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ARTICLE

Di-functional magnetic nanoflowers: A highly efficient support for immobilizing penicillin G acylase

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Abstract

The novel di-functional magnetic nanoflowers (DMNF) which had both epoxy groups and hydrophilic catechol as well as phthaloquinone groups capable of covalently coupling of penicillin G acylase (PGA) were characterized by scanning electron microscopy, transmission electron microscope (TEM), vibrating sample magnetometer, N_2 adsorption, and so on. The studies showed that DMNF possessed "hierarchical petal" structure of nanosheets had specific saturation magnetization of 39.7 emu/g and average pore diameter of 25.4 nm as well as specific surface area of 17.28 m^2/g. For hydrolysis of penicillin G potassium catalyzed by the PGA immobilized on DMNF with enzyme loading of 106 mg/g-support, its apparent activity reached 2,667 U/g, which benefited from the "hierarchical petal" and large pore structure of the magnetic DMNF leading to high enzyme loading and fast diffusion of substrate molecules to the immobilized PGA to reaction. The apparent activity of the immobilized PGA could keep 2,408 U/g (above 90% of its initial activity) after repeating use for 10 cycles. The magnetic immobilized PGA exhibited excellent operational stability due to covalently coupling of the enzyme molecules between the support by covalent interaction of the amino groups of PGA and the reactive groups of epoxy, catechol, and phthaloquinone groups on DMNF. Furthermore, the PGA displayed good acid and alkaline resistance as well as thermal stability by immobilization using DMNF.

KEYWORDS

di-functional reactive groups, "hierarchical petal" structure of nanosheets, immobilization, magnetic nanoflowers, penicillin G acylase

1 | INTRODUCTION

With the development of modern biotechnologies such as gene engineering and protein engineering, a variety of enzymes have been efficiently expressed and produced in large scale. Enzymes as biocatalysts could have been applied catalytic chemical reactions in the synthesis of pharmaceuticals and fine chemicals as well as degradation of organics in wastewater.[1] However, free enzyme is very sensitive to the environment and it is unstable in acid, alkali, high temperature, and organic solvent so that its catalytic activity was greatly reduced. Moreover, if free

enzyme was directly used in catalytic process, it is difficult to separate enzyme from reaction system to reuse. The immobilization technology of enzyme is an important subject in the field of industrial application of enzymes because it not only overcomes the above shortcoming but also maintains the unique catalytic activity of enzyme. Penicillin G acylase (PGA, EC 3.5.1.11) as a powerful biocatalyst plays a greatly important role in the manufacture of semi-synthetic β-lactam antibiotics. It can hydrolyze penicillin G to produce primary intermediate of nuclei-6-aminopenicillanic acid (6-APA) as well as can also catalyze the synthesis of semisynthetic penicillins with a high catalytic performance in mild reaction conditions.[2] It has long been the goal toward exploiting new-type supports and immobilization techniques to improve the catalytic efficiency of PGA.

In recent years, magnetic supports for immobilizing PGA have attracted extensive attention due to its easy and rapid separation, which was separated from mixture reaction system by a magnetic force, avoiding the loss of biocatalysts during separation and washing mechanical operation. In general, Fe_3O_4 nanoparticles are employed as magnetic sources to design and prepare magnetic supports.[3–6] Chen et al.[7] synthesized paramagnetic polymer microspheres as support immobilizing PGA through polymerization of glycidyl methacrylate, ally glycidylether, and methacrylamide on the surface of silica-coated Fe_3O_4 nanoparticles. The PGA immobilized on composite microspheres had initial activity of 430 U/g and retained 99% of its initial activity after recycling 10 times. In addition, immobilized PGA possessed high thermal stability and pH stability. Zhang et al.[8] synthesized functionalized magnetic mesoporous microspheres Fe_3O_4@SiO_2@m-SiO_2-DHA with specific saturation magnetization of 61.25 emu/g, which was prepared by modifying with ϑ-(2,3-epoxypropoxy)propytrimethoxysilane and dendritic hyperbranched amine (DHA) onto the surface of Fe_3O_4@SiO_2@m-SiO_2. The apparent activity of PGA immobilized on Fe_3O_4@SiO_2@m-SiO_2-DHA was approximately 475 U/g; meanwhile, the enzyme activity retention rate was 94.5% after cycling of 10 runs. Yang et al.[9] reported the aldehyde-functionalized mesoporous silica-Fe_3O_4 nanocomposites with a pore size of 7.4 nm via co-condensation of tetraethylorthosilicate and trimethoxysilylpropanal in the presence of triblock copolymer of Pluronic P123, NaCl, and Fe_3O_4 nanoparticles in the neutral solution. The magnetic immobilized PGA possessed considerable operation stability. So that, it retained 91.0% of initial activity after recycled for 10 times. As a potential biomedical material, magnetic inorganic nanocomposites not only serve as the supports for immobilizing enzyme but are also applied for drug and RNA transmission and multiple imaging agents.[10–13] With the rapid development of magnetic nanocomposites and the improvement of immobilization technology, the "enzyme-carrier" complex has both the new characteristics of the support and the efficient catalytic performance of the enzyme molecule. For example, the support possesses a unique morphology structure and the surface is rich in functional groups that are easy to covalently bind to PGA. Li et al.[14] reported a flexible dendrimer-grafted flower-like magnetic microcarriers with glutaraldehyde as crosslinking agent for PGA immobilization, and the catalytic activity of immobilized PGA maintained 82.14% after reused for 9 times. However, over-crosslinking of glutaraldehyde on enzyme and the reversibility of Schiff base chain cause many inherent difficulties to decrease activity recovery of immobilized enzyme.[15] The active epoxy group on the magnetic porous polymer microspheres can be covalently bound to the amino groups of PGA, so that the PGA can be immobilized on the magnetic polymer microsphere under mild conditions. It was considered that the porous supports with large specific surface area and pore volume are beneficial to reduce the contact limitation between the catalyst and the substrate as well as to the migration and diffusion of substrate molecules, especially for bulky PGA of 7 nm × 5 nm × 5.5 nm. The magnetic nanoflowers formed via the self-assembly of Fe_3O_4 nanochips possessed large pore size and specific surface area, and they could provide more opportunities for enzyme molecules to come into contact with the support surface in the immobilized.[16–19]

Herein, the novel magnetic composite nanoflowers with "hierarchical petal" structure and reactive groups of epoxy groups and hydrophilic catechol as well as phthaloquinone groups were obtained for immobilizing PGA. The catalytic performances of the PGA immobilized on the magnetic composite nanoflowers were studied for hydrolyzing penicillin G potassium salt to prepare 6-APA. In addition, the kinetic constants of the catalyst were determined. The influence of reaction temperature, pH value, and dopamine amount on the catalytic performance of immobilized PGA was also discussed.

