Electronic Supplementary Information (ESI)

Immobilization of amidase into a magnetic hierarchically porous metal-organic framework for efficient biocatalysis

Authors and Affiliation:

Chaoping Lin,[ab] Kongliang Xu,[ab] Renchao Zheng*[ab] and Yuguo Zheng[ab]

[a] *Key Laboratory of Bioorganic Synthesis of Zhejiang Province, College of Biotechnology and Bioengineering, Zhejiang University of Technology, Hangzhou 310014, P. R. China*

[b] *Engineering Research Center of Bioconversion and Biopurification of Ministry of Education, Zhejiang University of Technology, Hangzhou 310014, People's Republic of China*

***Corresponding author:**

Tel: +86-571-88320391; Fax: +86-571-88320884;
E-mail: zhengrc@zjut.edu.cn

1 Materials and characterization

1.1 Materials and methods

Ferric chloride ($FeCl_3$, 97%), sodium acetate (NaAc, 99%), sodium citrate, ethylene glycol (EG, 99%), polyethylene glycol (PEG, M_w=400), were purchased from Shanghai Ling Feng chemical reagents Co., Ltd. (Shanghai, China). Dopaminechloride, glutaraldehyde (GA 50%), dodecanoic acid, zirconium tetrachloride ($ZrCl_4$), and 2-aminoterephthalic acid (BDC-NH_2) were obtained from J&K Scientific Ltd. (Shanghai, China). Fluorescein isothiocyanate (FITC) was purchased from Aladdin Reagent Inc. (Shanghai, China). BCA protein assay kits were purchased from Jiangsu KeyGEN Biotechnology Co., Ltd. (Jiangsu, China). N-phenylacetyl-4-fluorophenylglycine and penicillin amidase were obtained in our previous work.[1] Ultrapure water (18.2 MΩ·cm) was purified using a Satorius AG arium system. All other chemicals and reagents used were of analytical reagent grade and commercially available.

1.2 Material characterization/Instruments

Scanning electron microscopy (SEM) images were acquired on JSM-7610F scanning electron microscopy (SEM) (Hitachi, Japan) and Gemini SEM 300 (Zeiss, German). Transmission electron microscopy (TEM) images were taken on a JEM-1010 transmission electron microscope (Hitachi, Japan). Powder X-ray diffraction (XRD) patterns were collected using an X'TRA deffractometer (ARL, Switzerland). Samples were prepared by dispersing 30 mg of sample on a flat, glass plate PXRD holder with a diameter of 2.5 cm. A stretched piece of plastic film was then used to spread, flatten and hold the sample in position for measurement. Fourier-transform infrared spectroscopy (FT-IR)

characterization was obtained on a Thermo Nicolet 380 spectrometer using KBr pellets (Wisconsin, USA) and FTIR spectra were collected from 500 cm^{-1} to 4000 cm^{-1}. Thermo gravimetric analysis (TGA) was conducted under a nitrogen atmosphere at a heating rate of 10 ^oC min^{-1} under continuous flow of nitrogen gas from room temperature to 700 ^oC (NETZSCH, Selb, Germany). The saturation magnetization curve was performed on a Physical Property Measurement System 9T (Quantum Design, San Diego, USA) at room temperature. Dynamic light scattering (DLS) was performed in Zetasizer Malvern Nano ZSP by dilution of each sample in ultrapure water. BET surface area was measured by N_2 adsorption and desorption using an ASAP 2020 apparatus (Micromeritics) and porosimetry system at 77 K surface area and pore size analyzer. Samples were activated under vacuum at 150 ^oC for 12 h with the activation port equipped on ASAP 2020. The confocal laser scanning microscopy analysis for Enzyme-FITC fluorescent images was obtained by confocal laser scanning microscope (CLSM) (C1-Si, Nikon, Japan) with excitation wavelength of 488 nm and emission wavelength of 520 nm.

1.3 High-performance liquid chromatography (HPLC) analysis

Quantitative analysis of substrate and products were conducted using a HPLC system (Shimadzu Co., Kyoto, Japan).[1]

2. Synthesis and characterization of hierarchical Fe_3O_4@MOF

2.1 Synthesis of Fe_3O_4 and PDA coated Fe_3O_4

Fe_3O_4 nanoparticles were synthesized by a solvent-thermal method as previously reported with some modification.[2] Typically, EG (80mL), PEG (2 g), $FeCl_3$ (1.3 g), NaAc (6.0 g), sodium citrate (1.0 g) and H_2O (75 μl) were sequentially added into a round-bottom flask. Then, the mixture was vigorous stirring at 100 ^oC until to obtain a homogeneous transparent solution. The suspension was transferred into Teflon-lined stainless-steel autoclave and kept 200 ^oC for 12 h without stirring then cooled to room temperature. The obtained magnetic black product was collected by a magnet after washed with water (3×50) and ethanol (3×50) and dried at 50 ^oC under vacuum for 12 h prior to subsequent use.

