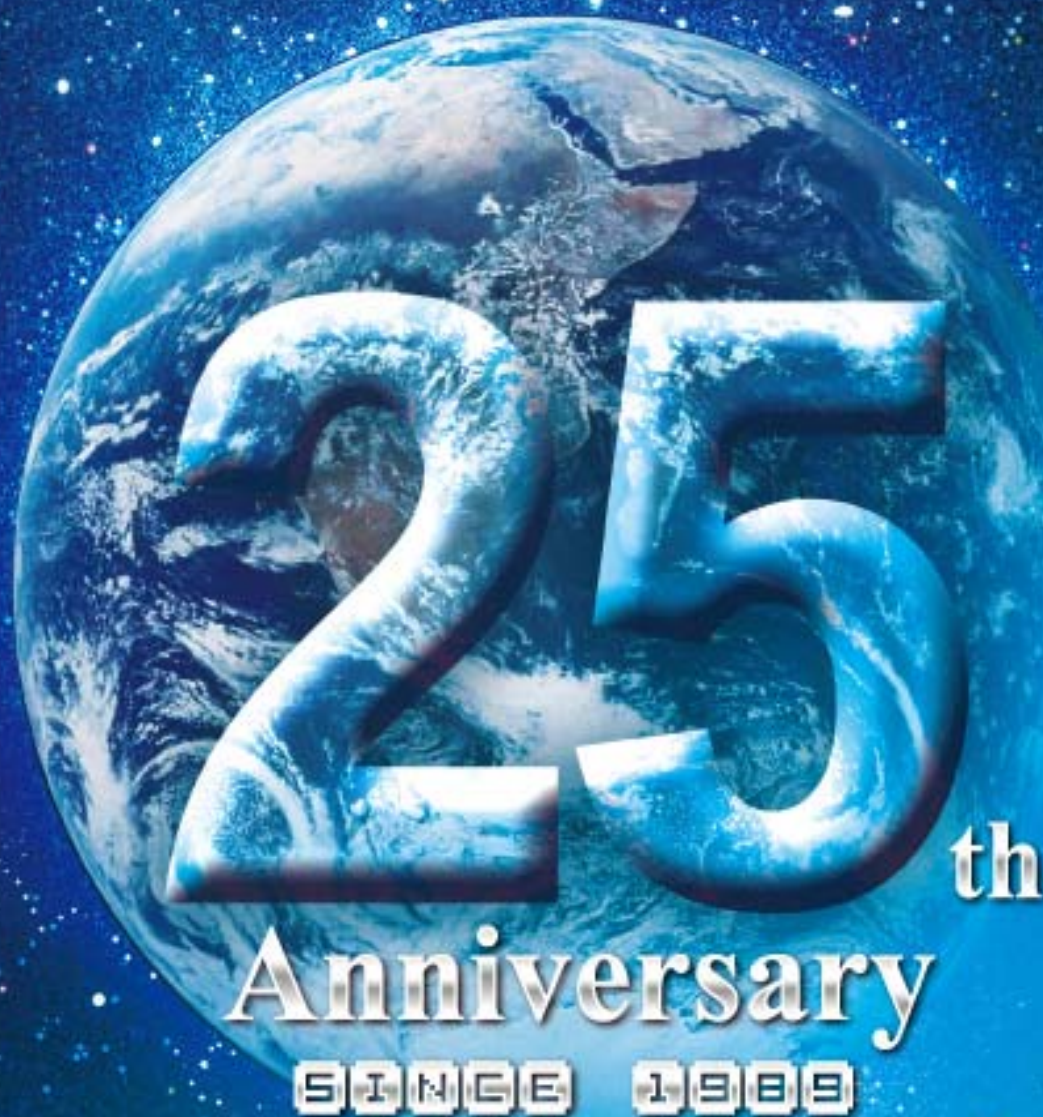


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## Mesoporous silicas synthesis and application for lignin peroxidase immobilization by covalent binding method

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### Abstract

Immobilization of enzymes on mesoporous silicas (MS) allows for good reusability. MS with two-dimensional hexagonal pores in diameter up to 14.13 nm were synthesized using Pluronic P123 as template and 1,3,5-triisopropylbenzene as a swelling agent in acetate buffer. The surface of MS was modified by the silanization reagents 3-aminopropyltriethoxysilane. Lignin peroxidase (LiP) was successfully immobilized on the modified MS through covalent binding method by four agents: glutaraldehyde, 1,4-phenylene diisothiocyanate, cyanotic chloride and water-soluble carbodiimide. Results showed that cyanotic chloride provided the best performance for LiP immobilization. The loaded protein concentration was 12.15 mg/g and the immobilized LiP activity was 812.9 U/L. Immobilized LiP had better pH stability. Acid Orange II was used to examine the reusability of immobilized LiP, showing more than 50% of the dye was decolorized at the fifth cycle.

