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Preparation of Polydopamine-Modified 3D Interconnected Macroporous Silica for Laccase Immobilization

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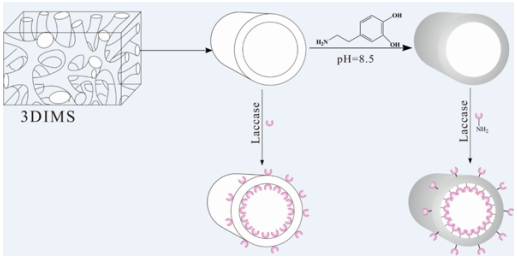
Abstract: Millimeter-sized polydopamine (PDA) modified three-dimensional interconnected macroporous silica (PDA/3DIMS) with high porosity, large pore volume and pore diameter was prepared and used as the support to immobilize commercial laccase (Lac) from Denilite II S. The skeleton of 3DIMS is constructed by silica nano-film, so the PDA/3DIMS is constructed by PDA/SiO_2/PDA sandwich nano-film. The immobilization conditions, incubation time, pH and enzyme concentration were optimized, and the properties of the immobilized laccase were investigated. The enzyme activity of Lac-PDA/3DIMS (295.9 U/g) was higher than that of Lac-3DIMS (222.2 U/g). The stability and reusability of the two immobilized laccases were both improved comparing with the free laccase. Lac-PDA/3DIMS exhibited higher retained activity (73.6%) than Lac-3DIMS (31.8%) after reused for ten times. Lac-PDA/3DIMS retained more than 84.0% initial activity after storage for a month, however, the free laccase and Lac-3DIMS were 7.1% and 48.6%, separately. These advantages revealed that PDA thin layer plays an important role to improve the enzymatic activity of immobilized laccase, and the PDA/3DIMS material is a very promising support for enzyme immobilization.



Keywords: laccase, immobilization, three-dimensional interconnected macroporous silica, nano-film.

1. Introduction

Laccase, a copper-containing oxidase, can catalyze and degrade benzenethiols, phenolic compounds, and ascorbate with concomitant reduction of dioxygen to two molecules water.[1,2] Due to its high catalytic activity and substrate specificity, laccase has gained extensive attention in various fields such as biosensor,[3] chemical synthesis,[4] and environmental protection.[5] However, low stability, high production cost and difficult separation from the reaction system of the free laccase limit its industrial applications.[6]

Enzyme immobilization is a valid method to stabilize enzyme structure, improve the reusability and the storage stability. There are plenty of methods for enzyme immobilization, such as physical adsorption, encapsulation, covalent binding, and cross-linking.[7-9] And numerous supports have been used to immobilize enzyme successfully, such as silica gel,[10] carbon nanomaterials,[11] polymer membrane,[12] mesoporous silica nanoparticles,[13] Cu-alginate matrices,[14] magnetic composite particles.[15] Which immobilization strategy is used usually depends on the properties, the nature of the support and enzyme, and complexity of the process. Nanoparticles, including porous and nonporous nanoparticles, have the advantages of high specific surface area and high enzyme loading.[11,13] Using porous nanoparticles as supports could increase the laccase loading, but the diffusion limitation is inevitable as the pore is easily to be blocked by the laccase. That may be the rate-controlling step in the catalytic reaction system. Besides, the nanoparticles are difficult to be separated from the reaction system.[16] Even the magnetic nanoparticles could be separated from the reaction system effectively by the external magnetic field, they are easy to aggregate. Millimeter-sized or submillimeter-sized catalysts are often applied in industry. However, when the pore is smaller, enzyme is either directly immobilized on the surface of the supports, or loaded in the pore to block the channel. Until now, there is still a great interest to develop novel supports for improving the activity and stability of immobilized enzyme. In comparison with nanoparticles, specific surface area of macroporous materials are lower, but the pore interconnectivity and surface accessibility are high.[17] Therefore, mass transport through macroporous materials is quite efficient. In addition, macroporous materials are easily to be prepared at low cost. Therefore, millimeter-sized macroporous materials could be used as promising candidates to immobilize laccase.

In this research, a novel millimeter-sized three-dimensional interconnected macroporous silica (3DIMS) constructed by nano-film was prepared. 3DIMS was modified by polydopamine (PDA) due to its lack of the active groups for firm binding of enzyme. Dopamine, a small bioactive molecule, could self-polymerize at weak alkaline solution. The generated PDA could adhere onto various types of substances, with the accompanied oxidization of catechol groups into quinone groups,[18,19] which can couple covalently with nucleophilic groups in enzyme. Cheap DeniLite II S laccase was immobilized on 3DIMS and PDA/3DIMS. The effects of incubation time, pH and initial laccase concentration

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on the immobilized enzyme activity were studied. The pH, thermal, storage and reusable stabilities of the free and immobilized laccase were also investigated, respectively.

