% of its initial activity at 50 °C for 10 h. At the same time the free enzyme was inactivated. The

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Keywords : D-amino acid dehydrogenase; Immobilization; Peptide linker; Metal-organic frameworks;Reduced graphene oxide;

1. Introduction

D-enantiomers of amino acids are rarely applied for protein synthesis during evolution. In recent years, D-amino acids have been increasingly used as intermediates to produce pharmaceuticals and fine chemicals [1]. D-amino acid can be environmentally produced by a one-step reaction using D-amino acid dehydrogenase (DAADHs) [2] . Several $NAD(P)^+$-dependent L-amino acid dehydrogenases have been reported for the synthesis of L-amino acids while D-amino acid dehydrogenases are less for D-amino acids [3]. Different from L-amino acids, D-amino acid are recognized as the "unnatural" amino acids, which means D-amino acids have unique potential in pharmaceuticals and fine chemicals while DAADHs are more likely unstable and with low catalytic efficiency [4]. Therefore, researchers long for obtaining DAADHs with high stability so that DAADHs can be widely applied in industrial production. $NADP^+$-dependent D-amino acid dehydrogenase (DAADH) from *Ureibacillus thermosphaericus* , which is meso-diaminopimelate dehydrogenase, for synthesis of D-leucine and D-isoleucine through site-directed mutagenesis was reported [5].

Akita et al. [6] reported an NADPH-dependent DAADH mutated from meso-diaminopimelate dehydrogenase for synthesis of D-branched-chain amino acids with high yields and optical purity. Meso-diaminopimelate dehydrogenase has been reported to be used for the synthesis of D-amino acid from 2-keto acids by one-step reductive amination [7]. Cui et al. [8] reported biosynthesis of D-phenylalanine by tri-enzymatic cascade from Proteus vulgaris meso-diaminopimelate dehydrogenase with 96.3 % conversion rate and $>$ 99 % enantioselectivity on a 3 L scale. However, DAADHs are not sufficiently stable [5]. Immobilization of enzymes would be a good way to solve the problem.

Metal-Organic Frameworks (MOFs) have extremely high specific surface area, abundant porosity, extraordinary functionality, and relatively high stability [9]. Immobilization of enzyme into metal–organic frameworks (MOFs) is performed through a one-step and facile method. Liang et al. [10] reported peptide-induced super-assembly of MOFs for programmed multi-enzyme cascade reactions, which showed 7.3-fold and 4.4-fold catalytic activity enhancements for the two-enzyme and three-enzyme cascade reactions, respectively. Sha et al. [11] reported that they stabilized enzyme cytochrome c by encapsulating it in a hierarchical mesoporous zirconium-based MOF, NU-1000 against denaturing organic solvents. Immobilized cytochrome c has significantly enhanced activity compared to the native enzyme. Gascon et al. [12] studied the use of a metal–organic framework (MOF) as a support for the*in situ* immobilization of alcohol dehydrogenase with enhancement of stability.

Peptides are applied as linkers for fusion enzymes. Peptides linkers would be beneficial to improve catalytic efficiency and stability of enzyme. Liu et al. [13] reported that a collagen-like polypeptide (CLP) and an elastin-like polypeptide (ELP) were fused to D-amino acid oxidase (DAAO).The catalytic efficiency of DAAO-CLP-ELP was 1.7-fold that of DAAO. Wang et al. [14] reported the MOF immobilized L-phenylalanine dehydrogenase. The stability of immobilized enzyme with peptide linker was greatly enhanced at 70-80 and pH 10-11. Du et al. [15] reported that an elastin-like polypeptide (ELP) was fused to D-amino acid oxidases (DAAO). ELP–DAAO exhibited a better solubility in aqueous solutions than DAAO, and its enzymatic activity is about 1.6 times than of DAAO. Adam et al. [16] reported that the role of peptide linker properties was investigated for fusions of a leucine zipper immobilization domain to a chimeric amine

dehydrogenase or a formate dehydrogenase, which aimed at providing a linker library. Song et al. [17] combined a zwitterionic peptide with iron metal-organic framework to develop a sensitive electrochemical enzyme sensing platform for T4 polynucleotide kinase detection.