**Key words:** mesoporous silica; lignin peroxidase; covalent binding; stability

**DOI:** 10.1016/S1001-0742(12)60008-4

### Introduction

Lignin peroxidase (LiP, EC1.11.1.14), a lignin-degrading peroxidase produced by white rot fungi, contains Fe as a group prosthetic and is a heme protein with molecular weight from 38 to 46 ku. It has a rather globular shape, with dimensions of about 5 nm × 4 nm × 4 nm (Choinowski et al., 1999) and is divided by the heme into a proximal and distal domain. LiP, performing as a family of multiple isoenzymes, has a high oxidation potential and operates H<sub>2</sub>O<sub>2</sub> mediated oxidation of various xenobiotic compounds, such as azo dyes, polycyclic aromatic hydrocarbons, and halogenated phenol (Ollikka et al., 1993; Joshi and Gold, 1996; Verma and Madamwar, 2002; Yu et al., 2006; Rubilar et al., 2008), which makes it own high application potential in pollution control. Nevertheless, employing free enzymes presents some important drawbacks, such as unstable activity under operational conditions, difficult to separate the product from the enzyme, which consequently results in inability of enzyme reuse.

Enzyme immobilization offers a solution to overcome these difficulties. A great deal of work has been carried

on LiP immobilization (Dezott et al., 1995; Podgornik and Podgornik, 2002; Li and Wen, 2009; Qiu et al., 2009). Immobilization of lignin peroxidases type I, II, III on activated silica was reported and results showed the three kinds of LiP can only attain 7%, 6% and 5% respectively of its initial activity after immobilization (Dezott et al., 1995). Two kinds of LiP isoenzymes, LiP H2 and LiP H8, were immobilized on CIM monoliths by covalent binding method. As the immobilization was conducted in alkaline conditions, activity of the immobilized LiP decreased sharply and the protein attached to the supports was 0.279 and 0.112 mg/L for LiP H2 and H8, respectively (Podgornik and Podgornik, 2002). From the previous work, it can be concluded that LiP immobilization was promising for LiP application, but it was still a challenge that the protein concentration attached and LiP activity immobilized on the support were low, moreover, activity of immobilized LiP decreased easily.

The carriers are essentially important for enzyme immobilization. Numerous materials were studied for enzyme immobilization (Lozinsky and Plieva, 1998; Lei et al., 2004). Mesoporous materials, owing to their uniform pore size, attracted much attention. Synthesis of mesoporous materials started from 1970s (Di Renzo et al., 1997). In 1992, scientists of Mobile Research and Development Cor-

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poration synthesized mesoporous molecular sieve M41S with huge specific surface area and regular channels for the first time (Kresge et al., 1992). Afterwards, studies about synthesis mechanism and conditions were carried on mesoporous molecular sieves and then mesoporous silicas (MS) as a new kind of mesoporous material were explored. Up to now, it is still a challenge to synthesize MS with even and controlled pore size as well as configuration (Liu et al., 2007).

By way of physical adsorption, some enzymes have been successfully immobilized onto mesoporous materials. The crude lipase was immobilized on SBA-15 mesoporous molecular sieve, with more than 90% of the lipase adsorbed to the SBA-15. This resulted in a more active lipase activity during hydrolysis of C-terminal protecting groups along with better stability when compared with free enzyme (Chen et al., 2002). Immobilization of globular enzymes, cytochrome c (bovine heart), papain (papaya latex) and trypsin (bovine pancreas), on the mesoporous molecular sieve MCM-41 was studied. It was found that physical adsorption on the hexagonal 4 nm pure silica phase of MCM-41 showed a clear dependence on the enzyme size and solution pH and the stability of the enzyme. The enzyme activity was conspicuously enhanced by immobilization (Díaz and Balkus, 1996). As the binding force between the enzyme molecular and the carrier is not very strong via physical adsorption, enzymes may easily leach from the carrier during repeated use (Yiu et al., 2001). One solution to reduce the degree of enzyme leaching from the mesoporous molecular sieves is to modify the mesoporous molecular sieves by using organic functional groups, such as  $-\text{NH}_2$ ,  $-\text{SH}$ ,  $-\text{CN}$ ,  $-\text{Cl}$ ,  $-\text{C}_6\text{H}_5$  to create stronger interaction between the enzyme and the carrier (Lu et al., 2007; Jung et al., 2010). Recent studies showed that modifying MS with  $-\text{NH}_2$  prior to enzyme immobilization via suitable covalent agent was an effective immobilization method (Shah et al., 2008; Shi et al., 2009).