2 | EXPERIMENTAL

2.1 | Materials

Penicillin G acylase (PGA, 862 U/ml) was provided by Zhejiang Shunfeng Haideer Co., Ltd., China. Methacryloxy propyl trimethoxyl silane (MPS) was supplied from Shanghai Macklin Biochemical Co., Ltd., China. $FeCl_3{\cdot}6H_2O$, potassium persulfate (KPS), urea, and ethylene glycol (EG) were purchased from sinopharm

Chemical Reagent Co., Ltd., China. Glycidy methacrylate (GMA) and Ethylene dimethacrylate (EDMA) were produced from Acros Organics, USA. Dopamine hydrochloride was provided by Ark Pharm, USA. Penicillin G potassium was purchased via Shanghai Aladdin Bio-Chem Technology Co., Ltd., China. The other reagents were of laboratory grade obtained from different commercial suppliers.

2.2 | Preparation of Fe_3O_4 nanoflowers

$FeCl_3{\cdot}6\ H_2O$ (0.1 g) and 3 g urea were dissolved in 40 ml EG and then stirred for 30 min at the temperature of 50°C. Afterward, the homogeneous solution was transferred to Teflon-lined autoclave and reacted at 200°C for 4 hr. After the reaction, the sediment was washed several times with water and ethanol to remove unreacted ions and organic solvent. Ultimately, the product was calcined at 400°C for 1 hr under the N_2 atmosphere at 5°C/min in tube furnace to obtain the Fe_3O_4 nanoflowers. Spherical Fe_3O_4 nanoparticles were prepared via a versatile solvothermal reaction.[20]

2.3 | Preparation of di-functional Fe_3O_4 nanoflowers

Fe_3O_4 nanoflowers (0.5 g) were dispersed into 320 ml ethanol and 100 ml deionized water, which was heated to 40°C in a water bath, and adding 5 ml MPS into the solution under the mechanical stirring. After being stirred for 12 hr, the precipitate was separated by a magnet and washed several times with ethanol and water. Then, 0.2 g microspheres abovementioned were dispersed in the mixture consisting of 0.1 g GMA and 2 g EDMA (5%) aqueous solution, and 10 g KPS (0.5%) aqueous solution was added into the mixture at 70°C for 6 hr with stirring. After the completion of polymerization, the polymeric EDMA-GMA magnetic nanoflowers (PGMN) were separated by a magnet and washed several times with ethanol and water, and then the products were dried in vacuum drying oven. Subsequently, 0.2 g PGMN was dispersed in Tris buffer solution (0.05 mol/L, pH 8.5) containing 5 mg of dopamine hydrochloride. The thin film of polydopamine on the surface of PGMN was formed after shaking at the temperature of 30°C for 12 hr. After that, the Fe_3O_4 nanoflowers modified by polymeric EDMA-GMA and polydopamine were separated in magnetic field and washed three times with water and ethanol. The di-functional magnetic nanoflowers were named as DMNF.

As a comparison, spherical Fe_3O_4 was used as support matrix and di-functionalized via the same method. The specific operation was as follows: 2 g of aqueous solution of EDMA (5%), 0.1 g GMA and 5 mg dopamine were sequentially modified on the surface of spherical Fe_3O_4 nanoparticles (0.5 g) under the same reaction conditions. The prepared di-functional spherical magnetic nanoparticles were named as DSMN.

2.4 | Preparation of immobilized PGA and testing of enzyme loading

Di-functional magnetic nanoflowers (DMNF) (0.2 g) were dispersed into the mixed solution containing 3.75 ml phosphate buffer solution (0.01 mol/L, pH 7.8) and 1.25 ml PGA original solution. After mechanical shaking at 30°C for 12 hr, PGA immobilized on DMNF were collected by a magnet and then washed with phosphate buffer solution three times to remove free PGA, and the filtrate was recovered for the determination of enzyme loading. The immobilized PGA on DMNF was denoted as PGA/DMNF.

As a comparison, PGA was immobilized onto different supports of Fe_3O_4 nanoflower, PGMN, and DSMN. Three kinds of carriers of 0.2 g were dispersed into the mixture containing 3.75 ml phosphate buffer solution (0.01 mol/L, pH 7.8) and 1.25 ml PGA original solution under the same immobilization conditions, correspondingly. The obtained immobilized PGA were labeled as PGA/Fe_3O_4 nanoflowers, PGA/PGMN, and PGA/DSMN, respectively.

The concentration of protein in PGA solution before and after immobilization was determined via Bradford's dye binding assay. A little diluted immobilized PGA filtrate and original enzyme solution were mixed with coomassie brilliant blue, respectively. Afterward, the absorbance of solution was determined at 595 nm by UV-3300 spectrophotometer, and the protein concentration of filtrates was calculated according to the standard curve. The enzyme loading of immobilized PGA was obtained from the following formulas:

$$\text{Enzyme loading Q (mg/g)} = \frac{(m_1 - m_2)}{m_s} \quad (1)$$

In Equation (1), where m_1 is the mass of added enzyme (mg); m_2 is the protein quality in total filtrate (mg); m_s is the mass of support (g).