$(RTHRK)_4$ is conductive peptide from Cytochrome P450, which can facilitate electron transfer. [18] Designing should be considered [19] so that $(RTHRK)_4$ can be connected appropriately to the N-terminus of DAADH. Immobilization of DAADH from *Ureibacillus thermosphaericus* by encapsulating them within MOF was proposed. Then, the hybrid materials of 2-methylimidazole zinc salt (ZIF-8) with reduced graphene oxide (RGO), such as ZIF-8/RGO and ZIF-8/RGO/Ni, were applied for enzyme immobilization. Catalytic activity, thermostability, reuse stability and morphological characterization of immobilized enzyme were investigated. Mechanism of immobilization of DAADH with peptide linker in ZIF-8 was investigated by multi-level interactions and kinetic study. Furthermore, *in situ*immobilization by ZIF-8/RGO/Ni was studied.

2. Materials and methods

2.1 Structures of DAADH

DAADH originating from*Ureibacillus thermosphaericus* was selected (PDB ID: 5gz6). An expression vector for DAADH_5gz6 with polypeptide linker (amino acid sequence: $(RTHRK)_4$) was constructed by amplifying the DAADH_5gz6 gene fragment infusion by PCR. The amplified gene fragment was digested with Nde I and Xho I and ligated with the expression vector*E.coli* BL21(DE3)/pET28a to generate pET28a/ DAADH_5gz6pep, which was then transformed into *Escherichia coli* strain Rosetta (DE3) (Novagen). The experiment used the plasmid extraction and gel recovery kit produced by Takara. Added 500 μL of recombinant expression strains to 500 μL 50% sterile glycerin, mixed and stored at -20 .

Cells were cultivated overnight at 37 and 200 r/min in 10 mL of LB medium containing 50 μg/L kanamycin. The culture solution was transferred to 100 ml of LB medium containing 50 μg /L kanamycin by volume fraction of 1 %, and cultured at 37 for 2-3 h until the optical density at 600 nm reached 0.6-0.8. Then added isopropyl-β-d-thiogalactopyranoside (IPTG) with final concentration as 1 mM, and continued to cultivate for 12 h at 25 .

2.2. Chemicals

Kanamycin and isopropyl-beta-D-thiogalactopyranoside (IPTG) were purchased from Trans-Gen Biotech (Shanghai, China). NADPH, ionic liquids, phenylpyruvate and all other chemicals were purchased from Sigma Chemical Company (Tianjin, China).

RGO is prepared from our laboratory. Methods: Add 0.05 g ascorbic acid to 1 mL, 1 mg/mL GO dispersion and stirred at room temperature for 12 h. The solution was centrifuged at 8000 rpm for 15 min and then was resuspended with deionized water, then centrifuged and resuspended in same condition, finally obtained 1 mg/mL RGO dispersion.

2.3. Enzyme extraction and purification

Cells were harvested by centrifugation, then suspended in potassium phosphate buffer (pH 7.4,10 mM), and disrupted by untrasonication (200 W, 3 s, 85 times) in ice water bath. The cell debris was removed by centrifugation (8000 rpm) for 20 min.

The DAADH obtained by recombination-induced expression was labeled with His-tag. Ni-NAT His Bind Resin column (Shanghai Qixing Company) was then equilibrated with 10 column volumes of loading buffer (20 mM Tris, 0.25 M NaCl, pH 7.4). Crude enzyme solution was collected by filtration through the membrane

filter (0.45 μm) and loaded into binding column. The column was then washed with ten column volumes of binding buffer (20 mM imidazole, 20 mM Tris, 0.25 M NaCl, pH 7.4). Finally, target protein was eluted with elution buffer (200 mM imidazole, 20 mM Tris, 0.25 M NaCl, pH 7.4) and was desalted and concentrated by Millipore ultrafiltration tube, the content of pure enzyme protein was 0.169 mg/mL.