Mesoporous materials provide great potential as carriers for the immobilization of enzyme because of the following unique chemical characters and advantages (Zhao et al., 1998; Lü et al., 2008; Hudson et al., 2008; Yang et al., 2009): (1) they have high specific surface area and porosity; (2) they have highly-ordered pore structure; (3) their pore diameters are even and can be adjusted among a wide range; (4) their macroscopic particles may have regular appearance or different morphology and can be adjusted; (5) they have high stability towards water and heat. However, up to now, no reports explored the immobilization of LiP on MS using covalent binding method.

The purpose of this article was to develop an effective method for LiP immobilization to enhance its applicability. Accordingly, in this research, MS with suitable morphologies and diameter were synthesized. Afterward, MS were functionalized and activated by 3-aminopropyl trimethoxy-silane (APTES), resulting  $-\text{NH}_2$  modified MS

(MS- $\text{NH}_2$ ). In a subsequent step, MS- $\text{NH}_2$  were activated by glutaraldehyde, 1,4-phenylene diisothiocyanate (PDC), cyanotic chloride (CC) and water-soluble carbodiimide (EDC) and then were used to covalently immobilize LiP. Protein loaded and activity of the immobilized LiP were examined in order to devise the best immobilization strategy. Properties of the immobilized LiP under the best strategy were studied. Dye-decolourization experiments were performed to demonstrate the effectiveness of the immobilized LiP.

## 1 Materials and methods

### 1.1 Materials

Pluronic P123 was purchased from Aldrich. Tetramethoxysilane (TMOS), EDC were obtained from J&K Chemical. 1,3,5-Triisopropylbenzene (TIPB), APTES and PDC were purchased from AlfaAesar. Cyanotic chloride was gained from Acros Organics and veratryl alcohol (VA) from Tokyo Kaseikogyo. Glutaraldehyde (GA) and Acid Orange II were purchased from Beijing Chemical Reagent Company. All other chemicals were of analytical grade. Deionized water was used through the experiments.

*Phanerochaete chrysosporium* BKM-F-1767 (ATCC24725) was cultured in a nitrogen-limited medium. The resulted cultures were harvested at the time when the maximum activity of LiP was detected and centrifuged at 12000 r/min for 30 min at 4°C. The resulting supernatant was concentrated by ultrafiltration in an Ultrafiltration Cell (Millipore). The concentrated supernatant was stored at 4°C and used as LiP.

### 1.2 Preparation and characterization of MS- $\text{NH}_2$

The typical synthesis procedure was as follows: 2.0 g P123 and 3.2 g ethanol were dissolved in 60 mL acetate buffer and the mixture was stirred in a water bath under 12°C until they were completely dissolved. Next, 5.0 mL TMOS and 1.0 g TIPB were added to the solution, which was stirred on a magnetic stirrer for 24 hr at the given temperature. Then the product was transferred to a Teflon-sealed container, autoclaved at 120°C for 48 hr under static condition. The synthesized MS were recovered by filtration, air-dried at room temperature overnight and calcined at 550°C for 6 hr for surfactant removal. Calcined MS (1 g) was dispersed in 20 mL toluene, with 4 mmol 3-aminopropyltriethoxysilane addition afterwards, heated with highly pure  $\text{N}_2$  reflux for 12 hr and then MS were filtered and washed by toluene and acetone excessively and dried at 80°C in vacuum for 24 hr, the product was MS- $\text{NH}_2$ .