2. Experimental

2.1. Materials

Bisphenol A epoxy resin (the epoxy value is 0.44) was bought from Nantong Xingchen Synthetic Material Co., Ltd. (Nantong, China). Tetraethoxysilane (TEOS, ≥98%) was bought from Shanghai Titan Scientific Co., Ltd. (Shanghai, China). Laccase (DeniLite II S) was purchased from Guangzhou Hainuo Biological Engineering Co., Ltd. (Guangzhou, China). Dopamine was purchased from Energy Chemical Industrial Corporation (China). 2,2'-Azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS) was purchased from Thermal Merle Biotechnology (China). All other chemicals such as, phosphoric acid (H_3PO_4), potassium dihydrogen phosphate (KH_2PO_4), and dipotassium phosphate (K_2HPO_4) *et al*., were analytical reagents.

2.2. Preparation of 3DIMS

The mixture of epoxy resin (16.0 g) and polyethylene glycol (29.0 g of PEG1000 and 5.0 g PEG2000) was heated and stirred until it became a clear solution. Then triethylenetetramine (4.6 g) was quickly added under strong stirring. The solution was poured into a polytetrafluroethylene tray and kept for 2.5 h at 70 °C. The resulting white product was washed repeatedly with deionized water to remove the PEG. The obtained macroporous polymer was cut into pieces (cubes with edge length of *ca*. 2 mm) and dried under vacuum for 12 h at 25 °C. The macroporous polymer was soaked in tetraethoxysilane for 3 h. Then the samples were exposed to $NH_3 \cdot H_2O$ atmosphere for 12 h in a sealed container to hydrolyze the infiltrated tetraethoxysilane. The polymer/silica composites were dried and calcinated at 600 °C for 30 min with the rate of 5 °C/min, the three-dimensional interconnected macroporous silica (3DIMS) was obtained.

2.3. Preparation of PDA/3DIMS

3DIMS was dispersed in dopamine solution with the concentration of 0.5 mg·mL^{-1}. After shaking for 2 h, the pH value of the solution was adjusted to 8.5 by tris(hydroxymethy) aminomethane. Then, the mixture was continued to shake for 12 h at room temperature. The resulting product was washed with deionized water repeatedly until the solution became colorless. The product was vacuumed for 12 h at 30 °C for further use.

2.4. Immobilization of laccase

0.1 g 3DIMS or PDA/3DIMS was added to 10 mL laccase solution prepared using 0.1 M phosphate buffer with a series of different concentrations, and the mixture was incubated at 30 °C for 8 h with shaking at 100 rpm. After that, the immobilized laccase was washed several times with the same phosphate buffer until no protein was detected in the supernatant.

2.5. Assay of enzyme activity

The activity of free and immobilized laccase was determined spectrophotometrically at 420 nm with 1.0 mM ABTS in 0.1 M phosphate buffer at 30 °C. Equivalent amount of free or immobilized laccase was added to the substrate solution and shaken for 5 min. The oxidation of substrate to $ABTS^+$ radicals was measured using UV-Vis spectrophotometer (T6, Beijing Purkinje General Instrument Co., Ltd., China), with the molar extinction coefficient of 3.6×10^4 L·mol^{-1}·cm^{-1}. One unit of enzyme activity was defined as the amount of enzyme, which catalyzed 1 μM of substrate per minute under the assay conditions. The relative activity was related to a percentage of the highest activity.

The effects of pH and enzyme concentration on laccase immobilization were evaluated under variety of pH (2.0-7.0) and enzyme concentration (15-80 mg·mL^{-1}).

2.6. Enzyme stability and kinetic parameters

Thermal stability was tested by incubating the free and immobilized laccase in 0.1 M phosphate buffer (pH 3.0 for free laccase, pH 4.0 for Lac-PDA/3DIMS and pH 6.0 for Lac-3DIMS) at different temperatures (25-50 °C) for 2.5 h.

The pH stability of free and immobilized laccase was studied by incubating them in buffers of pH in the range from 3.0 to 7.5 at 30 °C for 13 h.

The operating stability of the immobilized laccase was investigated in repeated batch experiments using ABTS as a substrate. The enzyme was incubated with 1.0 mM ABTS for 5 min (one cycle) in 0.1 M phosphate buffer at 30 °C. At the end of each oxidation cycle, the immobilized laccase was washed three times with the same phosphate buffer and the procedure was repeated with a fresh solution of substrate.

The storage stabilities of the free and immobilized laccase were evaluated by storing at 4 °C for 30 days with periodic measurement of residual activity.