2.4. Enzyme assay

The activities of DAADH and immobilized DAADH were determined by using phenylpyruvate as substrate in NH_4Cl-$NH_3{\cdot}H_2O$ buffer (200 mM, pH 9.5). The activity was measured by detecting the NADPH concentration at 340 nm by Infinite 200 PRO spectramicroplate reader (TECAN). The initial velocities was calculated as consumption rate of NADPH when the conversion rate of substrate was below 5%. Specific activity was recorded as U/mg enzyme. The amount of enzyme consumed for generation or consumption of 1 μmol of NADPH per minute was defined as one unit of enzyme activity. Specific activity (U/mg) is defined as the ratio of enzyme activity (U/mL) to protein concentration (mg/mL) at 30 . Parallel experiments were repeated four times. The relative activity was expressed as the maximal value of enzyme activity at a certain pH or temperature as 100%.

Fig.1

The chiral purity of D-phenylalanine was analyzed by HPLC. HPLC analysis with standard solution of L-phenylalanine and D-phenylalanine showed that the peak time of L-phenylalanine was about 25 min, and the peak time of D-phenylalanine was in 33 min (Fig.S1). HPLC detection condition for D/L phenylalanine was as follow: Chromatographic column: Philomon Chirex 3126 (D)-Penicillamine 250 nm×4.6 nm, 5 μm; mobile phase: 5% (v/v) acetonitrile and 95% (v/v) 2.0 mM, $CuSO_4$ aqueous solution; flow rate: 0.8 mL/min; detection wavelength: 254 nm.

Fig.S1

2.5. Preparation of immobilized enzyme

D-amino acid dehydrogenase immobilized in ZIF-8 (DAADH/ZIF-8) were prepared by mixing zinc nitrate solution (0.16 M, 500 μL), enzyme solution (500 μL) and 2-methylimidazole (0.16 M, 500 μL) together and then shaking at 4 , 200 rpm, 30 min. The mixing solution was centrifugated at 4 , 8000 rpm, 15 min. The supernatant was collected to measure the protein concentration for further determination of enzyme loading rate. The precipitate was washed with deionized water three times, and then resuspended it with PBS buffer (200 mM, pH 7.4).

D-amino acid dehydrogenase immobilized in hybrid materials of ZIF-8 and RGO (DAADH/ZIF-8/RGO) was prepared by mixing zinc nitrate solution (0.16 M, 500 μL), enzyme solution (500 μL), 2-methylimidazole (0.16 M, 500 μL) and RGO (1 mg/mL,500 μL). After shaking at 4 , 200 rpm for 4 h, the solution was centrifuged at 4 , 8000 rpm, 15 min. The supernatant was collected and measure the protein concentration for further determination of enzyme loading rate. The precipitate was washed with deionized water three times, and then resuspended it with PBS buffer (200 mM, pH 7.4).

One-step separation and immobilization of enzyme from crude solution was investigated. D-amino acid dehydrogenase immobilized in hybrid materials of ZIF-8, RGO and Ni (DAADH-ZIF-8/RGO/Ni-DAADH) was prepared by mixing zinc nitrate solution (0.16 M, 500 μL), $NiCl_2$ (0.08 M, 500 μL), enzyme solution (500 μL), 2-methylimidazole (0.16 M, 500 μL) and RGO (1 mg/mL,500 μL), and followed by the shaking, centrifugation and resuspending as above.

2.6. Morphology characterization

Morphology analysis of immobilized enzyme was carried out by scanning electron microscopy with a Zeiss Sigma scanning electronic microscopy (SEM) (Carl-Zeiss AG, Germany). JEOL JFC 1600 (JEOL, Tokyo,

Japan) was used to spray platinum on the sample to make it conductive.

