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Fabrication of enzyme reactor utilizing magnetic porous polymer membrane for screening D-Amino acid oxidase inhibitors

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ABSTRACT

In this work, a unique D-amino acid oxidase reactor for enhanced enzymolysis efficiency is presented. A kind of magnetic polymer matrices, composed of iron oxide nanoparticles and porous polymer membrane (poly styrene-co-maleic anhydride), was prepared. With covalent bonding D-Amino acid oxidase on the surface of the matrices and characterization of scanning electron microscope and vibrating sample magnetometer, it demonstrated that the membrane enzyme reactor was successfully constructed. The enzymolysis efficiency of the enzyme reactor was evaluated and the apparent Michaelis-Menten constants of D-Amino acid oxidase were determined (K_m was 1.10 mM, V_{max} was 23.8 mM min^{-1}) by a chiral ligand exchange capillary electrophoresis protocol with methionine as the substrate. The results indicated that the enzyme reactor could exhibit good stability and excellent reusability. Importantly, because the enzyme and the substrate could be confined into the pores of the matrices, the enzyme reactor displayed the improved enzymolysis efficiency due to the confinement effect. Further, the prepared enzyme reactor was applied for D-Amino acid oxidase inhibitors screening. It has displayed that the proposed protocol could pave a new way for fabrication of novel porous polymer membrane based enzyme reactors to screen enzyme inhibitors.

1. Introduction

D-Amino acid oxidase (DAAO) is an important enzyme which can catalyze the oxidative deamination of D-Amino acids. Some of the D-Amino acids including D-serine, D-aspartic acid and D-alanineare-candidates of physiologically active substances or biomarkers, so DAAO has played important role in pathophysiology of kidney disease, schizophrenia and depression [1–3]. DAAO is also related to the treatment of chronic pain due to hydrogen peroxide, which is a co-product of DAAO and is believed to contribute to pain hypersensitivity. During these pathophysiological processes, DAAO could mediate the metabolic reaction of some important metabolites [4–6] (such as D-serine, hydrogen peroxide). Importantly, DAAO inhibitors screening has been the considerable pharmacological approach in depression and other diseases therapy, and immunochemical investigation [5,7,8]. Moreover, the related study for exploring DAAO inhibitors as the potential medicines for disease treatment has attracted great interesting of researchers.

In recent years, a great number of methods for DAAO inhibitors screening have been reported including UV–vis absorption colorimetric method and fluorescence assay [9–14]. However, many assays utilized free DAAO in solutions [15], the reusability and stability of DAAO had rare been considered.

For improving DAAO enzymolysis efficiency and increasing its stability in screening DAAO inhibitors, some efforts have been devoted in finding new approaches. Among these developments, enzyme reactors which immobilized DAAO enzyme using many kinds of materials, such as sepabeads and inorganic nanoparticles, have provided many advantages in increased stability and reusability, easily operation and high enzyme efficiency convenience in handling. Interestingly, polymers also could be used for preparation of DAAO enzyme reactors [15–18]. Although previous work has demonstrated the good potential of polymer for DAAO enzyme immobilization [15], exploration of a wide diversity of polymer materials for fabrication of unique DAAO enzyme reactors is still desirable.

With the fast development of materials, magnetic membranes, which integrated magnetic nanoparticles into polymeric membranes, have been popular used. The magnetic membranes have provided

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dramatic properties based on the synergies of the nanoparticles and membranes, including response to magnetic field and obtaining well dispersed nanoparticles. Owing to the properties, the magnetic membranes have been applied as smart porous membrane valves, used for magnetic nanoparticles immobilization and electromagnetic shielding coating [19–21]. However, to our knowledge, the magnetic polymeric membranes have been rarely utilized for enzyme immobilization and applied as enzyme reactors.

In this study, a new kind of enzyme reactor which composed of magnetic nanoparticles and porous polymer membrane (MPPM) has been developed. The porous polymer membranes were fabricated by breath figure method [22,23] using poly styrene-co-maleic anhydride (PS-co-MAn). The membranes with ordered pores could provide multifunctional groups for DAAO immobilization. The MPPM based enzyme reactors have displayed both the merits of the magnetic nanoparticles and the porous polymer membranes: (1) providing multifunctional groups for enzyme immobilization and porous cavities for improving enzymolysis efficiency of DAAO, (2) exhibiting excellent reusability due to the magnetic property of iron oxide nanoparticles for fast separation, (3) displaying good stability compared to free DAAO in solution. All these advantages made MPPM based enzyme reactor as a good candidate for DAAO inhibitors screening. Using D-methionine (D-Met) as substrate, DAAO enzyme kinetics study has been carried out by chiral ligand-exchange capillary electrophoresis (CLE-CE) protocol. The enzymolysis efficiency of free DAAO and MPPM based enzyme reactor has been investigated. Furthermore, inhibition efficiency of various DAAO inhibitors including benzoic acid and its derivatives have been studied, indicating that the MPPM based DAAO enzyme reactors is promising for enzymolysis reaction.