2.5 | Characterization

The size and morphology of samples were obtained via scanning electron microscopy (SEM, JEOL JSM-6360LV)

and transmission electron microscopy (TEM, Hitachi HT7700). The magnetization curve was measured at room temperature under a varying magnetic field using a vibrating sample magnetometer (VSM, Lake Shore 7304). X-ray diffraction (XRD, K/MaX-3c) was used to investigate the crystal structure of the magnetic supports. The IR spectra were measured in the range from 4,000 to 400 cm^{-1} via Fourier transform infrared spectrometer with KBr pellets. Low-temperature N_2 adsorption was performed to determine the pore size and specific surface area of the magnetic microspheres using a Micromeritics Model 2010 ASAP apparatus.

2.6 | Testing of apparent activity for immobilized PGA

The apparent activity of the immobilized PGA for catalyzing hydrolysis of penicillin G potassium to produce 6-APA was determined by the titration method as follows[21]: The immobilized PGA were added into 10 ml phosphate buffer solution (0.01 mol/L, pH 7.8) and incubated under the temperature of 30°C for a period of time. Subsequently, 10 ml 8% (w/v) of penicillin G potassium solution (which has been preheated at 30°C) was added, and the mixture was titrated with 0.1 mol/L standard NaOH solution. Finally, the volume of NaOH aqueous consumed within 3 min was recorded. The apparent activity of immobilized PGA was calculated from the following Equation (2).

$$\mathrm{A(U/g)} = \frac{C \times V \times 1000}{m \times t} \quad (2)$$

In Equation (2), where C represents the concentration of NaOH standard solution (mol/L); V is the volume of consumed NaOH solution (ml); m is the dry weight of support (g); and t is the reaction time (min).

To test its reusability, at the end of each reaction, the PGA/DMNF was separated from the reaction system by magnet and washed with phosphate buffer solution for three times. The PGA/D2MNF was continuously used for 10 times of the hydrolysis reaction.

3 | RESULTS AND DISCUSSION

3.1 | Morphology of flower-like Fe_3O_4 nanostructures

The morphologic evolution of iron oxide precursor over time was investigated under keeping other reaction conditions the same as the typical experiment by SEM images and shown in Figure 1. Early in the reaction (0.5 hr), the sample was composed of a large number of Fe_3O_4 nanoparticles. When the reaction time was extended to 1.5 hr (Figure 1b), a large number of clustered "petals" could be observed at the expense of the nanoparticles. After reaction for 4 hr, it can be clearly observed that the three-dimensional nanostructures expressed flower-like morphology with no Fe_3O_4 nanoparticles, and the sample consisted entirely of 3D nanoflowers. Increasing the reaction time to 5.5 hr, the morphology of Fe_3O_4 nanoflowers did not change significantly. At the same time, a few Fe_3O_4 nanoparticles appeared around the 3D "layered petals." It was observed that the Fe_3O_4 nanoflowers formed at earlier stages were decorated with Fe_3O_4 nanoparticles again (Figure 1e), and no special "layered petals" reserved as shown in Figure 1f (8 hr).

3.2 | Characterization of DMNF

The SEM images were shown in Figure 2. As seen in the inset of Figure 2a, Fe_3O_4 nanoflowers with diameter of 3 μm were made of uniform sheet, which exhibited a unique appearance of 3D "layered petal." At the same time, it can be observed that the surface of DMNF became rough, but it still maintained unique 3D "layered petal" (Figure 2b), indicating that the introduction of polymeric GMA and polydopamine would not destroy the morphology of composite supports, which was in accordance with the synthesis of DSMN (Figure 2d). In addition, the pores of DMNF were mainly in the range of mesoporous size, which were significantly superior to traditional spherical microspheres (as shown in Figure 2c, d); thus, providing a larger contact area between PGA and supports and contributing to the reduction of mass transfer resistance in the process of catalysis.

Further observing the microstructure of Fe_3O_4 nanoflowers and DMNF, the high magnification of TEM images in Figure 3b were clearly exhibited "petal" morphology of DMNF. It can be seen that Fe_3O_4 nanoflowers were uniformly surrounded by thin coating, further demonstrating that the modification of polymeric GMA and polydopamine would not destroy the primary flower-like architectures of Fe_3O_4 nanoflowers. The "layered petal" support with larger specific surface area and mesoporous size is beneficial for the immobilization of PGA because it can increase the density of valid functional groups and provide a friendly biological micro-environment.

The crystal phases and compositions of corresponding samples of Fe_3O_4 nanoflower, PGMN, and DMNF were examined by X-ray diffraction, as shown in Figure 4. It can be seen that Fe_3O_4 nanoflowers were in an inverse

