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# The performance of mesoporous organosilicas with phenyl groups in Heme proteins immobilization

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A series of mesoporous organosilicas with different content of phenyl groups for immobilization of Heme proteins have been synthesized. A higher number of phenyl groups are conductive to immobilization of Heme proteins and improvement of the activity of the immobilized enzymes. The amount of immobilized horseradish peroxidase (HRP), myoglobin (Mb) and hemoglobin (Hb) is 35mg, 51mg and 244mg with 1g of mesoporous organisilicas respectively. In particular, the immobilization efficiency of Mb can reach 100%. The sensor utilizing HRP immobilized in mesoporous organosilicas is constructed on glassy carbon electrode. In the buffer solution (pH 6.0), the modified electrode shows an electrochemical response towards catechol.

## Introduction

Enzyme catalysts can be used in many fields such as pharmaceutical,[1] agriculture and food industry,[2, 3] and bioremediation.[4, 5] Enzyme-catalysed reactions have a rapid growth with the advantage of conforming to the trend of the development of green and sustainable chemistry.[6, 7] Nonetheless, the application of enzymes in industry is still limited by their low manipulative stability (denaturation and deactivation) under extreme environment (high temperature, low or high pH values and the presence of the organic solvents) and difficulties in recycling.[8, 9] Thus, the immobilisation of enzymes onto solid supports has gained wide attention.[10, 11, 12, 13, 14] Adsorption, covalent attachment, encapsulation and cross-linked enzyme aggregates are several normal methods for immobilization of enzymes onto the supports.[15] The physical adsorption is the most cost-effective and simple method is as a consequence of the interactions between the support surface and the outer shell of the enzyme.[16] Adsorption on the support may not impact the active site of the enzyme and it also may keep the activity of the enzyme.[17] The host-guest interaction during the adsorption is based on van der Waals forces, hydrogen bonding and hydrophobic interaction so the surface state of the materials plays a key role for successful immobilization and retention of activity.[18]

Because HRP is utilized as a reagent for biotransformation and organic synthesis as well as in treatment of wastewater, immunoassays and coupled enzymes assay,[19, 20] it is a promising candidate for industrial application. Nevertheless, it will cost a lot in recycling and reusing after utilizing the HRP as the enzymic catalyst. Additionally, HRP exhibits a short catalytic lifetime because of the influence from the environment during the reaction process.[21] Hence, HRP has been immobilized into many kinds of materials, including iron oxide nanoparticles,[22, 23] phospholipid bilayers polymers,[24] sol-gel and glass beads. Barbosa *et al* reported that the immobilized HRP on magnetite-modified polyaniline can be used for 13 cycles.[25] Yu *et al* used facile vapour deposition method to form a titania sol-gel thin film to immobilize HRP on glassy carbon electrode surface for production of an amperometric hydrogen peroxide biosensor.[26] Gomez *et al* immobilized HRP on glutaradehyde-activated aminopropyl glass beads to remove phenol.[27] Wang *et al* successfully immobilized HRP on silane-modified ceramics with the method of covalent bonding and cross-linking to remove oil from wastewater.[28] Ju *et al* prepared an electrochemical biosensor for phenol based on immobilization of tyrosinase-peroxedase on mesoporous silica.[29]

Recently mesoporous organosilicas have become a novel type of hybrid mesoporous materials with the dispersity of organic moieties in the framework.[30, 31] PMOs not only have the ordered structure, high surface area, and large pore volume, but also maintain the hydrophobicity due to the organic groups in the framework.[32] The types and the content of organic groups in the framework are also adjustable. All of these characteristics are conductive to immobilization of enzymes. However, there are only fewer reports about the immobilization of HRP onto mesoporous organosilica materials. Zhu *et al* studied the mesoporous organosilicas with –OH, –O– and –S– organic groups as supports for immobilization of HRP.[33-35] They stated

that the influence of organic groups is important for enzyme immobilization.

In this paper, our aim is to find simple and convenient immobilization supports for Heme proteins immobilization. We investigate the influence of different phenyl content in mesoporous organosilicas on Heme proteins immobilization. The results elucidate that these supports have the high Heme proteins loading and keep the enzymatic activity of immobilized HRP. The viability of using the immobilized HRP modified electrode as catechol sensor is demonstrated.

## Experimental

### Chemicals and reagents

Triblock co-polymer P123, myoglobin (Mb) and 3, 3′ 5, 5-tetramethylbenzidine (TMB) were purchased from Sigma-Aldrich. Bis(triethoxysilyl)ethane and diphenyldimethoxysilane were obtained from Gelest. Horseradish peroxidase (HRP) was purchased from Roche. 1, 3, 5-trimethyl benzene, hydrogen peroxide (30%) and hemoglobin (Hb) were purchased from Sinopharm.