2. Experimental section

2.1. Materials and chemicals

The chemical reagents for magnetic Fe_3O_4 nanoparticles synthesis including ferric chloride hexahydrate ($FeCl_3{\cdot}6H_2O$) and ferrous chloride tetrahydrate ($FeCl_2{\cdot}4H_2O$) were purchased from Xilong Chemical Company (Guangdong, P.R. China) and Tianjin Damao Chemical Reagent Factory (Tianjin, P.R. China), respectively. The chemical reagents for PS-co-MAn polymer synthesis including styrene and MAn monomers were purchased from Sinopharm Chemical Reagent Beijing Co., Ltd (Beijing, China) and Tianjin Guangfu Fine Chemical Research Institute (Tianjin, China), respectively. Azo-bis-isobutryonitrile (AIBN) was obtained from Shanghai Chemical Plant (Shanghai, China) and imidazole-1-carbodithioic acid phenyl ester (ICAP) was synthesized according to the reference [20].

D-methionine (D-Met) and other D,L-amino acid (D.L-AA) enantiomers, dansyl chloride (Dns-Cl), and D-Amino acid oxidase (DAAO, from porcine kidney) were provided by Sigma-Aldrich Chemical Co. (St. Louis, USA). Coomassie brilliant blue G-250, lithium perchlorate ($LiClO_4$), benzamide, benzoic acid, 4-hydroxybenzoic acid, 3-hydroxybenzoic acid, 4-aminobenzoic acid, 2-aminobenzoic acid, 4-nitrobenzoic acid, 3-nitrobenzoic acid, and 2-nitrobenzoic acid were purchased from Aladdin Chemistry Company(Shanghai, P.R. China). Sodium hydroxide, ammonium hydroxide, zinc sulfate, tris (hydroxymethyl) amino-methane (Tris), lithium carbonate, sodium chloride, sodium hydroxide, boric acid, hydrochloric acid, methanol, tetrahydrofuran (THF), diethyl ether and other reagents were all purchased from Beijing Chemical Corporation (Beijing, China). Throughout the experiments, Milli Q (Millipore Co., Massachusetts U.S.A) water was used.

2.2. Apparatus

The experiment for the AAs separation and DAAO activity study were carried out on the capillary electrophoresis (CE) system, which composed of HW-2000 chromatography workstation (Qianpu software, Beijing, China) and 1229 HPCE high voltage power (Beijing Institute of New Technology and Application, Beijing, China). The separation of the AAs was conducted by the uncoated bare capillaries of 60 cm (o. d. is 360 μm, i.d. is 75 μm, effective length 45 cm, Yongnian Optical Fiber Factory, Hebei, China).

The molecular weight and polydispersity index were determined by the gel permeation chromatography (GPC). An instrument composed by a Waters 1515 HPLC pump, a Waters 2414 detector and a set of Waters Styragel columns with THF as the eluent at a flow rate of 1.0 mL/min.

The polymer structure was characterized by Fourier transform infrared (FT-IR) spectra, which carried out on the Bruker Tensor-27 spectrophotometer (wave numbers ranging from 4000–400 cm^{-1}).

The morphology and porous diameters of the MPPM made by the breath figure method were investigated by S-4800 scanning electron microscope (SEM) from Hitachi Co. (Hitachi, Japan).

Thermogravimetric analysis (TGA) was carried out on a Heal force (Neofuge 23 R, Heal force development Ltd.) and the data were collected by a TA Instruments (pyris 1 TGA) in the temperature ranging from room temperature to 650 °C at a ramp rate of 20 °C/min.

2.3. Construction of MPPM based DAAO enzyme reactor

S-*co*-MAn was synthesized by reversible addition-fragmentation chain transfer (RAFT) method with a home-made droplets micro fluidic reactor, which composed of T junction and a tube (500 μm i.d. ×1.2 m, Fig. S1, Fig. S2). In a 50.0 mL boiling flask-3-neck, the polymerization solution was prepared including MAn: St:DATB: AIBN=100:1000:1:0.2 in molar ratio, and MAn is 10.0 mM, in 5.0 mL 1,4-dioxane. After degassed the oxygen by freeze-pump-thaw method, the reactant was draw into the droplets micro fluidic reactor with two pumps to realize the droplets synthesis. The reaction tube was heated to 80 °C and products were cooled to room temperature and collected. The products contained in 1,4-dioxane were separated from the oil and precipitated in excess of ether, filtered, and dried at 50 °C in a vacuum oven for 24 h.

The process for magnetic Fe_3O_4 nanoparticles synthesis was carried out according to the previously reported method [24]. Traditionally, in a flask, the $FeCl_3{\cdot}6H_2O$ (5.4 g, 20.0 mmol) and $FeCl_2{\cdot}4H_2O$ (1.98 g, 10.0 mmol) were dissolved in 100 mL deionized water, then using 25% ammonia, the pH of the reactant was adjusted to 12.0 and the solution reacted 3 h at vigorous stirring at 70 °C. The obtained magnetic nanoparticles were washed to remove the excess ammonia until the pH was neutral with water. Then, the products were washed with ethanol and dried at 45 °C for 24 h.

For preparation of MPPM used in the enzyme immobilization, typically, the magnetic Fe_3O_4 nanoparticles (10.0 mg/mL) were suspended in 30.0 mg/mL PS-co-MAn solution (dissolved in chloroform). Then, the mixed solution was cast onto a flat glass plate, which was placed in a closed humid environment (relative humidity 95%, prepared by a humidifier). The MPPM were obtained on the substrate after the chloroform evaporated.