Kinetic parameters (K_m and V_{max}) of free and immobilized laccase were determined by measuring initial rates of the reaction between laccase and ABTS (0.2-1.0 mM) in phosphate buffer (0.1 M, pH 3.0 for free laccase, pH 4.0 for Lac-PDA/3DIMS, pH 6.0 for Lac-3DIMS) at 30 °C. Kinetic parameters were obtained by fitting the data to the Michaelis-Menten equation using a nonlinear regression code.

2.7. Characterization

The structure of PDA/3DIMS and 3DIMS was observed by scanning electron micrographs (FE-SEM, SU-70, Ametek). The porosity, pore size distribution and pore volume were determined by mercury intrusion porosimetry (MIP, IV-9500, Micromeritics Instrument). The surface area was measured by BET (V-Sorb 2800P, Beijing Jinye Technology Co., Ltd). Elemental analysis was carried out on X-ray energy dispersive analysis spectrometer (EDX, SU-70, Ametek). The polymer content of the composite was measured with a thermo-gravimetric analysis (TGA, TGA/SDTA 851e, Mehler-Toledo) under N_2 protection. The Fourier transform infrared (FT-IR) spectra of the samples were obtained using

FT-IR spectrometer (PRO TEG E460 E.S.P Nicolet).

3. Results and discussion

3.1. SEM images

Figure 1 showed the SEM images of 3DIMS and PDA/3DIMS. It was clear that plenty of interconnected macropores exist in the 3DIMS, the pore size is between 300 and 700 nm, and the pore wall is constructed by silica nano-film, and the thickness of the film is uniform. As can be seen from Figure 1(d), the PDA/3DIMS composite retains the interconnected macroporous structure, the thickness of the pore wall is still uniform though it is thicker than that of 3DIMS, and the pore was not blocked, indicating that the PDA thin layer grew on the surface of the silica nano-film successfully.

3.2. FTIR spectra

The FT-IR spectra of 3DIMS and PDA/3DIMS were shown in Figure 2. In the FT-IR spectrum of 3DIMS, absorption bands at 804 cm^{-1}, 1104 cm^{-1}, and 3453 cm^{-1} were ascribed to the stretching vibration of Si-O, Si-O-Si, and -OH group, respectively. Comparing with the spectrum of 3DIMS, the band at 3453 cm^{-1} was stronger in the spectrum of PDA/3DIMS due to the superposition of -NH_2 with -OH group, the peaks at 804 cm^{-1} and 1104 cm^{-1} were weakened as the silicate was modified by PDA layer, and two new peaks appeared at 1601 cm^{-1} and 1355 cm^{-1} which could be attributed to the C=C resonance vibration in aromatic benzene ring and C-O-H bending vibration in phenol. The results suggested that 3DIMS had been modified by polydopamine successfully.

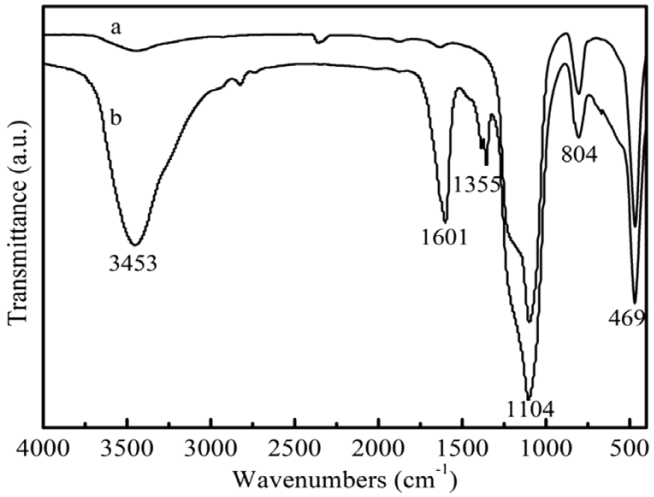

Figure 2. FTIR spectra of 3DIMS (a) and PDA/3DIMS (b).