Fe_3O_4 nanoparticles (1.0 g) were ultrasonically dispersed in100 mL of TRIS buffer (100 mM pH 8.5), followed by adding 100 mg of dopaminechloride. The mixture was stirred for 12 h, and the PDA coated Fe_3O_4 nanoparticles were collected by a magnet. The final nanoparticles were dried at 50 ^oC under vacuum for 12 h.

2.2 Synthesis of hierarchical Fe_3O_4@MOF

The core-shell hierarchical Fe_3O_4@MOF nanoparticles were prepared as follows. In brief, Fe_3O_4@PDA nanoparticles (0.5 g) were added into a mixture of $ZrCl_4$ (0.1 g) and dodecanoic acid (1.5 g) in a DMF (40 mL) mixture. Then, the mixture was treated by ultrasonication for 15 min, and BDC-NH_2 (0.04 g) was added, Subsequently, the mixture was placed in a preheated 100 ^oC oil-bath pan for 3 h. the reaction was then heated to 130 ^oC for 24 h. the product was collected by a magnet and then washed three times with DMF. The product was soaked in a mixture solution of DMF (80 mL) and concentrated hydrochloric acid (0.4 mL) at 90 ^oC for 24 h. After washing three times with DMF, the products were dried at 50 ^oC under vaccum for 12 h.

2.3 Synthesis of Fe_3O_4@[Cu_3(btc)$_2$], Fe_3O_4@MIL-100Fe, Fe_3O_4@ZIF-8

The magnetic MOFs including Fe_3O_4@PDA@[Cu_3(btc)$_2$], Fe_3O_4@MIL-100Fe, Fe_3O_4@ZIF-8 were synthesized according to previous reports and used for amidase immobilization.[3]

3. Immobilization of amidase on hierarchical Fe_3O_4@MOF and its properties

3.1 Immobilization of amidase on hierarchical Fe_3O_4@MOF

The immobilization procedure was performed as follows. The core-shell magnetic carriers (100 mg) were added into the phosphate buffer (10 mL, 2 M, pH 8.0) and then the mixture was treated by ultrasonication for 10 min. The amidase (50 mg) was added to the above mixture with stirring for 3 hour at 25 ^oC. Afterward, an amount of GA was added to the mixture and stirred to cross-link enzyme to the core-shell magnetic carrier. The immobilized enzymes were obtained after washing 3 times with phosphate buffer (100 mM, pH 8.0) and dried by vacuum freeze-drying.

The immobilization condition including the weight ratio of nanoparticle to enzyme, GA concentration, cross-linking time was further optimized. The protein content of all enzymes was analyzed by BCA assay Kit with bovine serum albumin as standard.

3.2 Immobilization of FITC-enzymes on hierarchical Fe_3O_4@hierarchifcal MOF

FITC solution in DMSO (10 mg/ml) was added into 1ml of an enzyme solution (2 mg/ml) in PBS buffer (pH 8.0), and the solution shaken for 8 h at 120 rpm at 25 ^oC. Subsequently, the suspension was filtered and the final suspension was preserved by light and kept on mechanical stirring at 25 ^oC. Enzyme and FITC were dissolved in 30 mL of phosphate buffer (50 mM, pH 8.0), and then the solution was stirred at 25 ^oC for 24 h (120 rpm). Afterwards, the mixture containing labeled proteins was dialyzed against distilled water for 48 h with a molecular weight cut-off of 8000-14000 Da. The amidases were subjected to lyophilization for obtaining the FITC-labeled amidases and the FITC-labeled amidases were immobilized on Fe_3O_4@hierarchical MOF as described as above to obtain an FITC-labeled immobilized enzyme.

3.3 Immobilization of amidase on different reported magnetic MOFs

100 mg magnetic MOFs including magnetic Cu-BTC, magnetic MIL-100Fe, and magnetic ZIF-8 were suspended in 10 mL phosphate buffer (2 M, pH=8.0) by a brief sonication, and 50 mg of amidase was added. The mixture was shaken at 200 rpm and 25 ^oC for 3 h. The immobilized enzyme was separated by magnet and washed by phosphate buffer (100 mM, pH=8.0) for three times. The enzyme content of supernatant was analyzed by BCA assay Kit using bovine serum albumin as standard.