To fabricate MPPM based DAAO enzyme reactor, the MPPM (10.0 mg), DAAO (2.5 mg) and $LiClO_4$ (2.0 mg) were suspended in 1.0 mL PBS buffer solution (100 mM, pH 8.2), then the mixture was vigorous stirred at 4 °C for different time (ranging from 1.0 to 5.0 h). Then the MPPM immobilized with DAAO was cleaned with PBS buffer using the magnet for three times. The amount of DAAO immobilized onto the MPPM could be determined by the Bradford assay [25], as described in Supporting Information. By detection the enzyme solution concentrations before and after immobilization, enzyme immobilized on the MPPM could be calculated.

2.4. Kinetic study of MPPM based DAAO enzyme reactor

Different immobilization time and concentrations have been studied for DAAO immobilization. Thus the activity of immobilized DAAO has been evaluated by the kinetics study, which calculated by the Michaelis-Menten's constant (K_m) and maximum rate (V_{max}), which monitored by the developed chiral ligand exchange capillary electrophoresis (CLE-CE) protocol (the separation conditions for CLE-CE method were displayed in supporting information). The typical substrate of DAAO, D-Met, was selected for kinetics study. In detail, the desired concentrations of D,L-Met dissolved and diluted with 50.0 mM Tri-HCl buffer (pH 8.6). Then 2.5 mg/mL D,L-Met substrate 50 μL mixed with 1.0 mg of DAAO-immobilized MPPM, in a 0.5 mL polypropylene tube and oxidized at 37 °C for 5 min and the MPPM separated using magnet. Then 20 μL of the supernatant solution was collected and further derived using Dns-Cl for CLE-CE analysis.

2.5. Screening DAAO inhibitors

The inhibition efficiency of nine classical DAAO inhibitors, such as benzamide, benzoic acid, 4-hydroxybenzoic acid, 3-hydroxybenzoic acid and so on, has been investigated in detail using the MPPM based DAAO enzyme reactor and the established CLE-CE method. The process could be summarized as below: 50 μL D,L-Met solution (2.5 mg/mL), 1.0 mg DAAO immobilized MPPM and 40 μL inhibitor solutions (200 μM) were mixed in a 0.5 mL tube and incubated at 37 °C for 5 min. The supernatant solution was collected and derived by Dns-Cl (Supporting Information, Section "Optimization of the separation conditions") for CLE-CE analysis after MPPM was retained by a magnet.

3. Result and discussion

3.1. Preparation of PS-co-MAn with droplets micro-fluidic synthesis system

PS-co-MAn was selected for fabrication of MPPM owing to its amphipathicity and multifunctional groups, which would construct porous membrane easily with breath figure method and DAAO enzyme immobilization. For efficiently obtaining PS-co-MAn polymer with controllable molecular weight, a series of PS-co-MAn was synthesized with a homemade droplets micro-fluidic synthesis device (Fig. S1) using RAFT polymerization method (Fig. S2) [26]. The molecular weight and polydispersity index (PDI) of the polymers PS-co-MAn obtained at different flow rate of oil phase and reactant phase were evaluated. Finally, the flow rate at 1.0 mL/h for reactant solution and 2.0 mL/h for oil phase were selected, and the obtained polymer PS-co-MAn with suitable molecular weight (30.4 kD) and good PDI (PDI=1.2) was further applied to construct the MPPM.

3.2. Construction and characterization of MPPM

The process for functional MPPM fabrication was presented in Fig. 1[26]. Firstly, the as prepared magnetic Fe_3O_4 nanoparticles were mixed with the PS-co-MAn solution [27]. Then, in a closed chamber (humidity higher than 95%), the mixture was cast onto a flat glass plate. After the chloroform solvent evaporated, the MPPM was successfully fabricated (Fig. 1A) [26]. The MAn block in the PS-co-MAn polymer provided groups which could easily react with the amino groups in DAAO enzyme for covalently immobilization. Thus, at very moderate condition, the DAAO could easily be immobilized onto MPPM (Fig. 1B).

FT-IR spectra of Fe_3O_4 nanoparticles and MPPM have been used for characterization of their structures and for proving successful fabrication of magnetic membrane. As displayed in Fig. 2, the typically stretching vibration absorption of Fe-O from magnetic Fe_3O_4 nanoparticles cores could be found at 590.1 cm^{-1} and the –OH absorption at the nanoparticles surface could be observed at 3415.7 cm^{-1}. The characterized stretching vibration absorption of CH_2 in benzene ring at 2920.1 cm^{-1} and C=C in benzene ring at 1450.1 cm^{-1}, 1493.2 cm^{-1}. The peak at 1778.3 cm^{-1}, 1853.5 cm^{-1} was ascribed to typical peak of C=O in maleic anhydride, which proved that the reactive MPPM has been successfully prepared.