3.3. MIP analyses

MIP analyses showed that the pore volume of 3DIMS is 5.6 $cm^3 \cdot g^{-1}$, the porosity is 90% and the average pore size is 350 nm (Figure 3). That was consistent with the results observed by SEM. Based on the N_2 adsorption and desorption isotherms, BET analysis showed that the BET surface area of PDA/3DIMS was 36.1 $m^2 \cdot g^{-1}$,

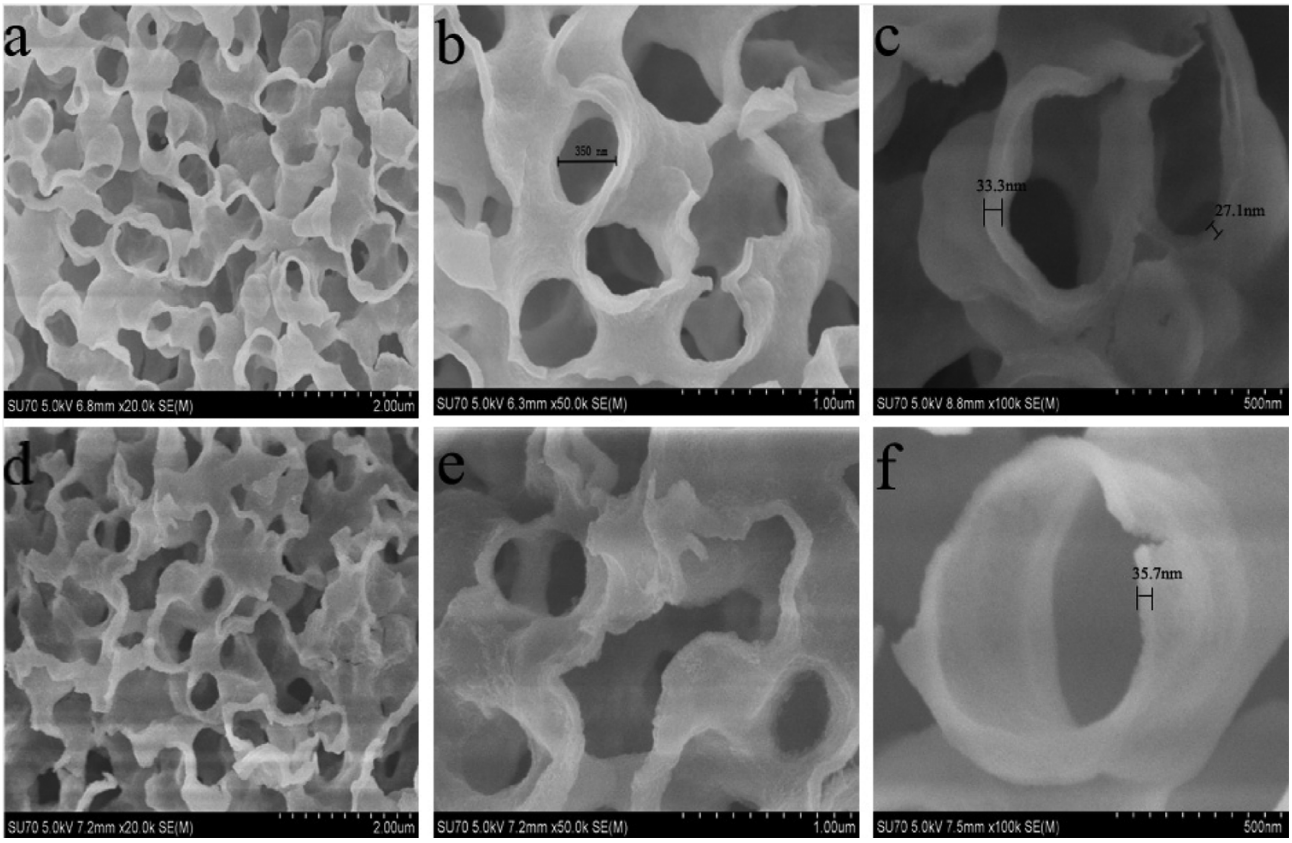

Figure 1. SEM images of 3DIMS (a, b, c) and PDA/3DIMS (d, e, f).

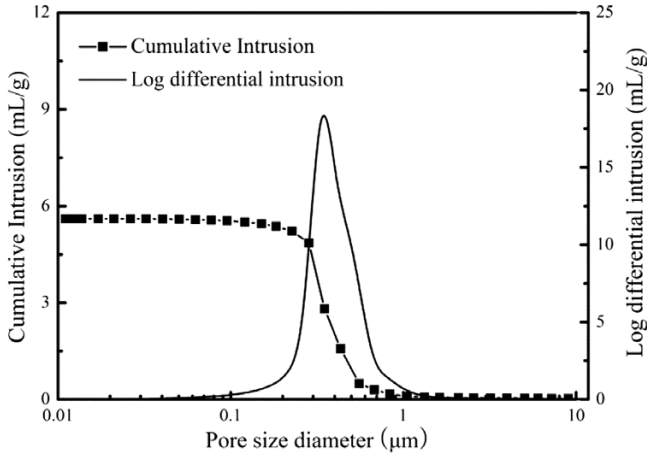

Figure 3. Macropore size distribution and cumulative intrusion pore volume of 3DIMS.

which was smaller than that of 3DIMS (45.9 $m^2 \cdot g^{-1}$). The decrease of the BET surface area was due to the partial blockage of mesopores and the increased weight of 3DIMS by the PDA modification.