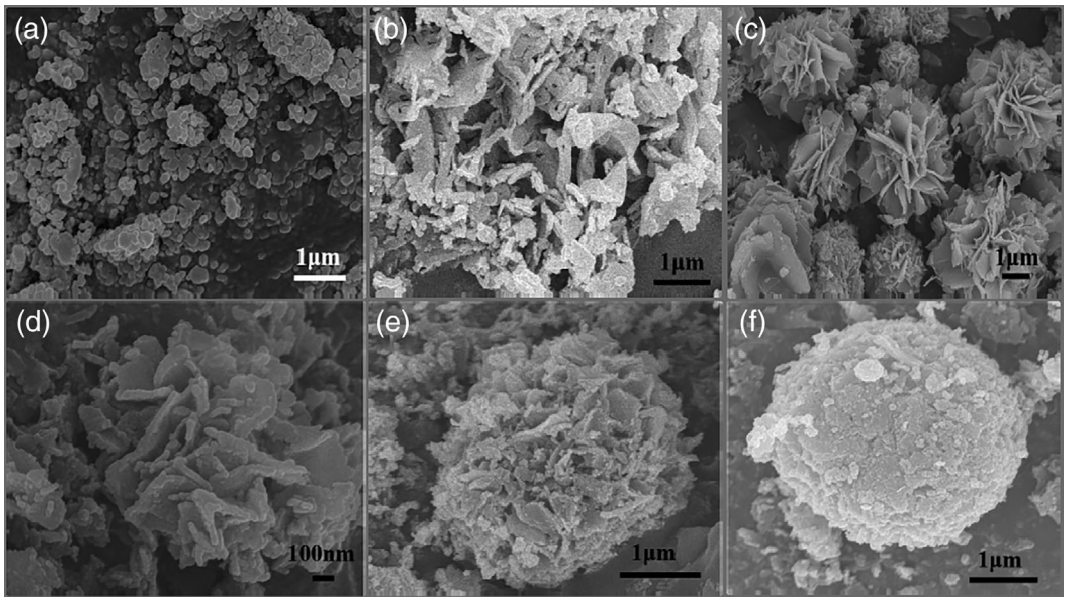
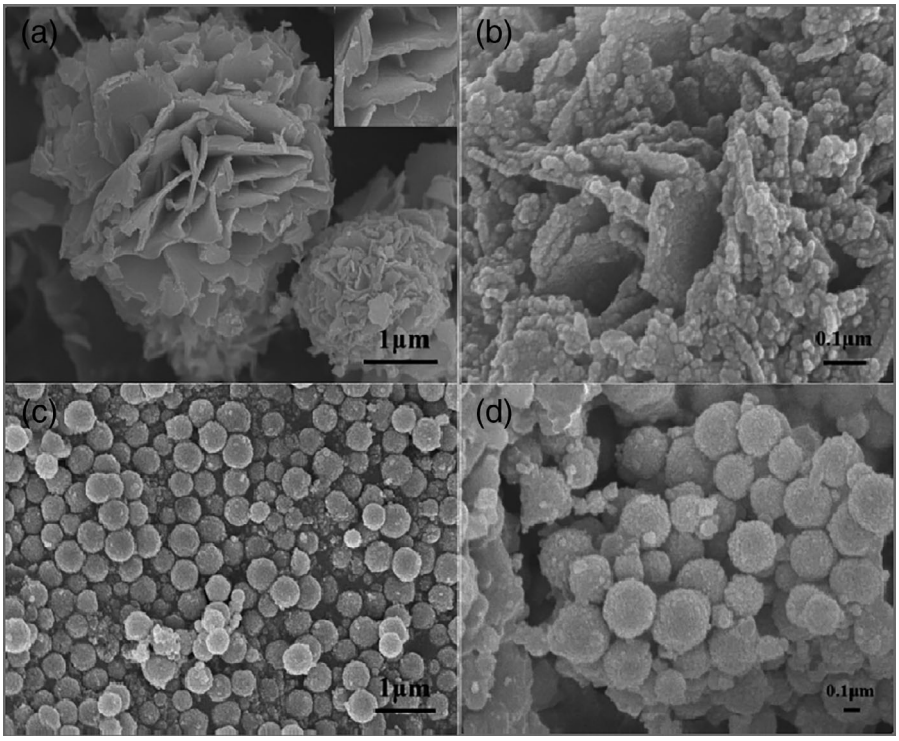


FIGURE 1 SEM images of Fe_3O_4 nanoflowers prepared for different reaction times of (a) 0.5 hr, (b) 1.5 hr, (c) 4 hr, (d) 5.5 hr, (e) 6.5 hr, (f) 8 hr

FIGURE 2 SEM images of Fe_3O_4 nanoflowers (a), DMNF (b), Fe_3O_4 spherical nanoparticles (c), and DSMN (d)



cubic spinel structure and the diffraction peaks at 2θ of 30.3°, 35.7°, 43.2°, 53.4°, 57.4°, and 62.9° were accurately corresponding to the indices of (220), (311), (400), (422), (511), and (440) crystal planes of Fe_3O_4 without other impurity peaks, which proved that good crystallinity of Fe_3O_4 can be obtained after calcined at 400°C.[22] The XRD patterns showed that Fe_3O_4 particles in PGMN and DMNF possessed favorable crystallinity owing to the peak position of samples basically the same as that of the standard Fe_3O_4 in Figure 4a, which indicated that the crystal structure of Fe_3O_4 did not change in the process of polymerization and coating. The broadening peak at 2θ in 10°–18° in Figure 4c belonged to the amorphous polymeric components in DMNF.

The FT-IR spectra of Fe_3O_4 nanoflowers, PGMN, and DMNF were presented in Figure 5. The characteristic

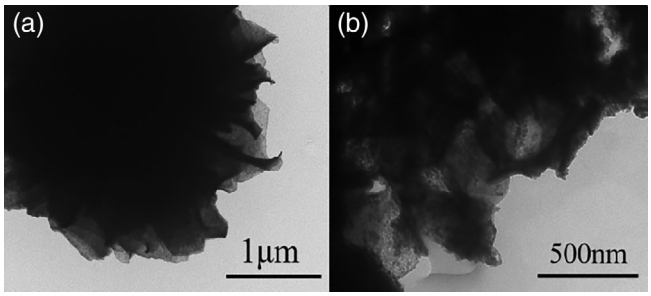


FIGURE 3 TEM images of Fe_3O_4 nanoflowers (a) and DMNF (b)