SEM images of the prepared MPPM are displayed in Fig. 3. After MPPM was fabricated by breath figure method, the polymer displayed relative uniform pores with pore diameters ranging from 0.8 to 1.6 μm (the data was obtained from the SEM images). These pores not only could provide numerous immobilization sites made by MAn groups, but also set up cavities which could improve the collision probability of the substrate and the immobilized enzyme. The SEM images of Fe_3O_4 nanoparticles (Fig. S3A) and MPPM fabricated with different polymer concentrations were displayed in Fig. S3. It should be noted that due to the relative low concentrations of the suspended magnetic Fe_3O_4 nanoparticles in the polymer solution, the magnetic Fe_3O_4 nanoparticles were almost invisible in the SEM images (Fig. 3, Fig. S3B, C). However, its presence still could be verified by magnetic properties study (Fig. 4B) using the vibrating sample magnetometer.

Then, the magnetic properties of the obtained magnetic Fe_3O_4 nanoparticles, MPPM and MPPM based DAAO enzyme reactor at room temperature were investigated. The maximum saturation magnetization values of Fe_3O_4, MPPM and MPPM based DAAO enzyme reactor were 58.8, 13.6 and 8.7 emu/g, respectively. Figs. 4A and 4B displayed that the magnetization of Fe_3O_4 nanoparticles decreased obviously when they were embedded in the MPPM and MPPM based DAAO enzyme reactor. However, the MPPM based DAAO enzyme reactor could be separated from the reaction solution conveniently and quickly due to its magnetic property (Fig. S4).

To well define the polymer membrane and the magnetic nanoparticles dispersed in MPPM, the thermo gravimetric analysis (TGA) of the Fe_3O_4 nanoparticles, MPPM and MPPM based DAAO enzyme reactor were carried out and the results were displayed in Fig. 5. After further analysis the spectra, it could be observed that the loss of residual water caused a slight weight loss at below 300 °C in the three samples. Then, obviously weight loss were found of MPPM and MPPM based DAAO enzyme reactor at about 420 and 560 °C, which ascribed to the decomposition of polymer and enzyme on the Fe_3O_4 nanoparticles and indicated the successfully fabrication of MPPM and enzyme immobilization.

3.3. DAAO immobilization on MPPM

Owing to the functional groups in the polymer and the pores in the membranes, the MPPM was applied for DAAO immobilization. The MAn groups could easily react with amino groups in the DAAO to form stable covalent bonds at very moderate conditions. To optimize the best immobilization condition, the effect of polymer concentrations on MPPM fabrication, the concentrations of DAAO and immobilization reacting times on immobilization efficiency have been studied in detail. The activity of the immobilized DAAO was evaluated by the percentage of substrate D-Met oxidized after incubated with MPPM based enzyme reactor, H (%), which was calculated by the following equation,

$$H(\%) = \frac{A_0 - A_{D-Met}}{A_0} \times 100\% \quad (1)$$

the A_0 and $A_{D\text{-}Met}$ are the values of D-Met peak areas before and after MPPM based enzyme reactor enzymolysis, respectively.

First, the polymer solutions with various concentrations (20.0–40.0 mg/mL) were mixed with 10.0 mg/mL Fe_3O_4 and were used for fabrication of MPPM. Then, different DAAO solutions ranging from 0.5 to 3.0 mg/mL were used for study the immobilization effect on the enzymolysis efficiency of the MPPM based enzyme reactor. Fig. S5A indicated that its enzymolysis efficiency was maximized when polymer

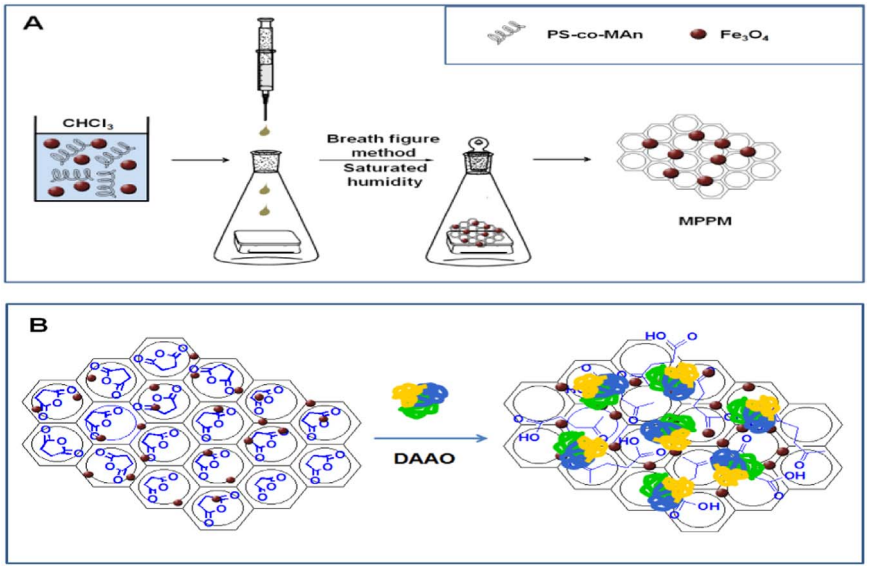

Fig. 1. Fabrication of MPPM (A) and schematic diagram of DAAO immobilization on MPPM (B). Conditions in (A): the polymer solutions (30.0 mg/mL) were mixed with 10.0 mg/mL Fe_3O_4 for fabrication of MPPM. Conditions in (B): 2.5 mg/mL DAAO reacted with MPPM for 3.0 h for enzyme immobilization.