3.4. TG analyses

Thermogravimetric analysis of 3DIMS and PDA/3DIMS was further determined to test the mass fraction of PDA. In Figure 4, the TG curve of 3DIMS showed a weight loss of 2% from 100 °C to 600 °C, corresponding to the elimination of water generated by the condensation of the Si-OH groups.[20] However, the weight loss of PDA/3DIMS was 8.4% within the same temperature range, which was more than that of 3DIMS. The weight loss at temperature of 270-420 °C is attributed to the removal of PDA in the PDA/3DIMS composite. Therefore the mass content of PDA in PDA/3DIMS is about 6.4%.

3.5. Optimization of the incubation time, pH and initial concentration for laccase immobilization

The incubation time of laccase loading on the support plays an important role in improving the stability of the laccase. Using PDA/3DIMS as the support, when the incubation time was 4 h, the reusability was poor (only 40.1% activity retained after 10 batch reaction) as the main interaction between the laccase and the support is physical adsorption. When the incubation time was prolonged to 8 h, the immobilized laccase still retained 73.6% of its original activity after the 10th reuse. Prolonging the incubation time more than 8 h did not improve the reusable stability obviously, and resulted in decrease of the activity of the immobilized laccase. Besides the industrial applications required good reusable stability, so the optimum immobilization time was chosen as 8 h.

The effect of pH on laccase immobilization on 3DIMS and PDA/3DIMS was determined in the pH range from 2.0 to 7.0, and the results are shown in Figure 5. The PDA layer in PDA/3DIMS contains plenty of catechol and quinone functional groups, and the quinone group can couple covalently with nucleophilic groups in laccase. Besides, comparing with free enzyme, enzyme immobilization on polyanionic surface often results in a more basic optimum pH and immobilization on polycationic surface often results in a more acidic pH.[21-23] When silica nano film with negative surface charge was modified by PDA layer, the zeta potential changed from negative to positive charge.[24,25] The laccase loaded on 3DIMS by physical interaction, while the immobilization process for laccase on PDA/3DMIS was a complex interaction of physical and chemical factors. So the optimum pH for laccase immobilization on 3DIMS and PDA/3DIMS changed from pH 6 to pH 4.

Figure 6 showed the activity of Lac-3DIMS and Lac-PDA/3DIMS under different initial concentration of laccase in the range from 15 $mg \cdot mL^{-1}$ to 80 $mg \cdot mL^{-1}$. It was clear that the enzyme activity of Lac-PDA/3DIMS increases with the increasing initial laccase concentration. When the initial concentration of enzyme increased to 70 $mg \cdot mL^{-1}$, the immobilized laccase reached the maximum activity. Continuing to increase the concentration of laccase, the activity of immobilized laccase began to decline. The activity of Lac-3DIMS reached the maximum at 20 $mg \cdot mL^{-1}$. The reason was that the number of the active site on the surface of the pore wall is limited, the activity of immobilized enzyme increased with increasing the concentration of laccase when the active site was not saturated. Once the active binding site was

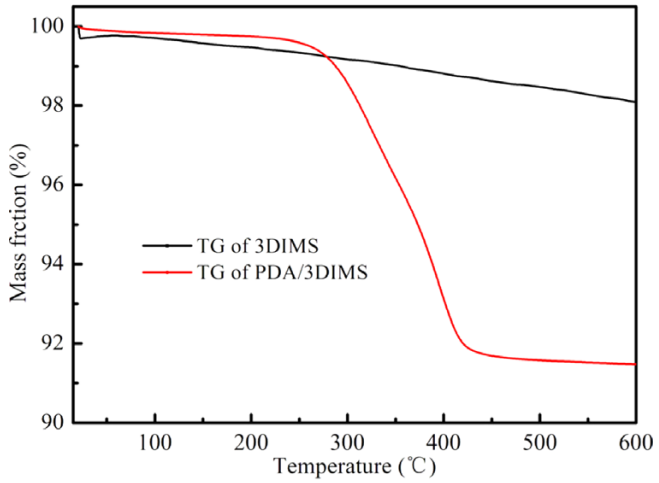

Figure 4. TG curves of 3DIMS and PDA/3DIMS.