2.7. Reusability

Reusability of immobilized enzyme was studied by recycling the enzymatic reaction for seven times. At the ending of each reaction, the immobilized enzyme was re-collected by centrifugation at 8000 rpm for 15 min (4 °C) and washed with PBS buffer (pH 7.4). Initial enzyme activity of first cycle was set as 100 %, and the remaining activity was counted for percent form (%) compared with that of the first reaction.

2.8. Thermostability

The enzyme was incubated in water bath at 50 °C for 10 h. The enzyme activity was measured by taking samples every 2 h (at 0,2,4,6,8,10 h). The relative enzyme activity was calculated by setting the enzyme activity of the unincubated enzyme solution as 100%.

3. Results and discussion

3.1 Effects of metal ions

Several divalent metal ions (Ca^{2+}, Mn^{2+}, Zn^{2+}, Mg^{2+}, Ni^{2+}, Cu^{2+} and Ba^{2+}) were selected to investigate the effect of metal ions on DAADH activity (Fig.2a). Ni^{2+} activated DAADH by 128.3%. Ca^{2+}, Ba^{2+}, Mg^{2+} inhibit the activity of DAADH. The effect of Ni^{2+} concentrations on enzyme activity was studied as shown (Fig.2b). Maximum enzyme activity was obtained with 15 mM Ni^{2+}, which was 161.2% of the initial activity .

Fig.2

Metal ions can interact with amino acid residues and change the three-dimensional structure of enzymes,which could affect the substrate channel that substrates access or egress [20].

The software Caver 3.0.3 was used to simulate the substrate channel. As shown in Fig.S2, the peptide linker (green) and the loop region were at the end of the enzyme molecule. Adding the peptide linker changed the substrate channel (blue), which may affect the enzyme activity [21].

Fig.S2

3.2 Effect of ionic liquids

Ionic liquids (ILs) are attractive in biocatalysis as ILs' unique chemical properties. Several ionic liquids ([EMIM]BF_4, [BMIM]Cl, [BMIM]BF_4) were selected and added into the reaction system with final concentration as 5 mM. [EMIM]$BF_4$and [BMIM]Cl reduced the relative enzyme activity (DAADH) to 47.7% and 68.6% respectively, while the activity of DAADH in [BMIM]BF_4 ionic liquid was 97.8% of the initial enzyme activity (Fig.3).

The electrostatic interactions between Ionic liquid and amino acid residues could affect enzyme activity [22]. Solvation effects is also a factor to be considered. [BMIM]BF_4 displays an excellent miscibility with water which could be beneficial to enzyme activity [23].

Fig.3

3.3 Recovery rate of immobilized enzyme

MOF ZIF-8 was selected for enzyme immobilization for its low cost and biocompatibility. DAADH were encapsulated into the ZIF-8 framework by a biomimetic mineralization. Enzyme immobilization by ZIF-8 and hybrid material of ZIF-8 with graphene (ZIF-8/RGO) were investigated. The loading rate of enzyme to both carriers are above 99%. Compared with free DAADH, the activity DAADH/ZIF-8 was only 60.0% while the activity of DAADH/ZIF-8/RGO was enhanced by 165.6% (Fig.4).

The results indicated that the addition of RGO can form hybrid material with higher biocompatibility, probably due to hydrophobic interaction between RGO and DAADH [24], which can increase the stability of DAADH/ZIF-8/RGO. ZIF-8/RGO is a good carrier because MOF ZIF-8 encapsulate DAADH to restrict the movements related to unfold [25] and RGO increase enzyme stability.

Fig.4

3.4 Optimal temperature

The optimal reaction temperature of free D-amino acid dehydrogenase with peptide linker was investigated. As it can be seen from Fig.5, the optimal temperature was 40 . Relative activity could keep above 80% at 40-80 , the relative activity was 97.7% at 80 . The results showed that the DAADH can maintain high catalytic activity under high temperature. In high temperature such as 70-80 , peptide linker cause conformation adjustment of enzyme, which may obtain high activity.