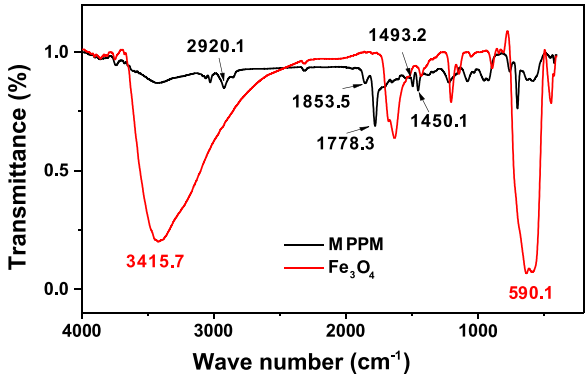

Fig. 2. FT-IR spectra of Fe_3O_4 and MPPM, respectively.

concentration at 30.0 mg/mL. Fig. S5B showed that the enzyme reactor modified with 2.5 mg/mL DAAO could reach the best enzymolysis efficiency. Finally, the enzyme immobilization time (1.0–5.0 h) has been optimized (Fig. S5C). The results displayed that the enzymolysis efficiency increased with enzyme immobilization time increasing from 1.0 to 3.0 h and decreased from 3.0 to 5.0 h. Thus, 3.0 h was chosen for reaction of MAn and amino group which could assure enough amount of enzyme for immobilization and for obtaining well enzymolysis efficiency. The $LiClO_4$ was applied in the enzyme immobilization process as the catalyst to active anhydride group and make it more easily for nucleophilic attack at the mild and efficient conditions [28].

The MPPM based enzyme reactor fabrication has been optimized. The amount of DAAO immobilized on the MPPM at the optimized condition has been investigated by the classical Coomassie blue-binding assay according to previous report protocols [26]. Firstly, Coomassie brillian blue solution (as described in Supporting Information, Section “Coomassie brillian blue G-250 solution”) was incubated with different DAAO solutions ranging from 1.0 to 3.0 mg/mL to obtain calibration curve by determining the absorbance at 595 nm (Fig. S6). Secondly, the DAAO solutions before and after the enzyme immobilization process were mixed with Coomassie brilliant blue G-250 solution to detect the amount of DAAO immobilized onto the MPPM. The results exhibited that the concentrations of DAAO solutions decreased from 2.5 to 1.0 mg/mL after immobilization process, which means 1.5 mg DAAO have been immobilized onto the MPPM. Considering that 10.0 mg MPPM were utilized, thus 0.15 mg

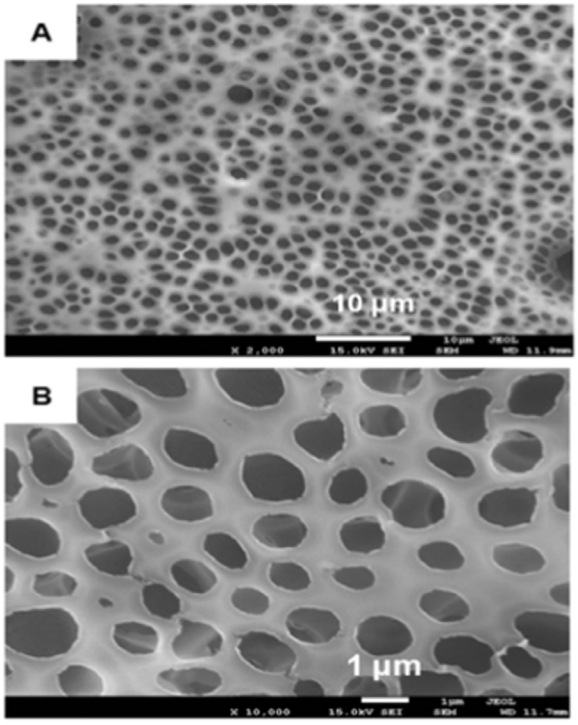

Fig. 3. SEM images of MPPM fabricated by 30.0 mg/mL polymer (A) and its high-resolution SEM image (B).

DAAO /mg MPPM was successfully loaded onto the materials. The large surface/volume ratio of MPPM has provided higher loading capacity for DAAO, which is good for the longevity and efficiency of MPPM based enzyme reactor.

3.4. Kinetic study of MPPM based DAAO enzyme reactor

The enzymolysis efficiency of MPPM based DAAO enzyme reactor was measured using D-Met as the substrate with chiral ligand exchange capillary electrophoresis (CLE-CE) technique. To remove the L-Met interferences in the real samples, CLE-CE method was constructed for separation of D- and L-amino acids due to its advantages of high efficiency, low cost and convenient manipulation. In this work, a kind