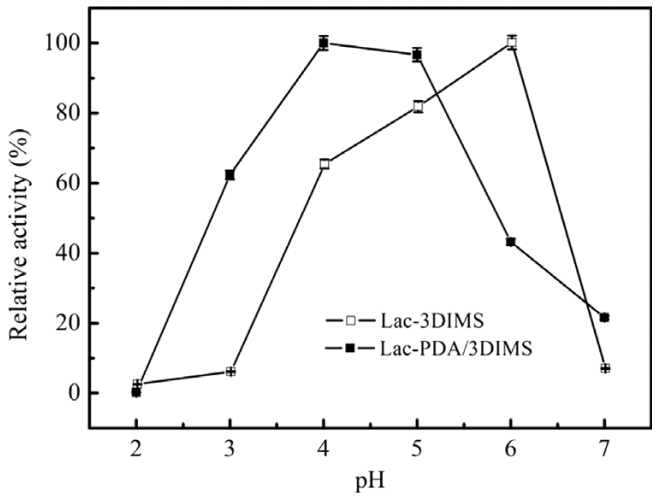

Figure 5. Effect of pH on laccase immobilization.

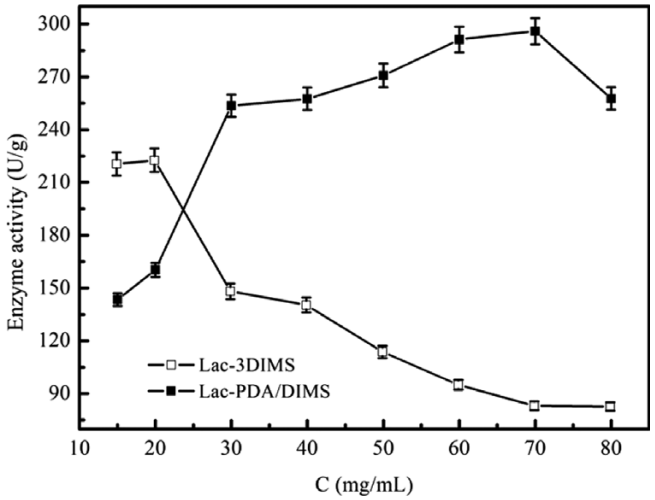

Figure 6. Effect of initial concentration on laccase immobilization.

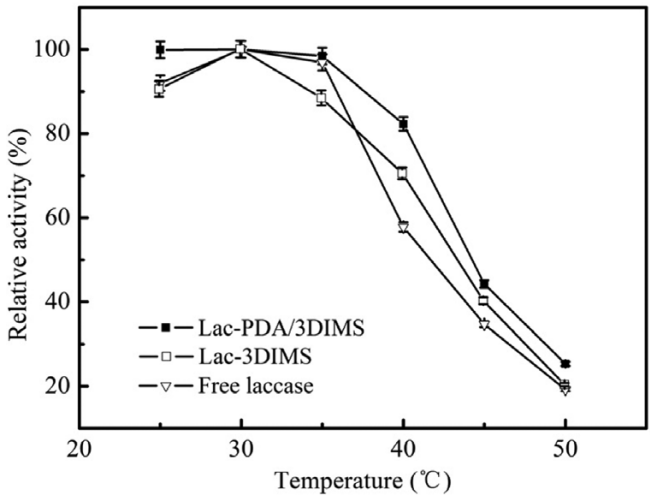

Figure 7. Thermostability of free laccase, Lac-PDA/3DIMS, and Lac-3DIMS.

saturated, continuing to increase the concentration of enzyme could cause the intermolecular space inhibition for the immobilized enzyme and subsequently restrained the dispersion of the substrate and product,[26] resulting in the decrease of activity for the immobilized enzyme. Similar phenomenon was also reported in the study of laccase immobilization on magnetic bimodal mesoporous carbon as a function of laccase concentration.[27] Therefore the activity of Lac-PDA/3DIMS (295.9 U/g) was higher than that of Lac-3DIMS (222.2 U/g) under the optimum initial concentration of laccase, and the recovery of enzyme activity for Lac-PDA/3DIMS and Lac-3DIMS is 40.8% and 107%, respectively.

3.6. Stability of free and immobilized laccase

The catalytic activity of enzyme is sensitive to pH and temperature, which limits the application of free enzyme in many fields. However, enzyme immobilization not only facilitates the enzyme separation from the reaction medium, but also improves the thermostability and extends the range of pH. So enzyme immobilization is an effective method to broaden its application fields.