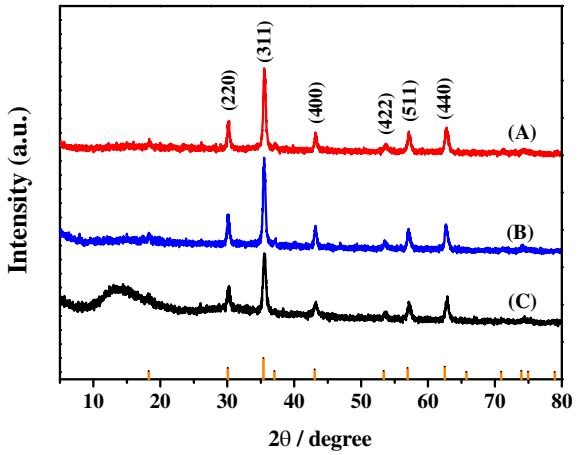


FIGURE 4 XRD patterns of Fe_3O_4 nanoflowers (a), PGMN (b), and DMNF (c)

absorption band located at 573 cm^{-1} in Figure 5a was assigned to the Fe-O bond, indicating that magnetic nanoflowers were Fe_3O_4. It can be seen from spectra in Figure 5b,c that the peak at 573 cm^{-1} of Fe_3O_4 could still be observed, illustrating that the introduction of polydopamine and polymeric GMA has no effect on the structure of Fe_3O_4, which is consistent with the discussion of SEM and XRD. The appearance of new peak in Figure 5b at 1,053 cm^{-1} may be attributed to the linkage between —OH of Fe_3O_4 and —O—Si—O— of MPS. Besides, the absorption peaks at 1,597 cm^{-1} and 1,730 cm^{-1} were related to the stretching vibration of C═C and carbonyl group, respectively. The absorption peak located at 930 cm^{-1} was corresponded to the epoxy group of polymeric glycidyl methacrylate,[23] which proved that glycidyl methacrylate was successfully modified on the surface of supports. Besides, the absorption bands at 1629 cm^{-1} and 1,384 cm^{-1} were associated with the stretching vibration of the carbonyl group and hydroxyl group on polydopamine.[24] The analytical results of FT-IR spectra confirmed that polymeric glycidyl methacrylate and polydopamine were successfully modified in magnetic nanoflowers.

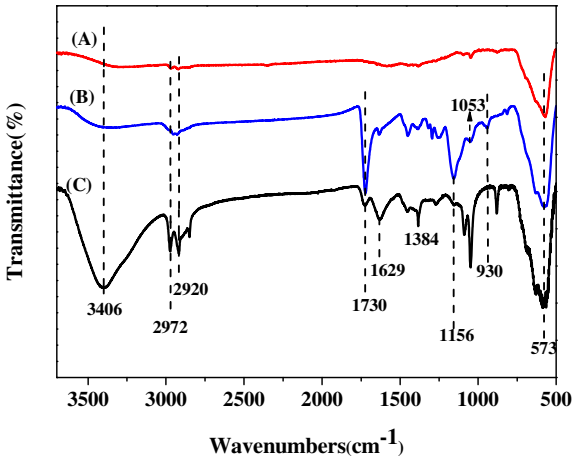


FIGURE 5 FT-IR spectra of Fe_3O_4 nanoflowers (a), PGMN (b), and DMNF (c)

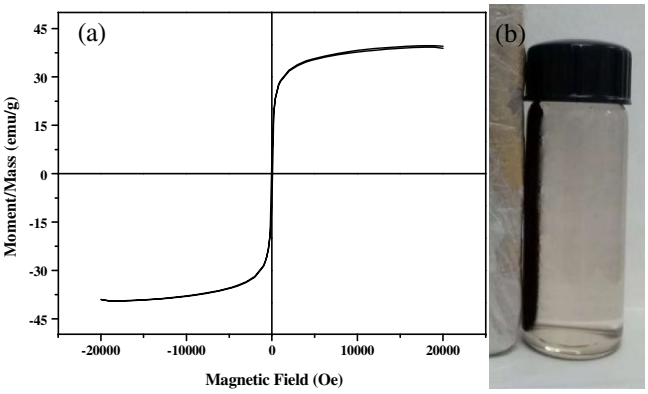


FIGURE 6 Magnetization curve of DMNF (a) and image of DMNF (b) in a magnetic field

Magnetic property characterization for DMNF is shown in Figure 6a in which there is no hysteresis in the magnetic curve with remanence and coercivity being zero, indicating that it possessed superparamagnetism. It was seen that the saturation magnetization of DMNF reached 39.7 emu/g, lower than that of Fe_3O_4 nanoflower matrix (61 emu/g).[16] However, as shown in Figure 6b, the DMNF particles dispersed in water are effectively separated by magnetic force. In our experiment, it was also observed that the DMNF particles did not appear to agglomerate during the immobilization process. This indicates that the magnetic self-agglomeration between the magnetic particles is weakened after the surface modification of Fe_3O_4 nanoflower by polydopamine and polymeric GMA.

The nitrogen adsorption–desorption isotherms (Figure 7) of Fe_3O_4 nanoflowers and DMNF showed the typical H3 hysteresis loop, indicating that pore structure of Fe_3O_4 nanoflowers and DMNF was slit pores piled up

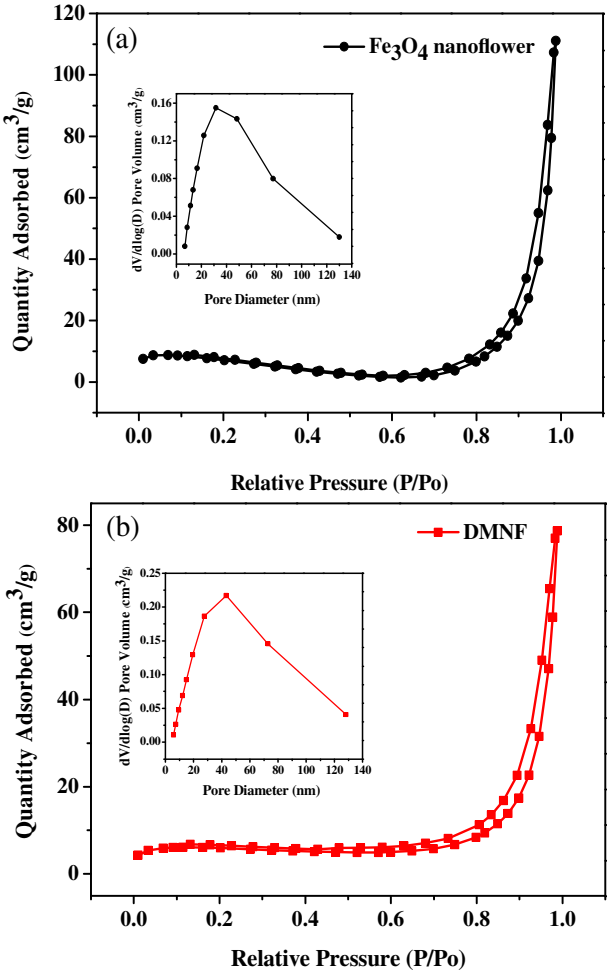


FIGURE 7 N_2 adsorption—desorption isotherm curves and corresponding pore size distribution of Fe_3O_4 nanoflowers (a) and DMNF (b)