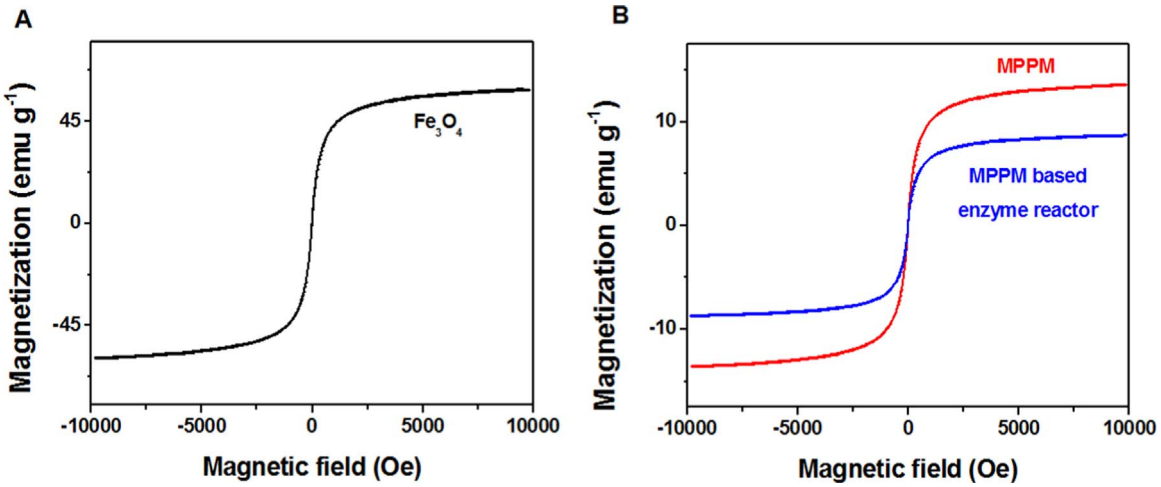

Fig. 4. The magnetization curves detected by vibrating sample magnetometer of Fe_3O_4 nanoparticles (solid line in Fig. 4A), MPPM (dash dot line in Fig. 4B) and MPPM based DAAO enzyme reactor (solid line in Fig. 4B) at room temperature.

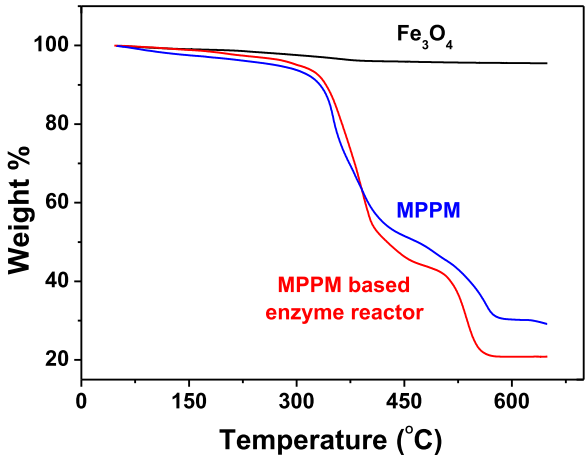

Fig. 5. The decomposition profiles for Fe_3O_4 nanoparticles (dot line), MPPM (solid line) and MPPM based DAAO enzyme reactor (dash dot line) analyzed by TGA.

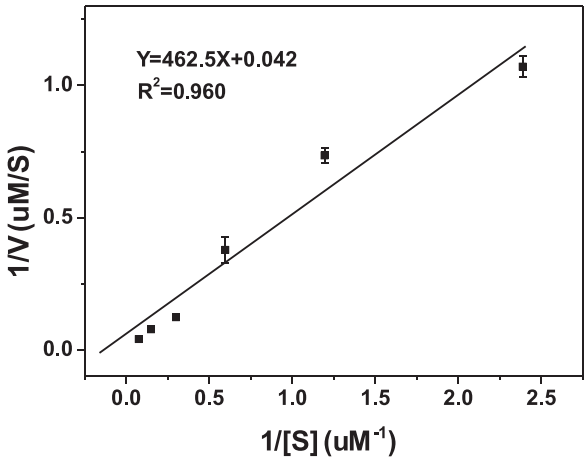

Fig. 6. Lineweaver-Burk plot for DAAO immobilized on the MPPM based enzyme reactor using D-Met as the substrate.

of new amino acid ionic liquids (AAILs) has been prepared and applied in CLE-CE system as chiral ligand coordinated with Zn(II). The detailed optimization processes for CLE-CE has been displayed in Supporting Information, including the effects of AAILs types (Fig. S7, Table S1), buffer pH (Fig. S8), ligand to Zn(II) ratio (Fig. S9), concentration of complexes (Fig. S10) on chiral separation efficiency. Importantly, using the proposed CLE-CE method, several pairs of amino acids have been separated successfully (Fig. S11, Table S2), including the substrate of DAAO (D,L-Met) Moreover, good linearity and favourable repeatability of Dns-D-Met were obtained (Supporting Information, Section "Quantitative analysis of D-Met"), which could be applied for enzyme kinetics study.

The enzyme kinetics study of MPPM based enzyme reactor and free DAAO in solution were evaluated by Michaelis constant (K_m) and maximum velocity (V_{max}) of the Michaelis-Menten equation in Eq. (2):

$$V = V_{max}[S]/(K_m + [S]) \quad (2)$$

where V and V_{max} are initial and maximum velocities, respectively, and [S] is target analyte concentration. The initial velocity means the reaction rate detected immediately after a short time period of the enzymolysis reaction triggered (5 min in this work). At this condition, the concentration of target analyte remained approximately constant.