Thermostability is an important parameter to evaluate the activity of the immobilized laccase. The effects of temperature on the activity of free and immobilized laccase were evaluated in the range from 25 °C to 50 °C. As demonstrated in Figure 7, the relative activity of Lac-PDA/3DIMS did not change significantly as the temperature increased from 25 °C to 35 °C. However, the relative activity of Lac-3DIMS and free laccase increased as the temperature changed from 25 °C to 30 °C, and the activity decreased remarkably when the temperature increased from 30 °C to 50 °C for free laccase, while the activity began to decline significantly at 35 °C for Lac-PDA/3DIMS and Lac-3DIMS. Similar cases had been reported on other enzymes such as Trametes versicolor laccase,[28] Candida rugosa lipase.[29] Besides, the relative activity of the free laccase dropped below 60% after incubation 2.5 h at 40 °C. In contrast, Lac-PDA/3DIMS kept about 83% of the relative activity under the same condition. That was higher than the relative activity of Lac-3DIMS (70.5%). It proved that Lac-PDA/3DIMS had a higher thermostability at higher temperature than free laccase and Lac-3DIMS. This result was attributed to multipoint attachments, which consequently lead to an increase in the activation energy for reorganization of the enzyme to an optimum conformation for binding to its substrate and preserve the structure of enzyme.[30]

The pH stabilities for free laccase, Lac-PDA/3DIMS and Lac-3DIMS were obtained as shown in Figure 8. The maximum activities for Lac-PDA/3DIMS and Lac-3DIMS were obtained at pH 6.5 and 7.0, which were higher than that of the free laccase (pH 5.5). The shift of the optimal pH could be caused by the ionic interaction between enzyme and the charged surface of support.[31] Bo Yu had reported that PDA surface was positively charged in acidic solution, which could adsorb the hydroxyl ion and reduce the hydroxyl ion concentration around the polymeric matrix surface. So there would be a lower pH value around the polymeric substrate surface than that of the bulk solution.[25] Moreover, immobilized laccase displayed higher relative activity than the free laccase at pH above 6.0. The relative activity was almost unchanged for Lac-3DIMS and Lac-PDA/3DIMS as the pH increased to 7.5, while free laccase just left 44.5%. The tolerability of the immobilized laccase to the pH improved greatly,

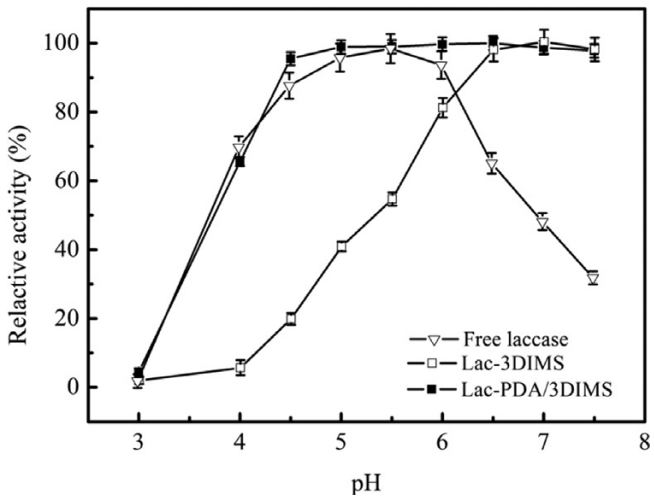

Figure 8. pH stability of free laccase, Lac-PDA/3DIMS, and Lac-3DIMS.

Lac-PDA/3DIMS was proved with an excellent adaptability in a wider pH region comparing with the free laccase and Lac-3DIMS due to the covalent bond between the laccase and PDA in PDA/3DIMS. It should be mentioned that the optimal pH for the immobilized enzyme is distinctly different from that for enzyme immobilization; the latter is related to the properties of the free enzyme rather than that of the immobilized enzyme. In the immobilization process, the free laccase is very sensitive to pH changes, as shown in Figure 5.

Unlike free enzyme, immobilized enzyme could be easily separated from the reaction solution and reused. That greatly decreased the cost of the enzyme for practical application.[32] The reusability of Lac-PDA/3DIMS and Lac-3DIMS was determined at optimum conditions. As shown in Figure 9, after five cycles, the relative activities of Lac-PDA/3DIMS and Lac-3DIMS were about 85.5% and 37.5%, respectively. Lac-PDA/3DIMS still retained 73.6% of the initial activity after 10 cycles, but the relative activity of Lac-3DIMS was just 31.8%. It can be explained that the main interaction between laccase and 3DIMS was physical adsorption compared with the strong force of covalent bond in Lac-PDA/3DIMS. The operational stability of Lac-PDA/3DIMS was better than that of the immobilized laccase on PAN-EFMs (75% activity retained after five cycles)[33] and poly microfiber (50% activity retained after 10 cycles).[34]

The storage stabilities of free laccase, Lac-PDA/3DIMS and Lac-3DIMS were illustrated in Figure 10. After storage for 15 days at 4 °C in phosphate buffer solution (0.1 M, pH 3.0 for free laccase, pH 4.0 for Lac-PDA/3DIMS, pH 6.0 for Lac-3DIMS), Lac-PDA/3DIMS retained 94.1% of its initial activity, the relative activity of Lac-3DIMS was just 62%, while free laccase lost nearly 84.5% of enzyme activity after the same period. The improved storage stability was attributed to the increasing stabilization of its active conformation by multipoint bond formation between the carrier and laccase molecule.[35-37] The results indicate that modification of 3DIMS with PDA provides a suitable microenvironment for laccase immobilization.