Fig.5

3.5 Optimal pH

The optimal pH value of DAADH in pH range of 8.5-12 was investigated. As shown in Fig.6, optimal pH for free enzyme was 10.0, while the activity was only 56.3% under pH 11. For immobilized enzyme, the optimal pH was 11.0, which indicated that the immobilized enzymes are robust in the alkaline solution. Moreover, DAADH/ZIF-8/RGO can maintain a relative activity of 70.5% with pH 12. The results indicated that the immobilized carrier (ZIF-8/RGO) can protect the structure of the enzyme under high alkaline conditions.

Many reported MOFs-enzyme composites exhibit unprecedented catalytic performances than those of free enzymes, including improved enzyme stability and recyclability, due to the protection of enzymes by highly ordered frameworks [9].

Fig.6

3.6 Reuse stability

The reuse stability of immobilized enzymes was investigated, as shown in Fig.7. After 7 consecutive cycles of reactions, the residual activities of DAADH/ZIF-8/RGO and DAADH/ZIF-8 were 64.3% and 45.6%, respectively. Enhanced stability of DAADH/ZIF-8/RGO probably as DAADH tends to combine with the hydrophobic surface of RGO. Hydrophobic effect greatly improves enzyme recovery activity and shows better reuse stability than immobilization by DAADH/ZIF-8.

Fig.7

3.7 Thermostability study

The thermostability of free enzyme and immobilized enzyme at 50 was investigated (Fig.8). The reductive amination activity of the immobilized enzymes has declined gradually over time. After incubation for

10h, DAADH/ZIF-8/RGO still retained 53.4% relative enzyme activity and had good thermostability stability. The residual enzyme activity of DAADH/ZIF-8 system was 37.8% while the free enzyme was almost inactivated. The results showed that immobilization improve thermostability stability of DAADH.

The enhancement of thermostability probably due to interaction between peptide and MOF which may restrict the conformational change of DAADH. The dissociation of subunit was prevented by cage effect of MOF immobilization. With large specific surface area, functional diversity and precise control of the particle size, MOFs are ideal carrier with biocompatible microenvironments for enzyme immobilization [26, 27].

Fig.8

3.8 Kinetic study of enzyme

Kinetic parameters of the free enzyme and immobilized enzyme were determined with phenylpyruvate concentrations ranging from 1.25 to 15.00 mM. Kinetic parameters were calculated based on the Michaelis-Menten equation. The Michaelis constants were calculated and showed (Table 1). Comparing with free enzyme, the K_m value of immobilized enzyme decreased, which indicated that the improvement of the affinity between enzyme (DAADH) and the substrate (Phenylpyruvate). V_{max}/K_m of DAADH/ZIF-8/RGO and DAADH/ZIF-8 were 1.3 and 1.6-fold of the free enzymes, respectively. The enhancement of kinetic parameters probably because ZIF-8's and ZIF-8/RGO's pore and inner-surface with functionality variations optimize the interior environment, which was beneficial to facilitate the substrate's diffusion [28].

Table 1

3.9 Morphology analysis

The morphology of the immobilized-DAADH was studied. SEM image indicated a mesoporous structure with pore sizes of different scales (Fig.9). This mesoporous structure probably facilitates the substrate's diffusion. ZIF-8 provides a biocompatible carrier for DAADH immobilization. The interaction of ZIF-8 and the peptide linker improved the stability of the immobilized enzyme. With the addition of RGO, the immobilized enzyme DAADH/ZIF-8/RGO showed amorphological layers.

The addition of RGO can form hybrid material (ZIF-8/RGO) with higher biocompatibility, which may due to hydrophobic interaction between RGO and DAADH [24]. ZIF-8/RGO composites can effectively protect the active conformation of enzymes and improve the stability of enzymes in extreme conditions such as high temperature and high pH value. The DAADH/ZIF-8/RGO is expected to be further applied in biocatalysis [29].