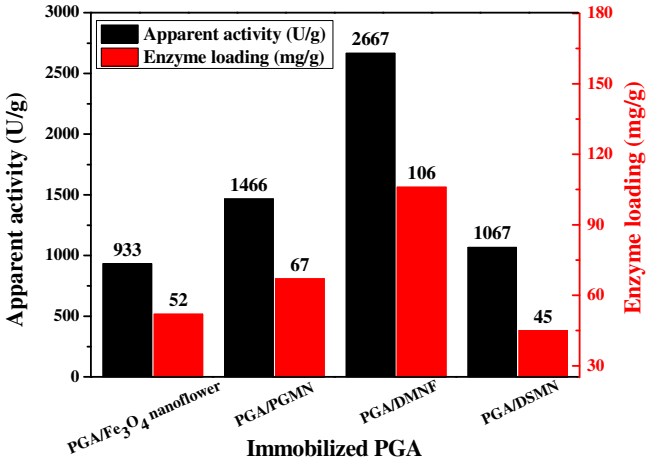


FIGURE 8 The apparent activity and enzyme loading of immobilized PGA

by plate-like particles,[17–19] which was consistent with the results of SEM images and TEM images. Moreover, it showed that Fe_3O_4 nanoflowers (35.3 nm) and DMNF (25.4 nm) both had large pore size; meanwhile, their specific surface area were 17.29 m^2/g and 17.28 m^2/g, respectively. Supports with "layered cross petal" architecture can be employed to immobilize PGA, which facilitates the contact with enzyme molecules and be in favor of the entrance of substrates to the internal surface of immobilized PGA thus producing higher diffusion efficiency of substrate and promoting the catalytic performance of PGA.

3.3 | The activity of PGA immobilized on nanoflowers

The results of apparent activity and enzyme loading of immobilized PGA for catalyzing hydrolysis of penicillin G potassium produce 6-APA are investigated and shown in Figure 8. It was obviously observed that enzyme loading (106 mg/g) of PGA/DMNF was significantly higher than protein content of PGA/Fe_3O_4 nanoflowers (52 mg/g) and PGA/PGMN (67 mg/g), which was mainly due to polydopamine film with more active functional groups for covalently coupled PGA formed on the surface of PGMN, illustrating that it could provide an excellent microenvironment for the immobilization of enzyme. The apparent activity of PGA/DMNF can be up to 2,667 U/g at optimal reaction conditions, which was 2.5 times as much as that of PGA/DSMN (1,067 U/g and 45 mg/g). There are some reports on the immobilization of PGA with functional magnetic carriers, which focus mainly on the functional groups of amino, aldehyde, carboxyl, and epoxy groups. Ling et al.[25] immobilized PGA on magnetic Fe_3O_4@chitosan nanoparticles by the Schiff base reaction and the immobilized PGA with protein loading of 6.2 mg/g-support was obtained under the optimal conditions. Shi et al.[26] used glutaraldehyde as a cross-linking agent to immobilize PGA on aminopropyl-functionalized silica-coated magnetic microspheres via the condensation of tetraethylorthosilicate and γ-aminopropyltriethoxysilane onto the surface of Fe_3O_4 microspheres. The result showed that the apparent activity of immobilized PGA was 384 U/g. Xue et al.[27] prepared magnetic hydrophilic polymer microspheres containing oxyalkyl groups for immobilizing PGA to produce 6-APA. Under the optimal reaction conditions, the apparent activity and enzyme loading of immobilized PGA were only 821 U/g and 65.3 mg/g, respectively. At present, PGA is mostly immobilized on spherical materials, and there is a lack of research on PGA immobilization by carriers with different morphologies. However, it is possible to reduce the resistance and improve the