### Synthesis of PMO materials

The cage-like pore mesoporous organosilicas were synthesized by using 1,3,5-trimethyl benzene as a swelling agent according to literature.[36] In a typical preparation, the block copolymer P123 was dissolved in a solution containing deionized water and concentrated HCl (37 wt. %) at 38°C under stirring for 2h. After addition of TMB, the mixture was stirred at 38°C for another 2h and the organosilane was added. After further stirring for 24h at 38°C, the reaction mixture was aged at 98°C for 48h under static conditions. The mixture was allowed to cool to room temperature and the white precipitate was filtered off and dried in air for at least 2 days. The template was removed by means of extraction with ethanol. The PMO that was synthesized by using bis(triethoxysilyl)ethane (BTEE) as bridged organosilane source was named MOS0 and by using the mixture of BTEE and diphenyldimethoxysilane (DPDM) two source precursors was named MOS-n (n is the mass percentage of DPDM/(DPDM+BTEE) in the initial sol mixture, listed in Table 1).

### Characterization

The nitrogen adsorption-desorption isotherms were obtained at -196°C on a micromeritics ASAP 2420 analyzer, and the sample was degassed at 120°C for at least 10h before measurement. The BET specific surface area was calculated in a relative pressure range of $P/P_0$=0.05-0.3. The FT-IR spectra of sample were obtained on a Bruker IFS 66/vS FTIR spectrometer, and the sample was mixed with KBr. Thermo-Gravimetric analysis of the extracted sample was performed on NETZSCH STA 449C analyzer in air from 30 to 800°C with a heating rate of 10 °C/min. The morphology and structure of the materials were analyzed by transmission electron microscopy (TEM) using FEI Tecnal G2 F20 s-twin D573 operated at 200 kV. Cyclic voltammograms were obtained from the CHI660C from CHI Instruments using a standard three-electrode cell.

### Enzyme immobilization and enzyme leaching

The immobilization of enzyme was performed according to the literature[33]: the powder support (50 mg) was added into a centrifuge tube with 5 ml buffer solution (pH 6.0) of HRP with the concentration of 2 mg $mL^{-1}$, and the mixture was shaken at 4°C for 16 h. The mixture was separated by centrifugation at 10,000 rpm for 5 min and the supports containing the encapsulated HRP were washed three times with 5 ml buffer solution. The amount of HRP immobilized HRP was determined by calculating from the difference in the supematant adsorbance at 401 nm before and after the addition of the support. The immobilization of Hb and Mb is implemented in the similar condition. To ensure that the number of enzyme molecules in the solution is consistent, the initial concentration of Hb and Mb is 3.2 mg $mL^{-1}$ and 0.84 mg $mL^{-1}$, respectively.

To check the stability of immobilized enzyme, the immobilized enzyme was washed with the same buffer solution and deionized water under stirring and then vacuum-dried. The suspensions were collected and measured to calculate the leaching amount enzyme.[33]

### Assay of free and immobilized enzyme

The TMB and hydrogen peroxide were used as substrate to evaluate the activity of free HRP and immobilized HRP. The reaction began through the addition of 2 μL hydrogen peroxide (0.2 M) to the mixture of 5 ml phosphate buffer solution (pH 6.0) containing TMB and same content free HRP or immobilized HRP. After reacting at room temperature for a period, this reaction was stopped by 2 M $H_2SO_4$ and measure the adsorbance of product at 450 nm using a UV-Vis spectrometer. The adsorbance at 450nm is defined as the specific activity of free HRP or immobilized HRP. All the samples were analysed in triplicate.

### Electrode modification

The working electrodes were fabricated as follow 5 μL of a certain amount of suspension was dropped onto a glassy carbon electrode. After drying, a 0.5 wt.% Nafion solution was coated on the sample[37].

## Results and discussion

### Characterization of the mesoporous organosilicas materials

Five mesoporous organosilicas materials with different content of phenyl groups have been explored for their capacity in the immobilization of enzyme. A series of mesoporous materials were synthesised using Pluronic P123 as a template, 1, 3, 5-trimethylbenzene as a pore expander and BTEE, DPDM as source precursors.

**Fig. 1a-e** show TEM images of sample MOS0, MOS1, MOS2.5, MOS5 and MOS10. The sample MOS0, MOS1, MOS2.5 exhibit uniform mesoporous structures. The sample



 

MOS5 and MOS10 are composed of the large hollow cage-like pores with multiple walls[36] with the content of phenyl groups increasing.