The results displayed in Fig. S12 and Fig. 6 exhibited Lineweaver-Burk plots for D-Met enzymolysis using free DAAO in solution and MPPM based enzyme reactor, respectively. Then the corresponding kinetic characteristics were calculated. The K_m and V_{max} were calculated to be 1.10 mM and 23.8 mM min^{-1} for MPPM based enzyme reactor and 0.26 mM and 8.33 mM min^{-1} for free DAAO in solution, respectively. The K_m data indicated that the structure and affinity of DAAO immobilized in MPPM based enzyme reactor was not significantly altered from those in free solution. The V_{max} of the MPPM based enzyme reactor was approximate four times higher than that of free DAAO, which presents that high enzymolysis rate for DAAO in MPPM based enzyme reactor could be obtained at the same condition of D-Met solution. It is proposed that the plenty pores in the MPPM based enzyme reactor could concentrate DAAO and its substrate in limited space, which would decrease the diffusion within the matrix, then the enzymolysis rate between DAAO and substrates increased obviously [29,30]. It should be noted that the V_{max} value of MPPM based enzyme reactor was higher than the reported ones (Table S3).

As the enzyme reactor contains magnetic nanoparticles, the most outstanding advantages of MPPM based enzyme reactor were that it could be applied repeatedly. To evaluate the reusability of MPPM based enzyme reactor, the same concentration of D-Met incubated with the same MPPM based enzyme reactor for different times, and the enzymolysis rates were summarized. As displayed in Fig. 7A, after eight runs repeatedly operated of enzyme enzymolysis, the immobilized enzyme in MPPM based enzyme reactor still could maintain 77.5% activity. Moreover, the storage stability of MPPM based enzyme reactor was studied by storing it in the Tris-HCl buffer (50.0 mM, pH 8.6) at 4 °C and the results were showed in Fig. 7B. After four weeks, the activity of MPPM based enzyme reactor kept 81.6%, while the activity of free

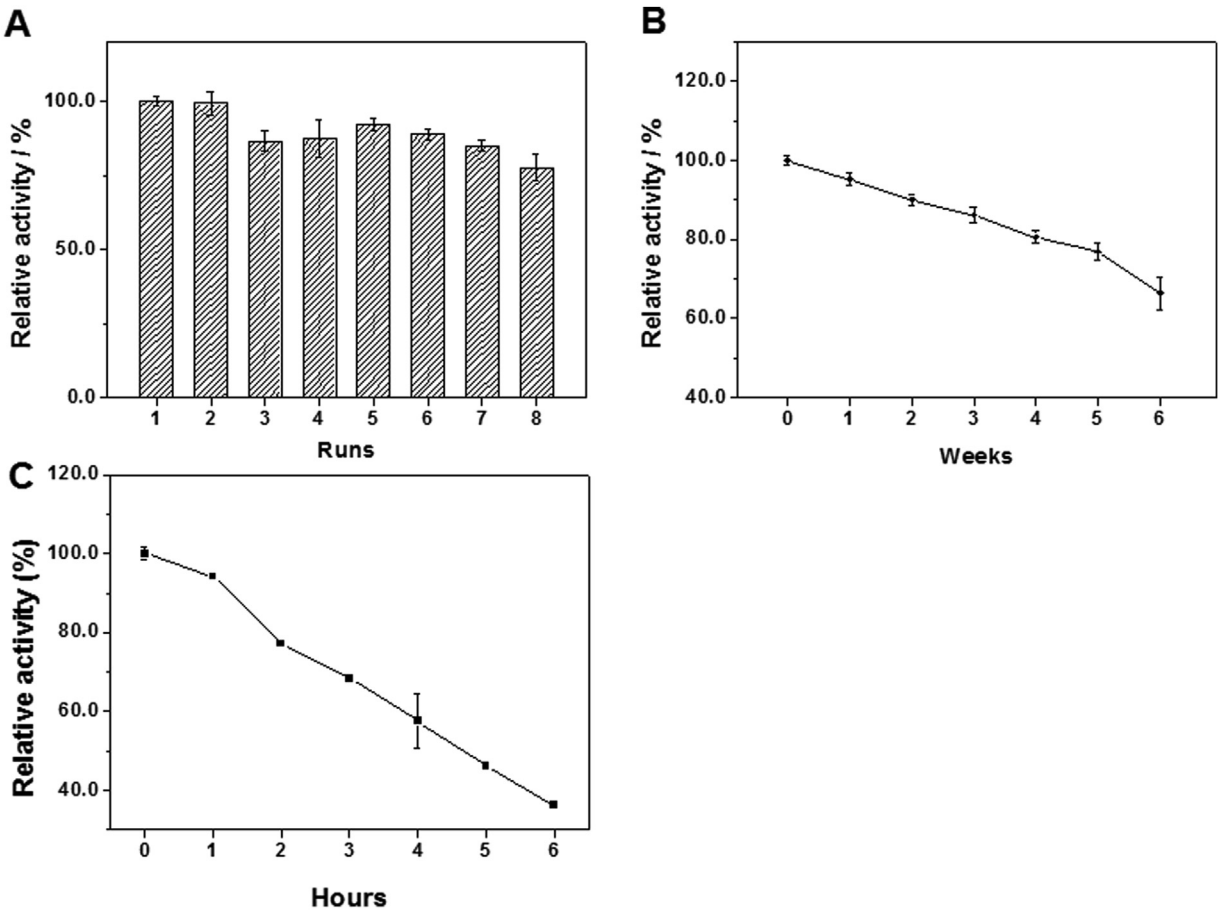

Fig. 7. Reusability (A), stability (B) of MPPM based DAAO enzyme reactor and stability of free DAAO in solution (C).