3.4 Activity analysis

The initial activity of the immobilized amidases (free amidases) were determined in a reaction reaction mixture containing 5 ml glycine-sodium hydroxide buffer (pH 9.0), N-phenylacetyl-4-fluorophenylglycine (50 mM), immobilized amidase (5 g/L). The reaction was conducted for 2 min at 40 ^oC with 200 rpm and then immobilized amidases were separated from the mixture solution by magnet. The product (*S*)-4-fluorophenylglycine concentration was analyzed with HPLC. One unit (U) immobilized enzyme activity was defined as the amount of immobilized enzyme that synthesizes 1 μmol of (*S*)-4-fluorophenylglycine per minute under the standard activity assay conditions. The specific activity, activity recovery (%), immobilization yield (%), relative activity (%) and enzyme loading were calculated using Eqs. (1)-(4):

$$\text{Activity recovery (\%)} = \text{Total activity of immobilized enzyme/ total activity of free enzyme} \times 100\% \quad (1)$$

$$\text{Immobilization yield (\%)} = \text{Immobilized protein/ total loading protein} \times 100\% \quad (2)$$

$$\text{Relative activity (\%)} = \text{residue activity /original activity} \times 100\% \quad (3)$$

$$\text{Enzyme loading} = \text{immobilized enzyme mass/ total mass of carriers (mg/g)} \quad (4)$$

3.5 Secondary structural analysis by FT-IR

The free and immobilized amidases were analyzed by FT-IR to determine its changes in the fractions of secondary structure. Further essential Thermo Scientific ™ OMNIC v.8.2 software was used to enhance the resolution of FT-IR spectra by taking the derivative of amide I region (1700-1600 cm^{-1}). The peak areas obtained under multi-component of amide I bonds were quantified using peak separation and analysis software with Peak Fit v4.12.

3.6 Properties of immobilized amidase

To investigate the organic solvent tolerance of immobilized amidase and compare that of free one, both immobilized and free amidase were incubated in organic solvents including dimethyl formanide (DMF), dimethyl sulfoxide (DMSO), methanol, and ethanol at 25 ^oC for 24h, respectively. Subsequently, the residual activity was measured by above activity assay method. Similarly, the extreme temperature tolerance of immobilized and free amides was investigated by incubating at 100 ^oC (80 ^oC) for 1h and then residual activities were analyzed.

The activities of immobilized and free amidase were assayed on the different temperatures (25-50 ^oC) and the pH values (7.0-11.0), respectively. The reactions were performed in a mixture of containing 5 ml glycine-sodium

hydroxide buffer (pH 9.0), N-phenylacetyl-4-fluorophenylglycine (50 mM), immobilized amidase (5 g/L). The thermal stability was investigated by measuring the residual activity of immobilized amidase and the free one after incubation in glycine-sodium hydroxide buffer (100 mM, pH 9.0) at 40 °C for 20 d. The storage stability was also inspected after storing at 4 °C in glycine-sodium hydroxide buffer (100 mM, pH 9.0) for 5 months and then activities were analyzed. The initial activity of immobilized and free form was considered to be 100% in all these experiments. The kinetic parameters immobilized and free amidase were assayed. The reactions were performed with various substrate concentrations (10-200 mM) at 40 °C and the kinetic parameters including Michaelis-Menten constants (K_M) and maximum reaction rates (V_{max}) were calculated from Lineweaver-Burk plot equation.[4]

3.7 Time-course of the biocatalytic process

The reaction was performed with the different substrate concentration (60-100 mM) in buffer at pH 9.0 for 2 h (100 rpm, 40 °C). Aliquot of the obtained mixture was withdraw at predetermined times and analyzed by HPLC.

3.8 Reusability of immobilized amidase

To evaluate the reusability of immobilized amidase**,** the reaction was performed with the mixture containing of 100 mM substrate, 10 g/L immobilized enzyme in buffer at pH 9.0 for 2 h (100 rpm, 40 °C). After each reaction, the immobilized enzyme was recovered by a magnetic and washed with distilled water, and the recovered immobilized enzyme was employed in repeated reuse as catalyst under the same conditions.

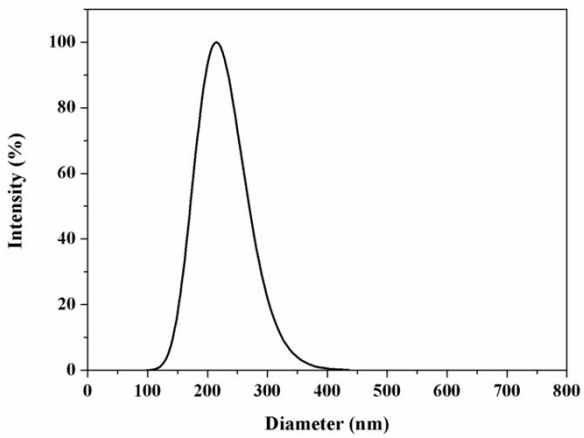

Figure S1. The DLS analysis of Fe_3O_4 nanoparticles.