MS and MS- $\text{NH}_2$  were characterized by several means. X-ray diffraction (XRD) was utilized to identify the crystal structure. XRD patterns were measured on an X-ray diffractometer (Rigaku D/maxrRB diffractometer, Japan),

performing the measurement at  $\lambda = 0.15418$  nm, Cu  $K\alpha$  radiation 40 kV 50 mA, each sample was scanned from  $0^\circ$  to  $5^\circ$  ( $2\theta$ ) with a scanning speed of  $1^\circ/\text{min}$ . The nitrogen sorption/desorption experiments were performed at 77 K on a Micromeritics ASAP 2010 system (Micromeritics Inc., USA). All samples were degassed for 12 hr at 573 K before measurement. The specific surface areas were measured by Barrett-Emmett-Teller (BET) and calculated using adsorption data in a relative pressure range  $P/P_0$ : 0.05–0.25. Pore size distributions were derived from the adsorption branch using the Barrett-Joyner-Halenda (BJH) method. The total pore volumes were estimated from the amounts adsorbed at a relative pressure  $P/P_0 = 0.99$ . Thermogravimetric analysis (TGA) was carried out on TA Instruments (TGA2050, USA). The sample was heated programmed from 40 to  $600^\circ\text{C}$  at  $10^\circ\text{C}/\text{min}$  in the atmosphere of 50 mL/min  $\text{N}_2$ . The infrared (IR) spectra were recorded in diffuse reflectance mode (spectral resolution  $4\text{ cm}^{-1}$ ; numbers of scans 100) using FT-IR spectroscopy.

### 1.3 Immobilization of LiP on MS-NH<sub>2</sub>

For blank control, 30 mg MS-NH<sub>2</sub> was dispersed in 2 mL LiP solution at  $4^\circ\text{C}$  and incubated for 12 hr. The suspension was centrifuged (6000 r/min, 15 min) to recover the immobilized LiP. Then the immobilized LiP was washed with tartrate buffer and freeze-dried to be the final sample.

For the enhancement by GA 30 mg MS-NH<sub>2</sub> was added into mixture of 1.5 mL tartrate buffer and 0.25 mL 25% GA, then the mixture was stirred for 4 hr, centrifuged and washed by tartrate buffer. Then the carrier was suspended in 2 mL LiP solution at  $4^\circ\text{C}$  under blending for 12 hr, the suspension was centrifuged (6000 r/min, 15 min) and the immobilized LiP was washed by tartrate buffer and freeze-dried to be the final sample.

For the enhancement by PDC mixing 0.5 mL pyridine and 4.5 mL dimethylformamide (DMF) and then 20 mg PDC was dissolved into the mixture. Then 30 mg MS-NH<sub>2</sub> was washed by methanol and acetone, and centrifuged to remove the washing solution. It was then added into 2 mL PDC containing mixture followed by blending for 4 hr. The suspension was centrifuged and washed with the buffer. Then the carrier was suspended in 2 mL LiP solution at  $4^\circ\text{C}$  under blending for 12 hr. The suspension was centrifuged (6000 r/min, 15 min) and the immobilized LiP was washed by tartrate buffer and freeze-dried to be the final sample.

For CC enhancement 30 mg MS-NH<sub>2</sub> and 30 mg CC were suspended in 2 mL toluene under blending for 4 hr. Then the mixture was washed by toluene, acetone and tartrate buffer. Then the carrier was suspended in 2 mL LiP solution at  $4^\circ\text{C}$  under blending for 12 hr. The suspension was centrifuged (6000 r/min, 15 min) and the immobilized LiP was washed by tartrate buffer and freeze-dried to be the final sample.

For EDC enhancement 30 mg MS-NH<sub>2</sub> and 2 mg

EDC were suspended in 2 mL LiP solution at  $4^\circ\text{C}$  under blending for 12 hr, the suspension was centrifuged (6000 r/min, 15 min) and the immobilized LiP was washed by tartrate buffer and freeze-dried to be the final sample.

### 1.4 Determination of activity and protein concentration of immobilized LiP

Lignin peroxidase activity was measured by the same method reported by Tien and Kirk (1988). One unit (U) of LiP activity is defined as the amount of enzyme catalyzing the formation of 1  $\mu\text{mol}$  of veratraldehyde per minute. The amount of the enzyme immobilized on the support was calculated based on the difference between the amount of protein added and reclaimed in the supernatant and washing buffer. Protein concentration was determined by the Bradford method. The crude LiP we prepared was 2838 U/L in activity and 0.19 mg/mL in protein concentration with  $\text{pH} = 4.0$ .

### 1.5 Effect of pH and temperature on the characteristics of immobilized LiP

Tartrate buffer with different pH levels (1.91, 2.6, 2.89, 3.2, 3.51, 3.98, 4.53, 5.01, and 5.5) was used in order to assay the activity of free and immobilized LiP. The highest activity of LiP was taken as 100%.