Kinetic parameters (K_m and V_{max}) of the free and the immobilized laccase are shown in Table 1. The Michaelis constant (K_m) reflects the binding affinity of the enzyme to the substrate.

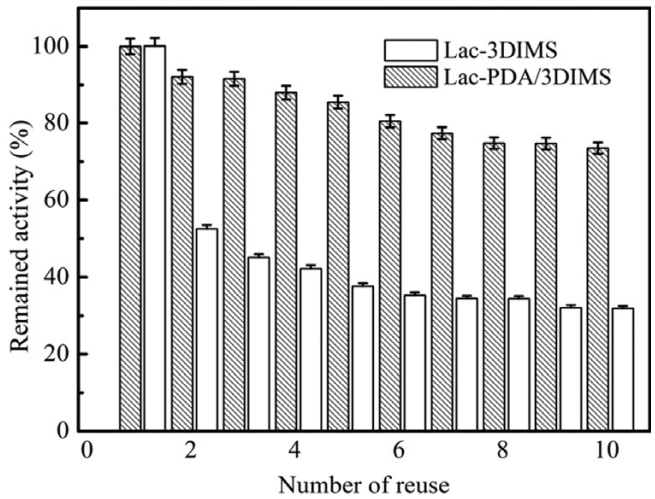

Figure 9. Reusability of Lac-PDA/3DIMS and Lac-3DIMS.

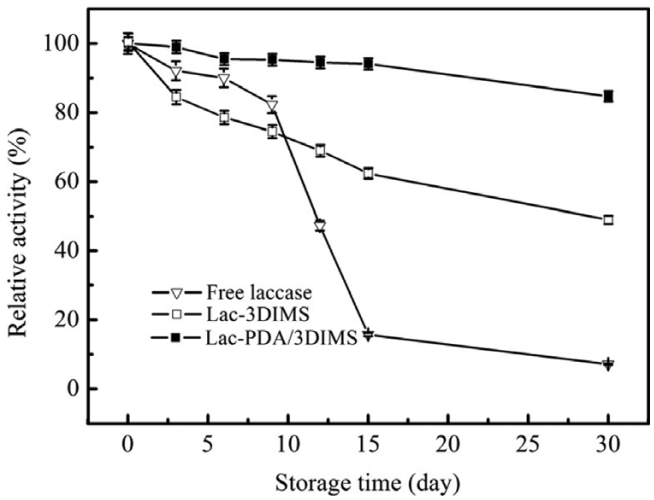

Figure 10. Storage stability at 4 °C of free laccase, Lac-PDA/3DIMS, and Lac-3DIMS.

Table 1. Kinetic parameters of free and immobilized laccase

Form of enzyme	V_{max} (mM·min^{-1})	K_m (mM)	V_{max}/K_m (min^{-1})
Free laccase	2.865	0.082	34.94
Lac-3DIMS	2.454	0.173	14.18
Lace-PDA/3DIMS	2.924	0.164	17.83

Smaller K_m value means higher affinity between enzyme and substrate. The K_m value of free laccase was 0.082 mM, which was lower than that of Lac-PDA/3DIMS (0.164 mM) and Lac-3DIMS (0.173 mM). The V_{max} of Lac-PDA/3DIMS was 2.924 mM·min^{-1}, and it was higher than that of free laccase (2.856 mM·min^{-1}) and Lac-3DIMS (2.454 mM·min^{-1}), separately. The change in the affinity of the laccase to substrate could be attributed to conformational change of laccase in the immobilization process and low accessibility of the substrate to the active site of the immobilized laccase.[15]

4. Conclusions

The results obtained in this study demonstrated that the laccase was immobilized on 3DIMS and PDA/3DIMS, respectively. Because of the strong interaction between laccase and PDA, the thermostability and the acid and alkali resistance of Lac-PDA/3DIMS had been significantly improved compared with the free laccase and Lac-3DIMS. Moreover, the operational and storage stabilities of Lac-PDA/3DIMS were greatly elevated. Besides, Lac-PDA/3DIMS was prepared by industrial laccase, which reduced the production costs greatly. And, Lac-PDA/3DIMS, as a millimeter-sized biocatalyst, can be easily recovered and recycled. With these favorable enzymatic properties, this research serves as an important step forward promoting the industrial application of immobilized enzyme biocatalyst.

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