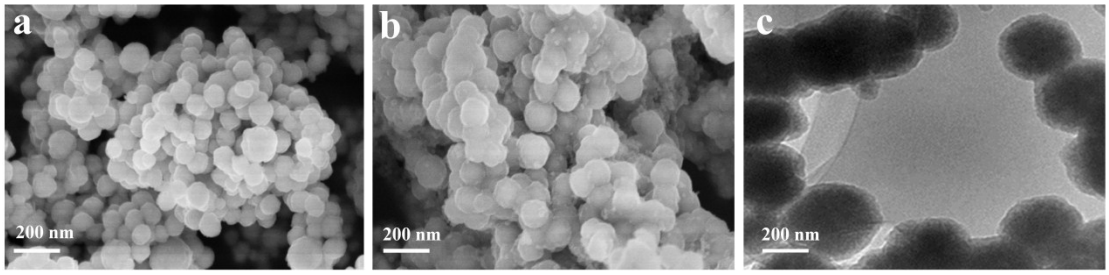

Figure S2. (a) SEM of PDA coated Fe_3O_4; (b) SEM of Fe_3O_4@hierarchical MOF; (c) TEM of PDA coated Fe_3O_4.

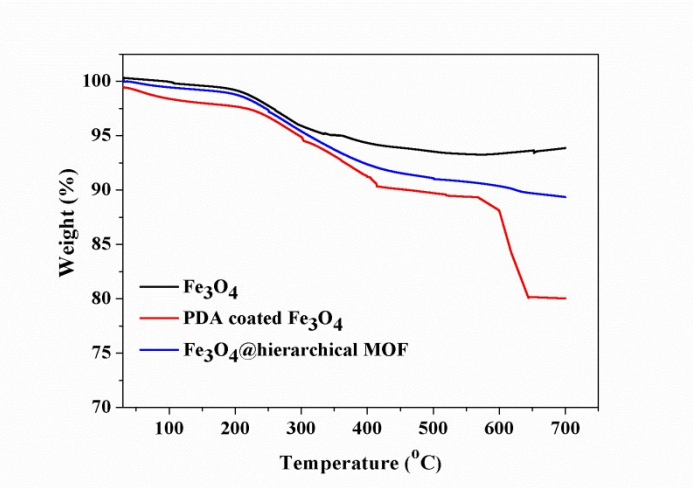

Figure S3. The TGA analysis of Fe_3O_4, PDA coated Fe_3O_4 and Fe_3O_4@hierarchical MOF.

The weight loss of Fe_3O_4@hierarchical MOF is less than that of PDA coated Fe_3O_4 when the temperature heated to ~580 °C, which means that a material with high thermal stability (*i.e.* MOF) has been coated on the PDA layer, demonstrating the formation of MOF shell.

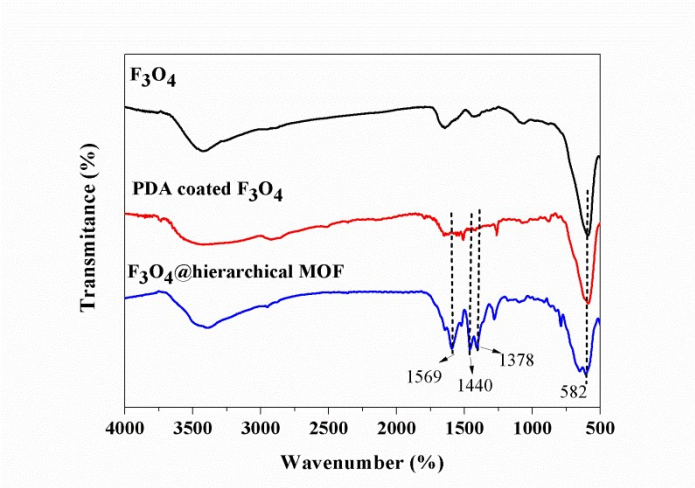

Figure S4. FT-IR spectra of Fe_3O_4, PDA coated Fe_3O_4, and Fe_3O_4@hierarchical MOF.

Compared with the FT-IR spectrum of PDA coated Fe_3O_4, 3 new sharp peaks at 1569 cm^{-1}, 1440 cm^{-1} and 1378 cm^{-1} have been observed from Fe_3O_4@hierarchical MOF, which corresponds to C=O from the carboxy group of organic ligand. This evidence proved that the MOF has been successfully coated on the surface of PDA coated Fe_3O_4.

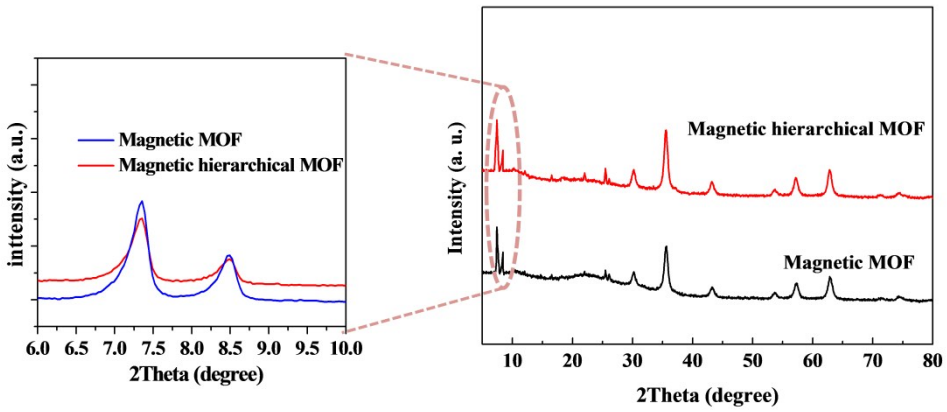

Figure S5. XRD patterns of magnetic MOF and magnetic@ hierarchical MOF.