The reaction solution without LiP and  $\text{H}_2\text{O}_2$  was placed into the water bath (temperature was set to 27.6, 32.5, 37.0, 44.5, and  $54.4^\circ\text{C}$ , respectively) for 10 min to achieve an isothermal state. Subsequently, free or immobilized LiP together with  $\text{H}_2\text{O}_2$ , was added into the reaction solution rapidly and the light absorbance of each reaction solution was recorded. The highest activity of LiP was taken as 100%.

### 1.6 pH, thermal stability and reusability of immobilized LiP

pH stability was evaluated by incubating free and immobilized LiP in tartrate buffers with different pHs (1.9, 2.5, 4.5) at  $30^\circ\text{C}$ . The residual activity of free and immobilized LiP was assayed at given time intervals. The initial activity of LiP was taken as 100%.

Thermal stability was evaluated by putting free and immobilized LiP into a water bath at different temperatures ( $30^\circ\text{C}$ ,  $45^\circ\text{C}$ ). At given time intervals, 0.05 mL free or immobilized LiP containing solution was taken out respectively for activity assay at  $30^\circ\text{C}$ . The initial activity of LiP was taken as 100%.

Acid Orange II was chosen as a model dye for studying the reusability of immobilized LiP. Its concentration was 0.25 mmol/L. The percentage of decolorization ( $r_d$ , %) was calculated by the following equation to evaluate the ability of free and immobilized LiP in dye decolorization.

$$r_d = \frac{A_0 - A}{A_0} \times 100\%$$

where,  $A_0$  was the initial absorbance of the dye at 484 nm

and  $A$  was its absorbance at the given time at the same wavelength.

## 2 Results and discussion

### 2.1 Characterization of MS and MS-NH<sub>2</sub>

The XRD results are shown in **Fig. 1a**. The XRD pattern for MS showed three diffraction peaks in the low angle of 0.5°–2.0°, which can be exactly indexed to the (100), (110) and (200) reflections, standing of a hexagonal symmetry lattice ( $p6mm$ ) (Zhao et al., 1998). The XRD pattern for MS-NH<sub>2</sub> was nearly the same as that for MS, indicating that the hexagonal structure was not destroyed after modification with APTES.

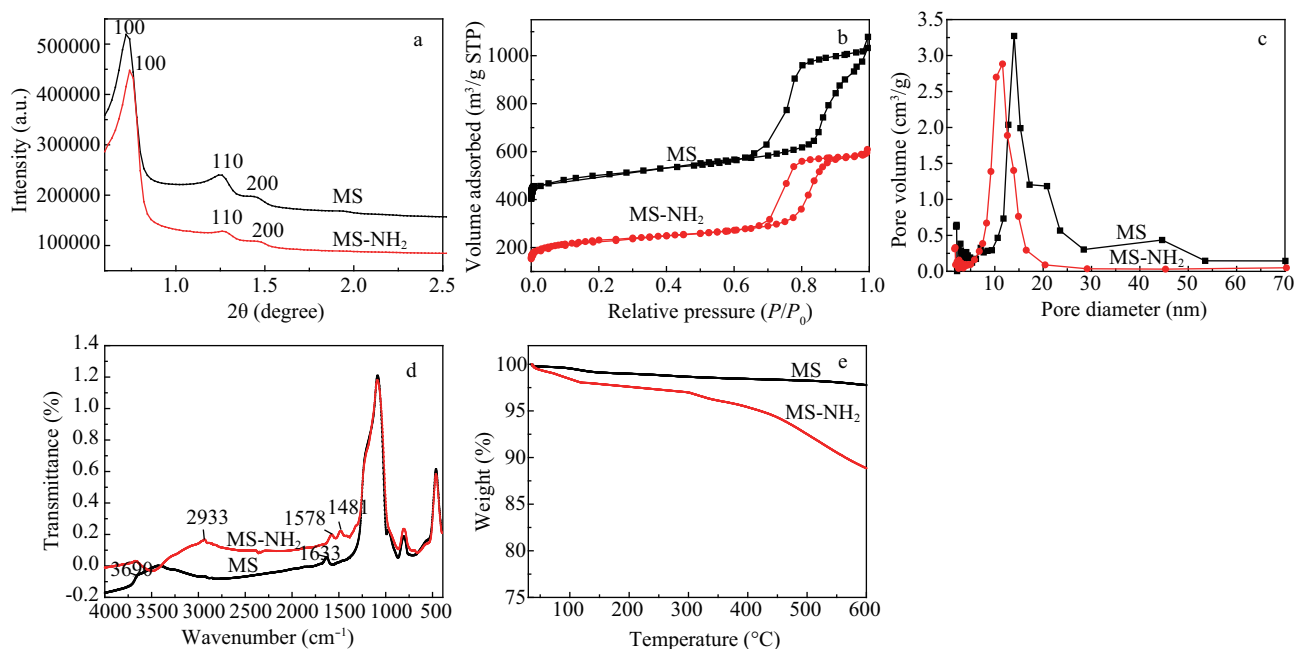
The nitrogen adsorption-desorption isotherm of MS and MS-NH<sub>2</sub> are shown in **Fig. 1b**. The results showed that the isotherms were type IV according to the International Association of Chemistry Societies classification, and had H1 hysteresis loops with the sharp adsorption and desorption branches at a relative pressure  $P/P_0$  of 0.6–0.8, which indicated that both samples had a narrow pore size distribution (Cao et al., 2009).