Fig.9

Mechanism of ZIF-8/RGO for DAADH immobilization was thought about multi-level interactions include coordination bonds, hydrogen bonds, electrostatic interactions and hydrophobic effect (Fig.10). There will probably be coordination bonds, hydrogen bonds and electrostatic interactions between DAADH and ZIF-8. Controllable immobilization of DAADH within ZIF-8 was crucial for achieving efficient catalysis and high stability. As for DAADH and RGO, it will be hydrophobic effect, which can improve the stability. ZIF-8/RGO is showed to be a good carrier for DAADH and the DAADH/ZIF-8/RGO has potential in biocatalysis

. **Fig.10**

3.10 One-step separation and immobilization of enzyme by bimetal MOF

One-step separation and immobilization of DAADH from crude enzyme solution by bimetal based MOF was studied. Integrate of separation and immobilization of enzyme directly from cell lysis solution in a single biomineralization step. Bimetal-based MOF was developed to immobilize DAADH by nickel (Ni) and zinc

(Zn) ions. ZIF-8/RGO/Ni composite for *in situ* immobilization of D-amino acid dehydrogenase obtained 1.5-fold activity of free enzyme. Kinetic parameters of DAADH-ZIF-8/RGO/Ni-DAADH were calculated and the results confirmed the enhancement of activity (Table 2).

Table 2

Thermostability indicated that after incubation at 50 for 6 hours, DAADH-ZIF-8/RGO/Ni-DAADH maintained 70% activity (Fig. 11). The enhancement of activity is consistent with previous literature that a novel multi-metallic system was reported to exhibit higher catalytic efficiency compared with individual metal-based hybrid materials [30]. Bimetal-based MOF displays potential in immobilization with high enzyme loading capacity, stability, and recyclability [31].

Fig.11

4. Conclusion

MOFs, with extremely high specific surface area, abundant porosity and extraordinary multi-functionality, hold a great promise as matrices for enzyme immobilization with biocompatibility and high protein loading capacity and enhance the cost-efficiency of biocatalysis. The D-amino acid dehydrogenase connected with $(RTHRK)_4$ was constructed. Combined with peptide linker and MOF, efficient enzyme immobilization was achieved by encapsulation of DAADH via a facile one-step biomimetic mineralization strategy. These results indicated the interactions of MOF and peptide linker substantially improved the catalytic efficiency and stability of DAADH. After 10 hours of incubation at 50 degC, DAADH/ZIF-8/RGO retained 53.4% relative activity while that is 37.8% for DAADH/ZIF-8, and the free enzyme was almost inactivated. After 7 recycles, the residual activities of DAADH/ZIF-8/RGO and DAADH/ZIF-8 were 64.3% and 45.6%, respectively. Furthermore, one-step separation and immobilization by DAADH-ZIF-8/RGO/Ni-DAADH was carried out with 1.5-fold activity enhancement. Hybrid materials of MOF and carbon materials also can be extended for the multi-enzyme immobilization, which have potential in biocatalysis.

Abbreviations

DAADH, D-amino acid dehydrogenase; **MOF,** Metal-Organic Frameworks; **ZIF-8,** 2-methylimidazole zinc salt; **RGO** , Reduced graphene oxide; **DAADH/ZIF-8,** D-amino acid dehydrogenase immobilized in ZIF-8. **DAADH/ZIF-8/RGO** , D-amino acid dehydrogenase immobilized in hybrid materials of ZIF-8 and RGO;**DAADH-ZIF-8/RGO/Ni-DAADH,** D-amino acid dehydrogenase immobilized in hybrid materials of ZIF-8, RGO and Ni. **SEM,**scanning electronic microscopy.