Table S1. Analysis Parameters of XRD of magnetic MOF and magnetic hierarchical MOF

	FWHM (7.45)	FWHM (8.42)	Crystallinity
Magnetic MOF	0.266	0.232	7.2%
Magnetic hierarchical MOF	0.27	0.241	6.7%

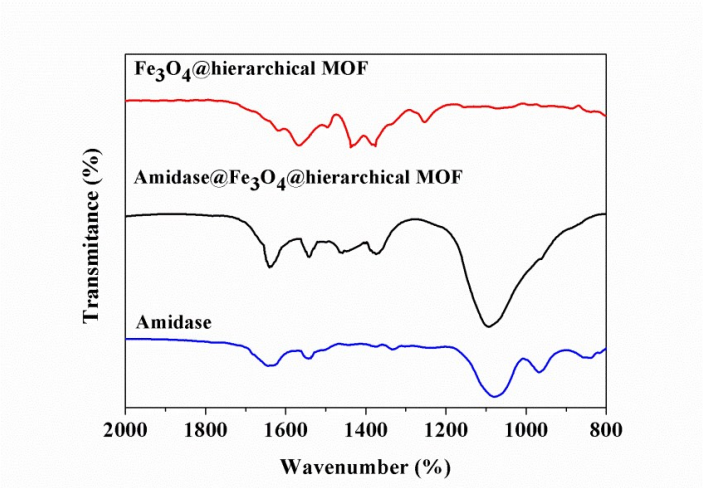

Figure S6. FT-IR spectra of Fe_3O_4@hierarchical MOF, amidase@Fe_3O_4@hierarchical MOF, and amidase.

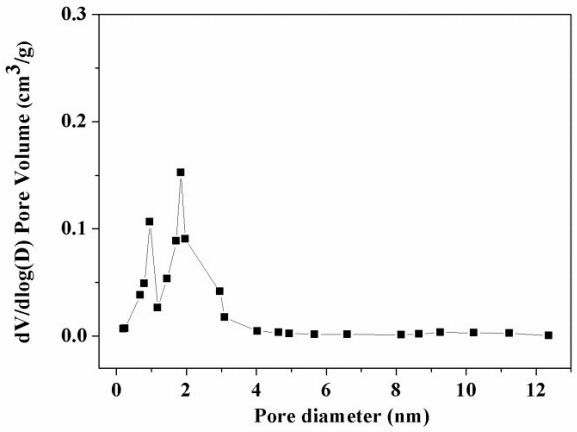

Figure S7. Pore size distribution curves of amidase@hierarchical Fe_3O_4@MOF.

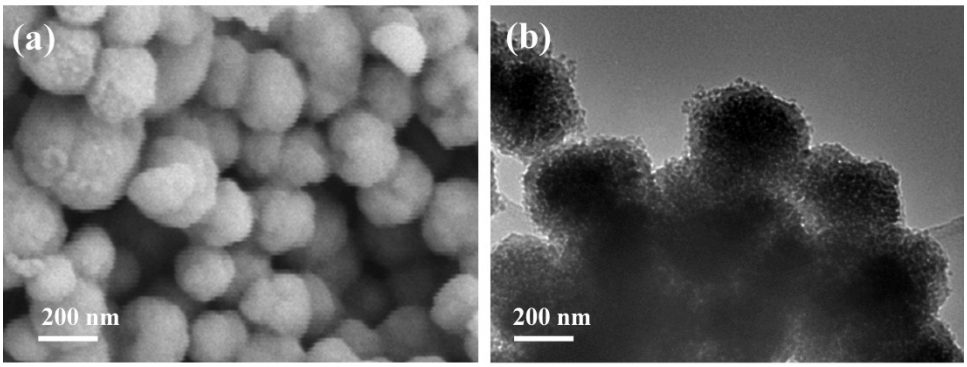

Figure S8. SEM (a) and TEM (b) of amidase@Fe_3O_4@hierarchical MOF.

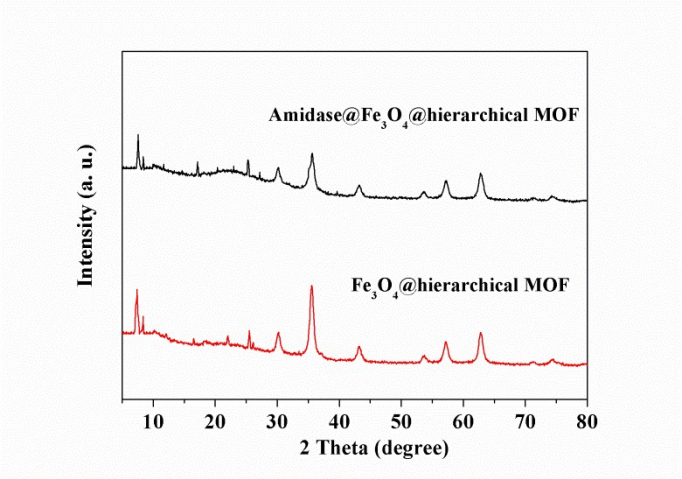

Figure S9. The XRD profile of amidase@hierarchical Fe_3O_4@MOF.