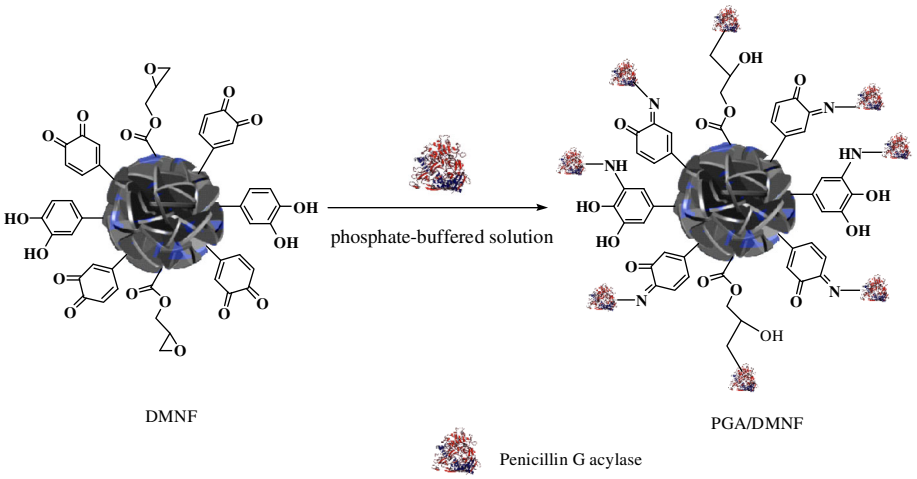


FIGURE 9 The schematic diagram of covalent interaction between PGA and DMNF

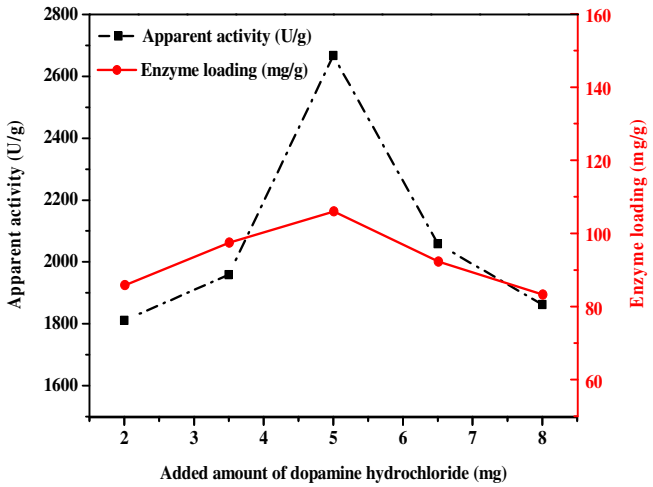


FIGURE 10 Effect of dosage of dopamine hydrochloride on catalytic performance of PGA/DMNF

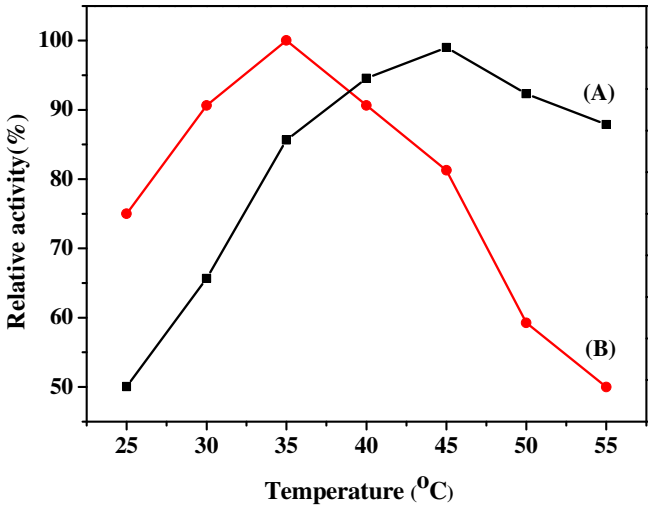


FIGURE 11 Effect of temperature on activity of PGA/DMNF (a) and free PGA (b)

diffuseness in immobilization of bulky PGA molecules of by controlling the morphologies of carriers. It was observed that the PGA-immobilized DMNF with 3D "hierarchical petal" displayed high apparent activity of 2,667 U/g for hydrolyzing penicillin G potassium to produce 6-APA.

Using the PGA immobilized on the di-functional magnetic nanoflowers with mesoporous-size having high activity of 2,667 U/g and 106 mg/g-support protein loading, where higher than other supports for immobilized PGA, illuminating that there were two kinds crucial design, that is, larger pore structure and effective functional groups. The schematic diagram of covalent interaction between PGA and DMNF is shown in Figure 9. The reactive epoxide functional groups of glycidyl methacrylate can be directly covalent coupling the amino groups of PGA via ring opening reactions and polydopamine film could also offer an easy way to combine the amino groups of PGA by means of aldolic condensation or Schiff base reactions to enhance the structural rigidity and operational stability of PGA.[28–29]

The effect of dosage of dopamine hydrochloride on catalytic performance of PGA/DMNF was performed. As shown in Figure 10, with the dosage of dopamine rising, the apparent activity and enzyme loading of PGA/DMNF first increased and the maximum activity and enzyme loading were both obtained at 5 mg. The apparent activity and enzyme loading of PGA/DMNF reached 2,667 U/g and 106 mg/g-support. As the dosage of dopamine hydrochloride increased to 8 mg, the apparent activity and enzyme loading of PGA/DMNF decreased to 1863 U/g and 83.6 mg/g-support, respectively.

The optimal temperature of free PGA and PGA/DMNF was determined in the temperature range of 25–55°C (Figure 11). It can be observed that the optimum temperature of free PGA was obtained at 35°C, while

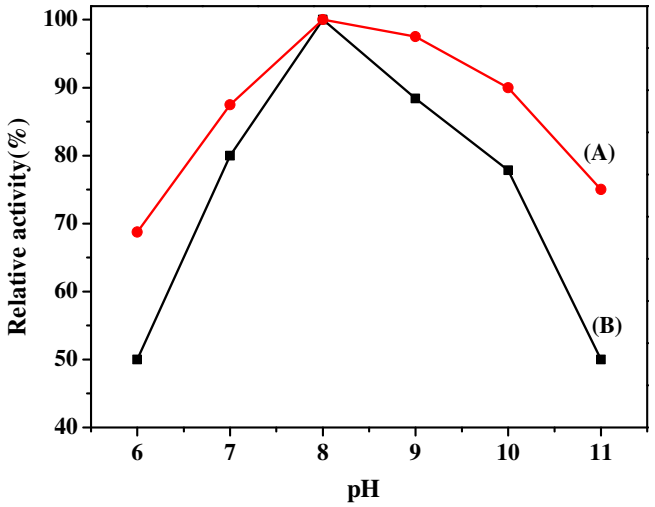


FIGURE 12 Effect of pH on activity of PGA/DMNF (a) and free PGA (b)

PGA/DMNF which showed maximum activity, was shifted to 45°C. When the temperature of free PGA higher than 35°C with the relative activity falls sharply, the activity of PGA/DMNF still increased with the raise of temperature until 45°C, demonstrating that the heat resistance of PGA/DMNF is better than that of free PGA. Also, it was obvious that the temperature was as high as 55°C; PGA/DMNF maintained quite a high relative activity (89%), which was higher than its counterparts, free PGA (50%). This reaction trend may be explained such that the enhanced intermolecular interaction between PGA and the support, making PGA structure more rigid and protecting the active site from damage.[30]