CRediT authorship contribution statement

Hangbin Lei : Methodology, Investigation, Analysis, Interpretation and Writing- original draft. **Qian Zhang** : Methodology, Investigation and Analysis. **Xiaoyan Xiang** : Investigation, Data curation, Validation. **Liang Jiang** : Investigation, Analysis. **Shiyan Wang** : Investigation, Validation**Lingxuan Duan** : Investigation, Validation. **Shizhen Wang** : Conception, Design, Funding acquisition, Supervision, Analysis and Interpretation, Drafting the manuscript, reading and final approval of the version to be published.

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Conflict of interest statement

The authors have declared no conflict of interest.

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Table:

Table 1 Kinetic parameters for reductive amination of immobilized enzyme

Enzyme	$\mathbf{V_{max}}$ **(mM/min)**	$\mathbf{K_m}$ **(mM)**	$\mathbf{V_{max}/K_m(min^{-1})}$
DAADH	0.605	2.332	0.259
DAADH/ZIF-8	0.683	1.587	0.430
DAADH/ZIF-8/RGO	0.457	1.387	0.329

Reaction conditions: Phenylpyruvate, 1.25-15 mM; NADPH, 0.2 mM; pH, 10.0; temperature, 30 °C.

Table 2 Kinetic parameters of one-step immobilization by ZIF-8/rGO/Ni-DAADH

Enzyme	Vmax (mM/min))	K_m (mM)	$V_{max}/K_m(min^{-1})$
DAADH	0.605	2.332	0.259
ZIF-8/RGO/Ni-DAADH	0.970	2.116	0.458

Figure:

Fig.1 Asymmetric reductive amination of phenylpyruvate catalyzed by DAADH

Fig.2 a.Effect of metal ions on enzyme activity of DAADH. b.Effect of Ni^{2+} concentration on enzyme activity of DAADH.

Reaction conditions: Phenylpyruvate, 5 mM; NADPH, 0.2 mM; Metal ions, 10 mM, pH, 10.0; temperature, 30 °C.

Fig.3 Effect of ionic liquids on DAADH activity. Reaction conditions: Phenylpyruvate, 5 mM; NADPH, 0.2 mM; pH, 10.0; temperature, 30 °C.

Fig.4 Activity recovery rate of immobilized enzyme.

Reaction conditions: Phenylpyruvate, 5 mM; NADPH, 0.2 mM; pH, 10.0; temperature, 30 °C.

Fig.5 The optimum reaction temperature for reductive amination of DAADH.

Reaction conditions: Phenylpyruvate, 5 mM; NADPH, 0.2 mM; pH, 10.0; temperature, 30-80 °C

Fig.6 Optimal pH for reductive amination of immobilized enzymes.

Reaction conditions: Phenylpyruvate, 5 mM; NADPH, 0.2 mM; pH, 8.5-12.0; temperature, 30 °C.

Fig. 7 Reuse stability of immobilized enzyme.

Reaction conditions: Phenylpyruvate, 5 mM; NADPH, 0.2 mM; pH, 10.0; temperature, 30 °C.

Fig.8 Thermostability of free enzyme and immobilized enzyme at 50 .

Reaction conditions: Phenylpyruvate, 5 mM; NADPH, 0.2 mM; pH, 10.0; temperature, 50 degC.