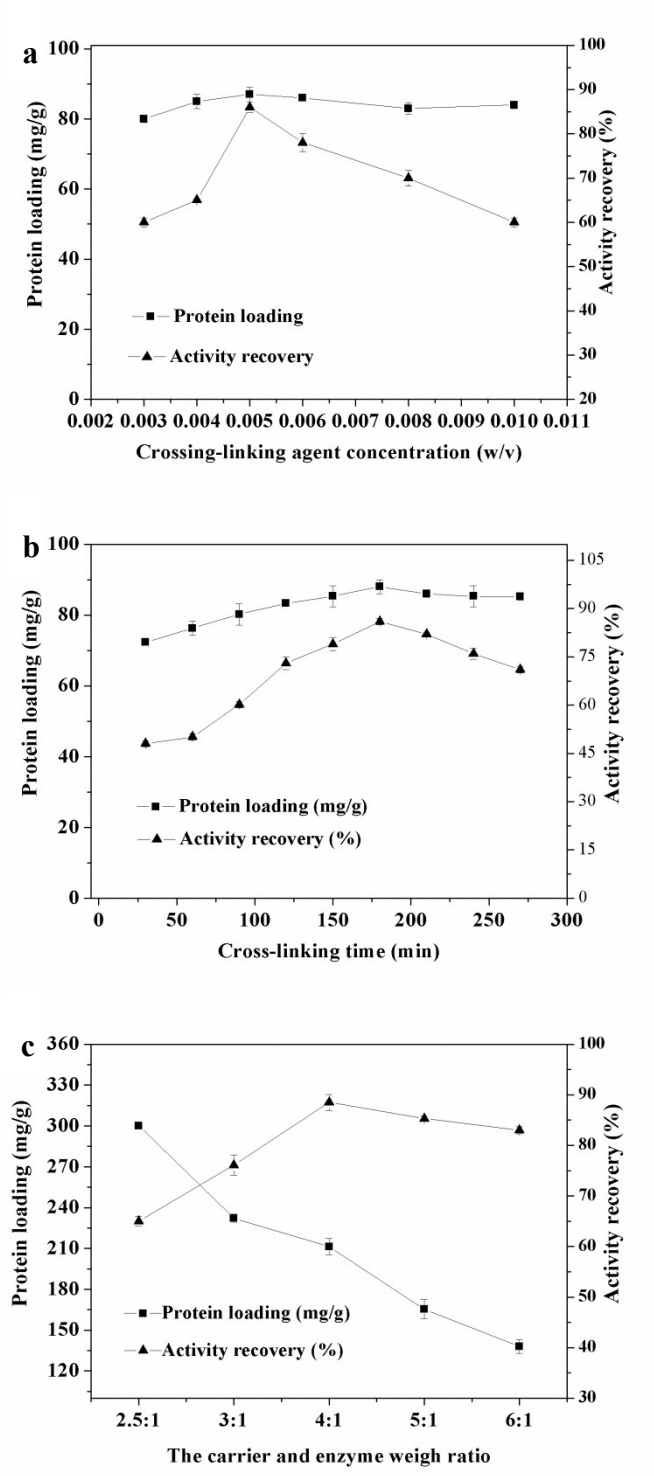

Figure S10. (a) The effect of glutaraldehyde concentration on immobilization efficient, (b) the effect of cross-linking time on immobilization efficient, (c) the effect of magnetic carrier and amidase mass ratio on immobilization efficient.