DAAO in solution was less than 40.0% within six hours (Fig. 7C), which indicated the stability of the enzyme reactor was greatly improved comparing with free DAAO in solution.

3.5. Application of MPPM based enzyme reactor for DAAO inhibitors screening

Due to the important property of DAAO, it has been selected as a therapeutic target for many diseases. Thus the inhibitors screening of DAAO has attracted researcher's attention extensively. Enzyme reactors have provided convenient existence form of enzyme for determining and identifying enzyme inhibitors. It has been reported that benzoic acid and its derivatives were classical and effective inhibitors of DAAO [15]. To evaluate the application function of MPPM based enzyme reactor of DAAO, the inhibiting efficiency of benzoic acid and its derivatives, including benzoic acid, have been investigated using MPPM based enzyme reactor. According to the reference [31], inhibition efficiency could be calculated by the following equation:

$$I\% = (C_I - C_E)/(C_0 - C_E) \times 100\% \quad (3)$$

in which I% is the inhibition efficiency, C_0 refers to the concentrations of D-Met in the absence of DAAO and inhibitor, C_I is the residual concentration of D-Met in the presence of inhibitor, C_E is the residual concentration of D-Met without inhibitor.

As reported in the reference [15], benzoic acid was the much popular used, thus inhibitory curve of benzoic acid to MPPM based enzyme reactor of DAAO activity was successfully calculated by determine the inhibition efficiency of benzoic acid at different concentrations, ranging from 5.0 μM to 900.0 μM. Fig. 8A showed that with the concentrations of D-Met increasing, its inhibitory effect on activity of DAAO enzyme reactor increased and reached a plateau. The results

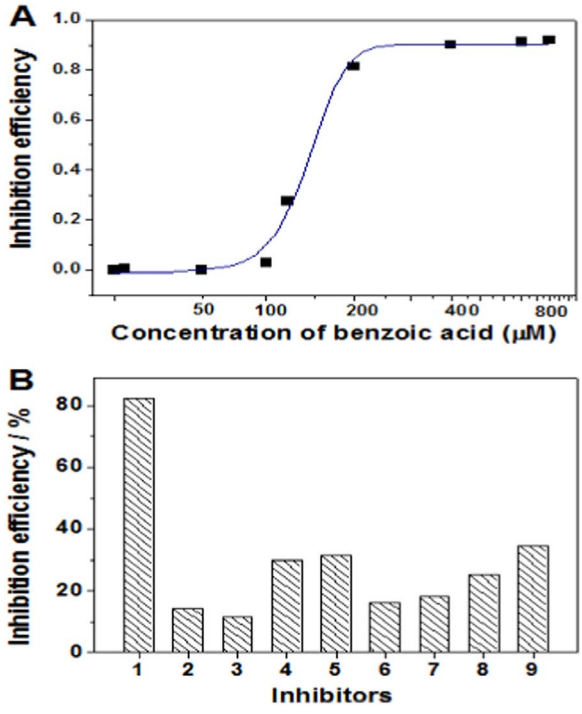

Fig. 8. The dose-dependent relationship of benzoic acid and DAAO activity inhibition (A) and the inhibition efficiency of different inhibitor at 200.0 μM screening by MPPM based enzyme reactor (B). Inhibitors: 1. benzoic acid; 2. 4-hydroxybenzoic acid; 3. 3-nitrobenzoic acid; 4. 4-aminobenzoic acid; 5. benzamide; 6. 4-nitrobenzoic acid; 7. 3-hydroxybenzoic acid; 8. 2-nitrobenzoic acid; 9. 2-aminobenzoic acid.

indicated that I% reached 82.4% when the concentration of benzoic acid at 200.0 μM, then almost remained constant with the concentration of benzoic acid increasing. Then, eight kinds of benzoic acid derivatives as inhibitor at concentration at 200.0 μM were incubated with the DAAO modified on the enzyme reactor and D,L-Met for 5 min, then the I% of each inhibitor has been calculated and displayed in Fig. 8B. The data indicated that benzoic acid exhibited the highest I% than other derivatives [15] and demonstrated that the MPPM based enzyme reactor of DAAO has good potential in screening DAAO inhibitors.

4. Conclusion

A kind of magnetic membrane DAAO enzyme reactor has been successfully constructed for enzyme inhibitors screening. Based on the merits of magnetic nanoparticles and porous polymer membrane, the enzyme reactor has exhibited its properties in enhanced enzymolysis efficiency due to the confinement effect. Moreover, compared with free DAAO solution, the proposed enzyme reactor displayed good stability and well reusability. This approach has great potential in constructing unique polymer membrane based enzyme reactors and screening enzyme inhibitors for drug discovery.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.talanta.2016.12.055.

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