These results were in agreement with the pore size distribution curves shown in **Fig. 1c**. After modification, the isotherms of MS-NH<sub>2</sub> still retained the characteristic step as that of MS, but the capillary condensation step shifted slightly to a relative lower pressure, which meant the surface functionalization led to a decrease in its average pore diameter. The phenomenon was confirmed by the BJH pore size distribution curves of the functionalized MS and the parent MS. Nearly no difference existed in the shape

of pore size distribution curve. However, the pore size of MS-NH<sub>2</sub> (11.4 nm) was smaller than that of MS (14.13 nm) obviously.

FT-IR spectra of MS and MS-NH<sub>2</sub> are shown **Fig. 1d**. An IR band more than 3690 cm<sup>-1</sup> could be assigned to an –OH stretching mode arising from the Si–OH groups on the surface of MS and MS-NH<sub>2</sub> (Shah et al., 2008). It can also be assigned to the vibrational modes of the inactive Si–OH hydroxyl groups on the silica surface or may be assigned to the isolated and geminal hydroxyl groups. For MS-NH<sub>2</sub> sample, the occurrence of weak absorption bands at 2933 cm<sup>-1</sup> was assigned to the asymmetric and symmetric stretching vibrations of CH<sub>2</sub> (Shi et al., 2009). Moreover, the absorption peak at 1481 cm<sup>-1</sup>, which was attributed to the symmetric NH<sub>2</sub> bending vibration, was observed clearly. The appearance of these bands showed that the reaction was occurring between APTES and silanol groups on the MS surface.

TGA curves of MS and MS-NH<sub>2</sub> are shown in **Fig. 1e**. MS had a slight weight loss with increasing temperature, due to dehydration and/or dehydroxylation on the surface. The same phenomenon was also observed in cubic Ia3d mesoporous silicas (Lü et al., 2007). In contrast, MS-NH<sub>2</sub> had two notable periods of weight loss. The first weight loss at the temperature range of 100–300°C was due to the dehydration reaction between –NH<sub>2</sub> and the neighboring surface silanols. The second weight loss at the temperature range of 300–600°C might be attributed to the decomposition of the aminopropyl groups, which confirmed the presence of aminopropyl groups on MS-NH<sub>2</sub>.



**Fig. 1** Characterization of mesoporous silicas (MS) and MS-NH<sub>2</sub>. (a) XRD patterns for MS and MS-NH<sub>2</sub>; (b) nitrogen adsorption-desorption isotherms of MS and MS-NH<sub>2</sub>; (c) BJH pore size distribution curves of MS and MS-NH<sub>2</sub>; (d) FT-IR spectra of MS and MS-NH<sub>2</sub>; (e) TGA curves of MS and MS-NH<sub>2</sub>.

## 2.2 Immobilization of LiP on MS-NH<sub>2</sub>

**Table 1** shows the results of LiP immobilization by four covalent binding agents: GA, PDC, CC, and EDC.

The blank sample acquired the minimum protein loading rate and LiP activity, indicating that the inactive -NH<sub>2</sub> on MS-NH<sub>2</sub> was difficult to interact with the active groups in LiP. Immobilization with CC showed the highest loading rate of 12.15 mg/g and LiP activity of 812.90 U/L, indicating that the active groups of CC had good interaction with LiP and little influence on LiP structure and active sites. Relatively lower loading rate and LiP activity were gained with other covalent agents. This was especially true when the agents were PDC and EDC, in which circumstances the activity was zero. It may be PDC destroyed the three-dimensional structure of LiP, resulting in an absolute loss of activity. The inactivation caused by EDC might be caused by two reasons: (1) before combination with -NH<sub>2</sub> of MS-NH<sub>2</sub>, EDC interacted with -COOH at the LiP active center leading to LiP internal bound (Freire et al., 2001); (2) products produced by EDC and -COOH of LiP were unstable, it would be hydrolyzed rapidly unless it reacted with -NH<sub>2</sub> in LiP. Therefore immobilization using CC by covalent binding was a favorable strategy.