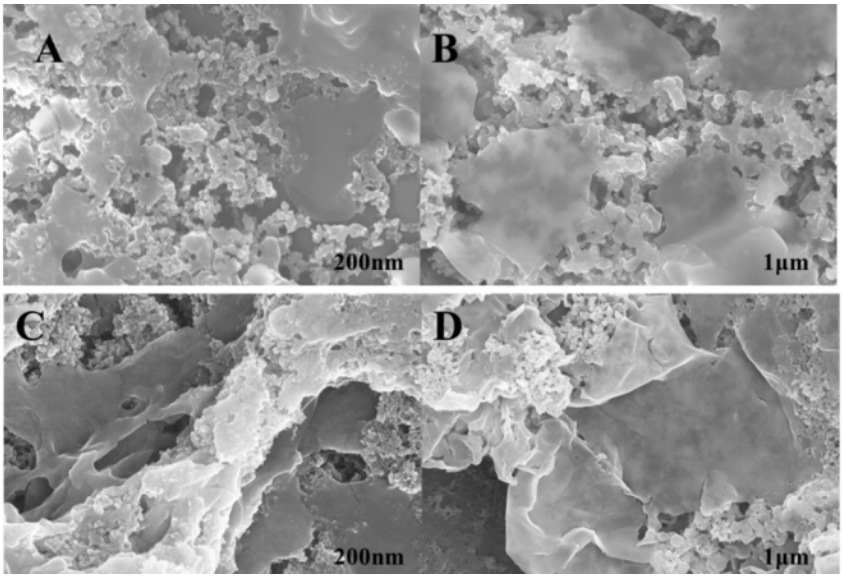

Fig.9 SEM characterization of immobilized enzyme morphology (A),(B) DAADH/ZIF-8; (C),(D) DAADH/ZIF-8/RGO

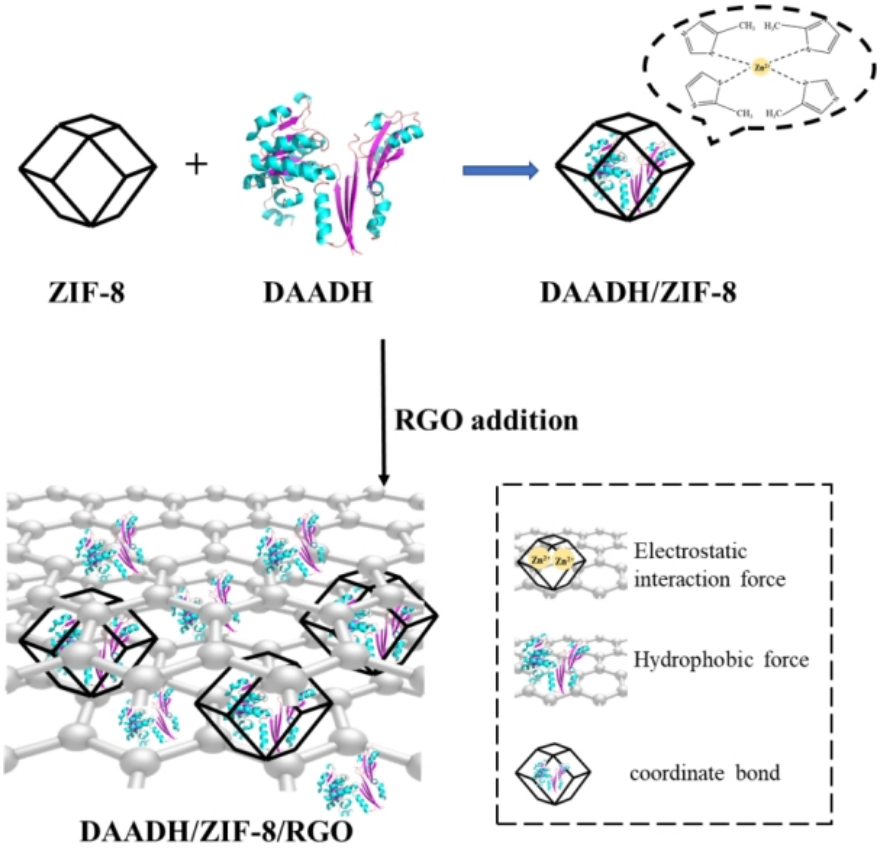

Fig.10 Mechanism of ZIF-8/RGO immobilized DAADH

Fig.11 Thermostability of reductive amination of immobilized enzyme.

Reaction conditions: Phenylpyruvate, 5 mM; NADPH, 0.2 mM; pH, 10; temperature, 50