In this study, the optimal pH of free PGA and PGA/DMNF was also investigated with the pH values varied from 6 to 11 and was seen in Figure 12. With the enhancement of pH values, it was obviously that free PGA and PGA/DMNF showed similar trends and their maximum relative activity were both obtained at pH of 8. It was worthy to note that PGA/DMNF could tolerate harsher pH condition than free PGA, especially at pH of 11. The PGA/DMNF retained 75% of its initial activity, while that of its counterpart using free PGA as biocatalyst was only 50%. The main reason for this phenomenon could be attributed to the introduction of polymeric GMA and dopamine, whose "synergistic effect" made the connection between PGA and DMNF more stable, thus exhibiting the excellent acid and alkaline resistance. Besides, when the pH was as high as 9–11, the activity of free PGA and PGA/DMNF fell sharply. The possible reason is that the pH directly affects the surface charge distribution of PGA, which leads to the change of enzyme microstructure and affects the binding of PGA and support.[31–32]

Running title (a short but clear subtitle, less than 50 characters)

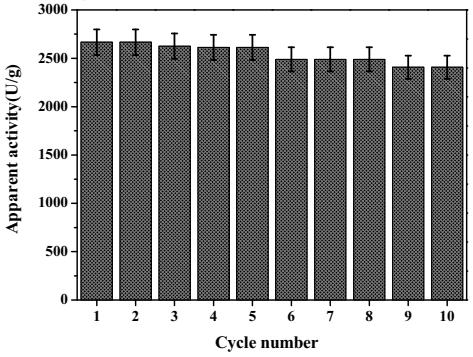


FIGURE 13 Reusability of PGA/DMNF

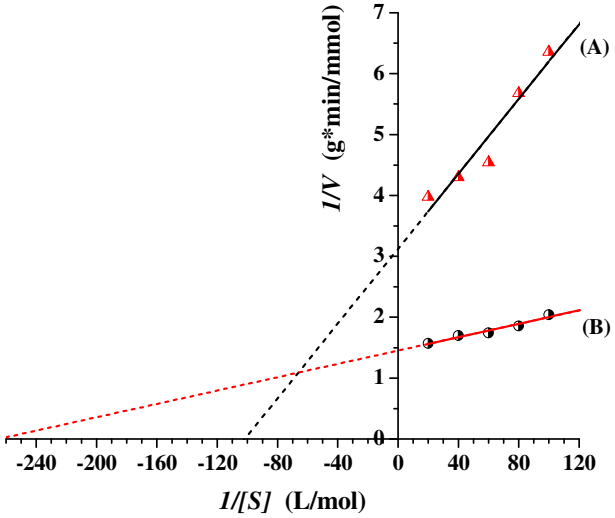


FIGURE 14 Lineweaver-Burk plots of PGA/DMNF (a) and free PGA (b)

3.4 | Operational stability of PGA/DMNF

As illustrated in Figure 13, the PGA/DMNF were used for consecutive cycles to catalyze hydrolysis of penicillin G potassium salt, and its apparent activity was 2,408 U/g retaining above 90% of its initial activity after 10 repetitions, which was higher than the activity retention rate obtained by Jiang's group after repeated use for 9 times.[33] The results exhibited the relatively excellent stability and catalytic activity owing to the co-polymerization of GMA and dopamine, which benefited from the covalent coupling between their functional groups of epoxy groups, hydrophilic catechol and phthaloquinone and the amino group of PGA, meanwhile they provide a more suitable microenvironment for the for immobilized enzyme. Overall, PGA immobilized on DMNF using di-functional coupling method exhibited better adaptability to operational and economical.

TABLE 1 Apparent kinetics parameters of PGA/DMNF and free PGA

Enzyme preparation	K_m (mol/L)	V_{max} ($mol \cdot L^{-1} \cdot min^{-1}$)
Free PGA	3.8×10^{-3}	9.34×10^{-3}
PGA/DMNF	9.86×10^{-3}	3.39×10^{-3}

3.5 | Kinetic constant K_m and V_m of PGA/DMNF and free PGA

The hydrolysis rates of penicillin G potassium catalyzed by free PGA and PGA/DMNF were investigated at different substrate concentrations in Figure 14. The apparent K_m constant and the V_{max} are calculated via Lineweaver-Burk plots. As seen in Table 1, the K_m value for PGA/DMNF (9.86×10^{-3} mol/L) was much higher than that for free PGA (3.8×10^{-3} mol/L), indicating the affinity of immobilized PGA to the substrate is lower than that of free PGA, while the V_{max} value for PGA/DMNF (3.39×10^{-3} $mol \cdot L^{-1} \cdot min^{-1}$) was lower than the corresponding value for free PGA (9.34×10^{-3} $mol \cdot L^{-1} \cdot min^{-1}$). The result manifested that the diffusion of substrate in DMNF carriers was restricted after immobilized PGA, and the main reason in the decrease of the V_{max} for immobilized PGA might be attributed to the steric hindrance effect between enzyme molecules and between carriers.[34]

4 | CONCLUSIONS

The magnetic composite nanoflowers with active epoxy groups and hydrophilic catechol as well as phthaloquinone groups were successfully designed and prepared via the polymerization of GMA and coating of dopamine, which possessed a mesopore size of 25.4 nm and unique appearance of layered 3D flowers. The results showed that the dosage of dopamine played an important role in the catalytic performance on preparation of 6-APA with penicillin G potassium salt catalyzed by PGA/DMNF. Moreover, the apparent activity and enzyme loading were obtained of PGA immobilized on different kinds of supports, in which PGA/DMNF possessed the apparent activity of 2,667 U/g and enzyme loading of 106 mg/g-support as well as exhibited superparamagnetic properties, that is, 39.7 emu/g. Meanwhile, the immobilized PGA exhibited superior acid, alkaline resistance, and thermal stability to the free, and it displayed considerable reusability after cycling of 10 runs with above 90% of its initial activity under magnetic condition. It is obvious that PGA/DMNF is a promising and efficient biocatalyst in industrial application.

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