**Table 1** Comparison of LiP immobilization via different covalent agents

Covalent agent	Loading rate* (mg/g)	Activity of immobilized LiP (U/L)
Blank	4.89	64.52
GA	9.34	9.34
PDC	11.13	0.00
CC	12.15	812.90
EDC	6.38	0.00

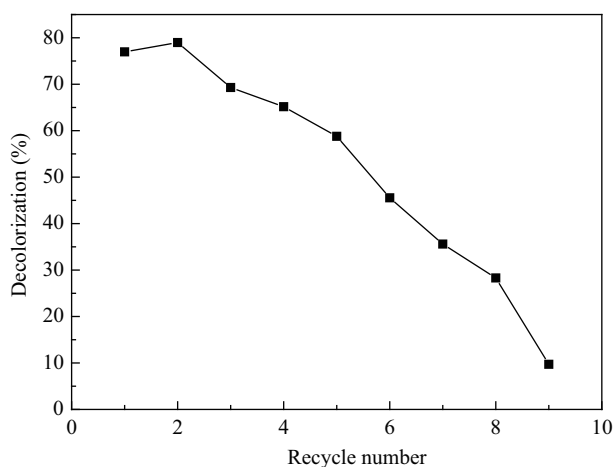
\* Protein amount loaded on per gram MS-NH<sub>2</sub>.

## 2.3 Properties of immobilized LiP

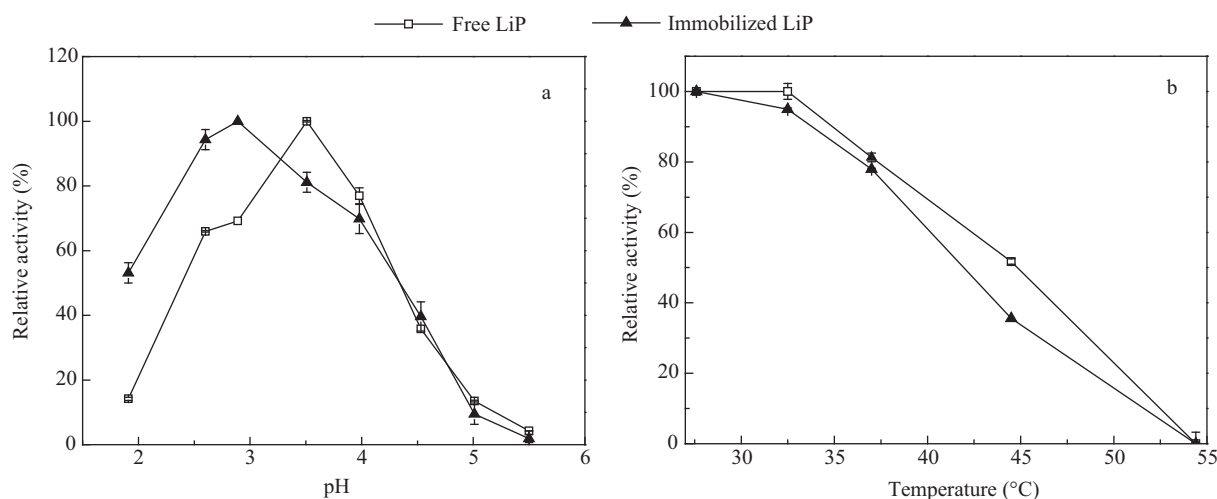
### 2.3.1 Effect of pH and temperature

Free LiP and immobilized LiP had similar pH-activity profile shown in **Fig. 2a**. Free LiP showed its highest activity at pH 3.5 and relatively high activity at pH from 2.5 to 4.0. After immobilization, the optimum pH for LiP became 2.8. Immobilized LiP kept 50% of its initial activity even in strong acidic condition (pH 1.9). It was concluded that because of being immobilized by covalent agent CC, the net charge of the LiP molecular altered, resulting in a change of LiP conformation and loss of activity (Matsuyama et al., 1993). At relatively high pH (4.0–5.0), free and immobilized LiPs had similar activity.