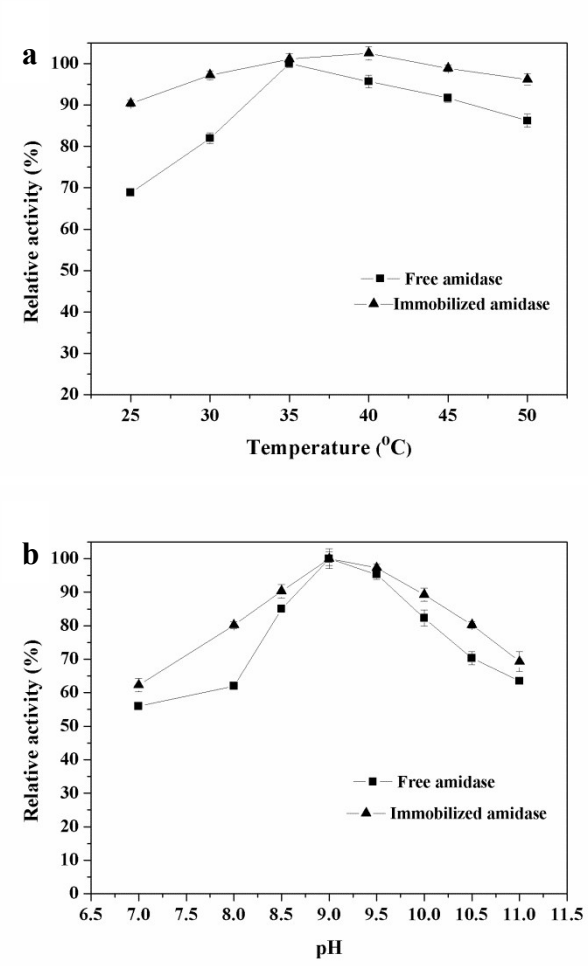

Figure S11. Effects of temperatures (a) and pHs (b) on the activity of immobilized and free amidase.

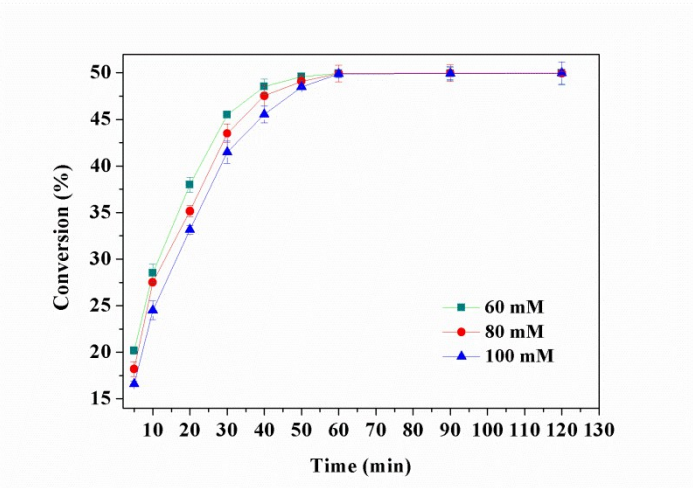

Figure S12. Time course of kinetic resolution of *N*-phenylacetyl-4-fluorophenylglycine using immobilized amidase at different substrate loading.

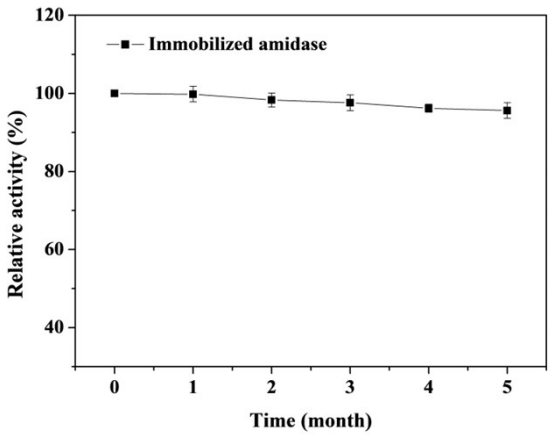

Figure S13. Storage stability of immobilized amidase.

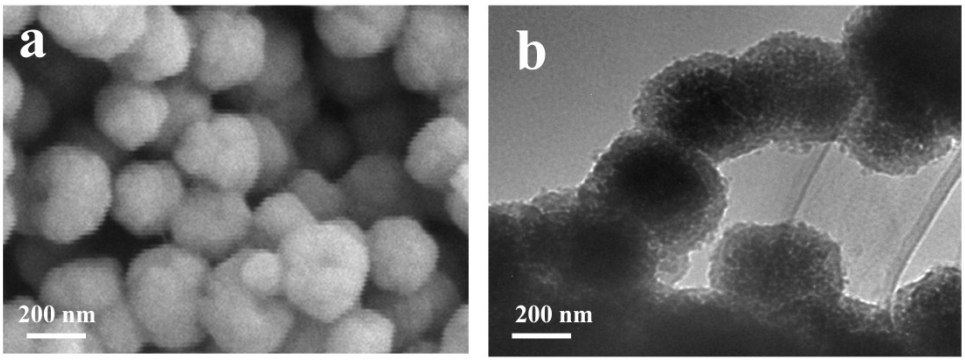

Figure S14. SEM (a) and TEM (b) of amidase@Fe_3O_4@hierarchical MOF after 15 cycles.