Temperature-activity profile of both LiPs was nearly the same between 27–55°C (**Fig. 2b**). In the temperature range of 27–35°C, both LiPs had high activity, showing that moderately high temperature was good for LiP activity. With temperature rising, activities of both LiPs descended sharply, indicating both LiPs were sensitive to higher temperature.



**Fig. 4** Immobilized LiP reusability for dye decolorization.



**Fig. 2** Effect of pH (a) and temperature (b) on the activity of free LiP and immobilized LiP.



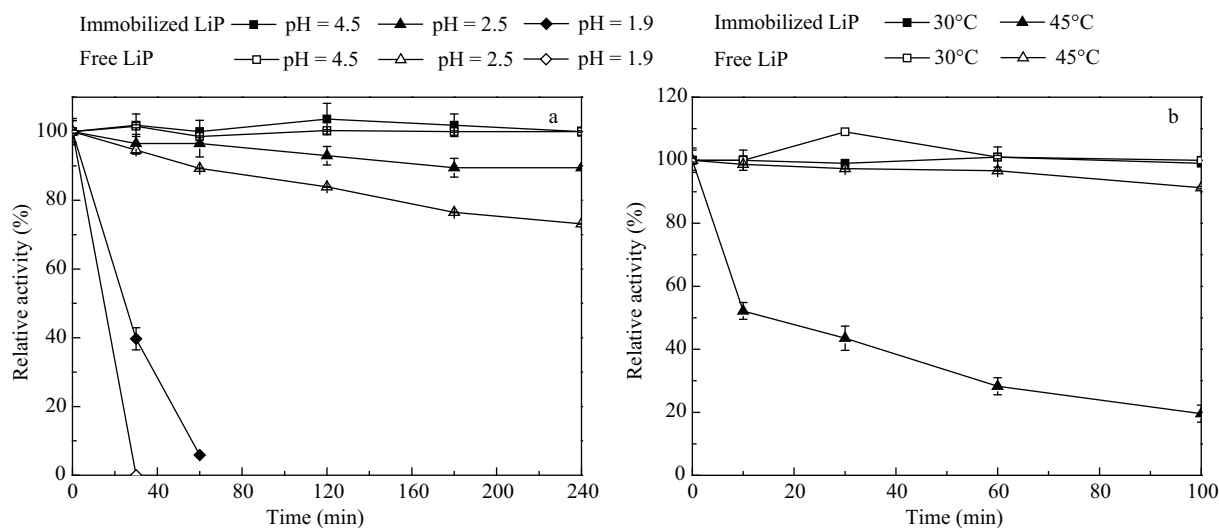


Fig. 3 pH and thermal stability of free LiP and immobilized LiP. (a) pH stability; (b) thermal stability.

### 2.3.2 pH and thermal stability

Seen from **Fig. 3a**, when pH = 4.5, both LiPs had similar time-activity profile with no obvious activity decline within 4 hr, when pH = 2.5, activity of both LiPs declined as time went on and 4 hr later, free LiP kept 74% of its initial activity while immobilized LiP kept 90% and when pH = 1.9, activity of free LiP cannot be detected while immobilized LiP kept 40% of its initial activity after 30 min immersion. To summarize, immobilized LiP had better stability in different pH environments than free LiP.

Free and immobilized LiPs were incubated at different temperatures and results are shown in **Fig. 3b**. At 30°C, free and immobilized LiPs had negligible activity loss. At 45°C, free LiP showed little activity loss during a 100-min period. While immobilized LiP was more sensitive to rising temperature, showing a sharp decline in activity after 10 min and remaining only 20% of its initial activity after 100 min. Immobilized LiP using CC showed decreased thermal stability. It may be due to the interaction between CC and the enzyme, resulting in a change of the enzyme structure, exposing more active sites outside and making it more sensitive to high temperature.

### 2.3.3 Reusability

As shown in **Fig. 4**, the results for repeated use of immobilized LiP for dye decolorization indicated that immobilized LiP had good reusability. The initial decolorization rate was 77% and above 50% by the fifth cycle.

## 3 Conclusions

The spherical mesoporous silicas with two-dimensional hexagonal pores were fabricated, modified and activated. LiP was successfully immobilized on the active MS-NH<sub>2</sub> by covalent binding method. Immobilized LiP had better pH and relatively weaker thermal stability than free LiP and had good reusability. Furthermore, immobilized LiP

exhibited good performance in dye decolorization, with more than 50% of the dye decolorized at the fifth cycle.

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