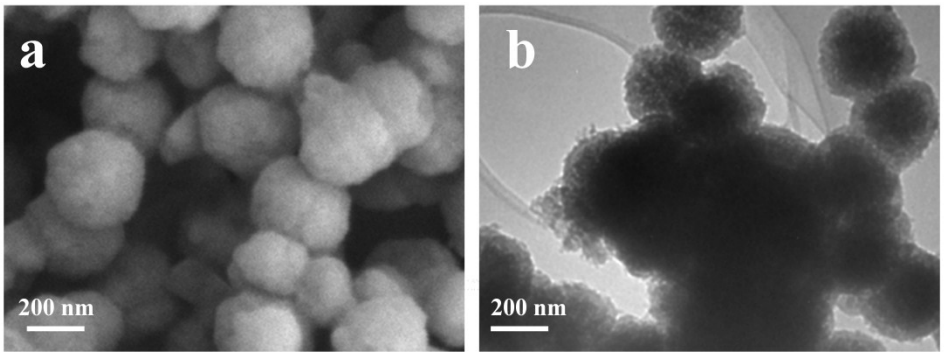

Figure S15. SEM (a) and TEM (b) of amidase@Fe_3O_4@hierarchical MOF after 5-month storage.

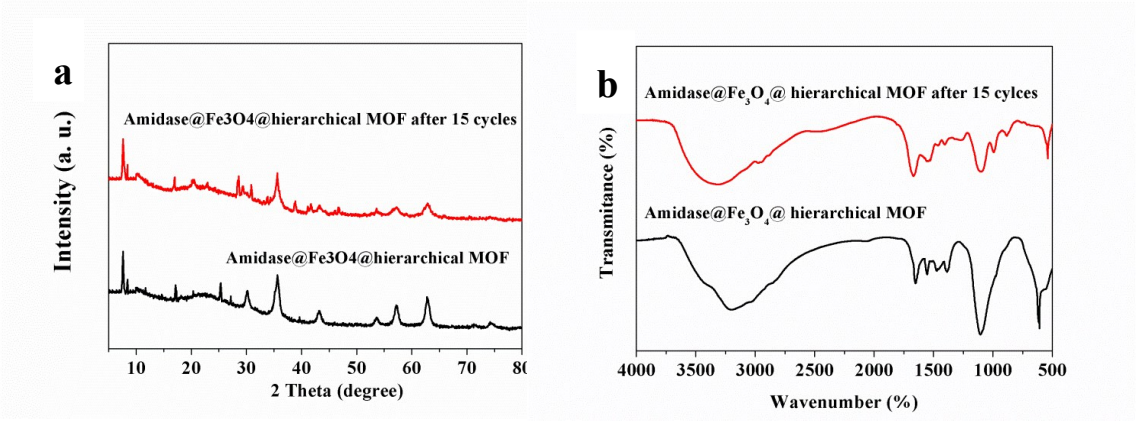

Figure S16. (a) The XRD of immobilized amidase and after 15 cycles, (b) FT-IR spectra of immobilized amidase and after 15 cycles.

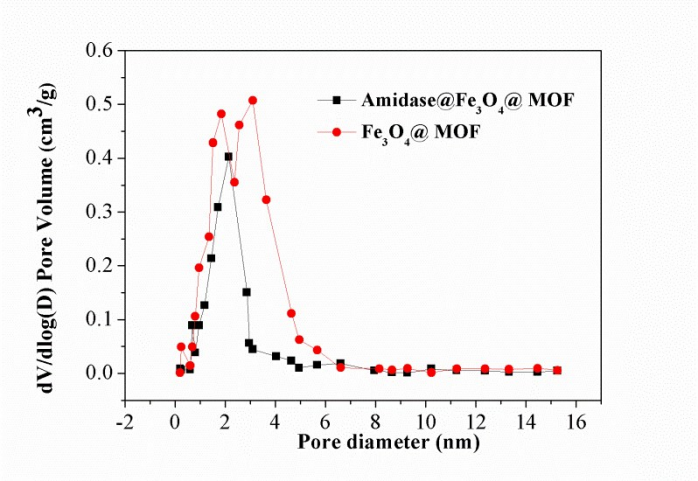

Figure S17. Pore size distribution curves of amidase@ Fe_3O_4@MOF.

References

1 C. Lin, X. Tang, R. Zheng and Y. Zheng, *Bioorg. Chem.*, 2018, **80**, 174.

2 Y. Dong, B. Wen, Y. Chen, P. Cao and C. Zhang, *RSC Advances*, 2016, **6**.

3 (a) M. Zhao, X. Zhang and C. Deng, *Chem. Commun.*, 2015, **51**, 8116; (b) Y. Wu, Y. Ma, G. Xu, F. Wei, Y. Ma, Q. Song, X. Wang, T. Tang, Y. Song, M. Shi, X. Xu and Q. Hu, *Sensor. Actuat. B-Chem.*, 2017, **249**, 195; (c) C. Hou, Y. Wang, Q. Ding, L. Jiang, M. Li, W. Zhu, D. Pan, H. Zhu and M. Liu, *Nanoscale*, 2015, **7**, 18770.

4 N. Hashemifard, A. Mohsenifar, B. Ranjbar, A. Allameh, A. S. Lotfi and B. Etemadikia, *Anal. Chim. Acta*, 2010, **675**, 181.