**Table 1.**The synthetic conditions and textural properties of supports[a]

| Sample | MOS0 | MOS1 | MOS2.5 | MOS5 | MOS10 |
|---|---|---|---|---|---|
| Mass percentage of DPDM in the initial sol mixture | 0% | 1% | 2.5% | 5% | 10% |
| $S_{BET}/m^2\ g^{-1}$ | 868 | 844 | 781 | 734 | 663 |
| $V_P/cm^3\ g^{-1}$ | 1.24 | 1.21 | 1.06 | 0.88 | 0.72 |
| $d_P$/nm | 9.2 | 9.5 | 9.3 | 7.6; 18 | 4.7; >50 |
| Mass percentage of DPDM in the framework | 0% | 0.8% | 1.3% | 5.3% | 9.5% |
| Zeta-potential in pH 6.0 solution/mV | -6.8 | -6.3 | -6.0 | -8.3 | -10.6 |

[a] $S_{BET}$, BET surface area; $d_P$, pore diameter calculated from adsorption branch with improved KJS method; $V_P$, pore volume at $P/P_0 = 0.92$

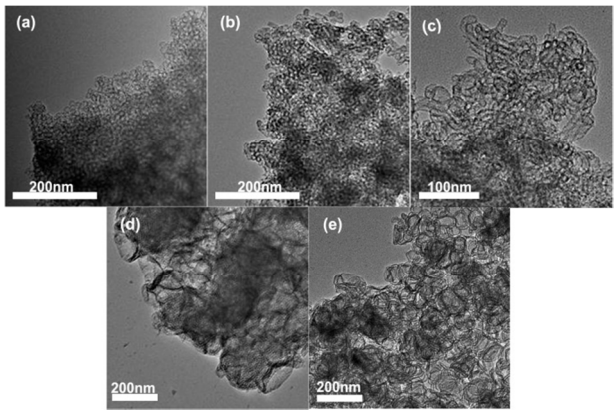


**Fig.1** TEM images of the supports: (a) MOS0, (b) MOS1, (c) MOS2.5, (d) MOS5 and (e) MOS10.

Fig. 2 shows $N_2$ adsorption-desorption isotherms of the sample MOS0, MOS1, MOS2.5, MOS5 and MOS10. The sorption isotherms of sample MOS0, MOS1, MOS2.5 have the characteristics of type IV isotherm suggesting the mesoporous system.[36] The sorption isotherms of the sample MOS5 and MOS10 have the characteristics of type IV and type II isotherm. The increasing adsorption of nitrogen at a higher relative pressure demonstrates the larger pores in these materials. A small capillary condensation step at relative pressure $0.6 < P/P_0 < 0.8$ indicates the mesoporosity.[38]

As listed in Table 1, these five materials have high BET surface area. The sample MOS5 and MOS10 emerge on two pore size distributions. The smaller one is due to the existence of multilamellar pores in the walls of the large cages and the larger one is due to the existence of cage-like pore. These results are consistent with the TEM images.

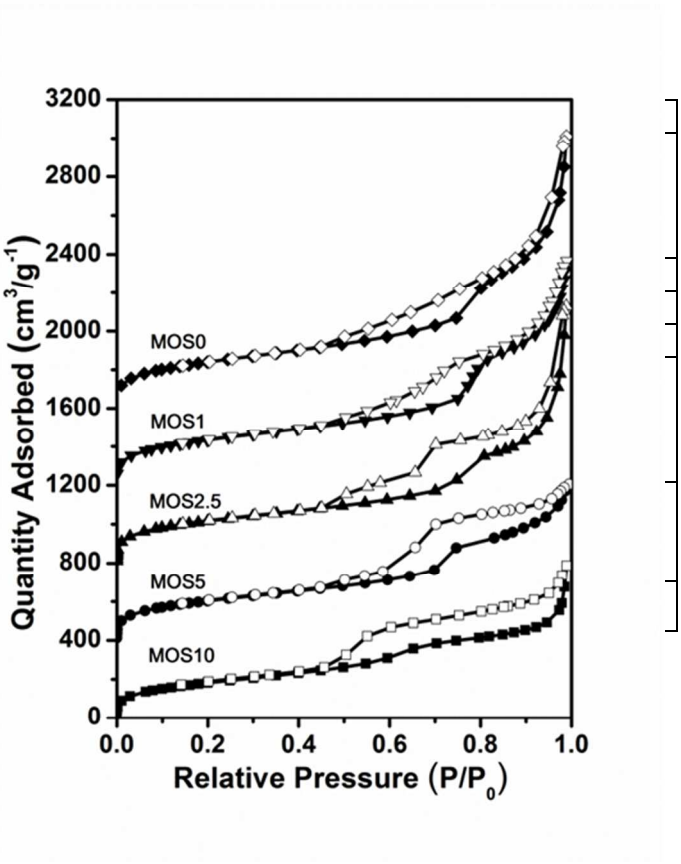


Fig. 2 $N_2$ adsorption-desorption isotherms of supports. Isotherms are offset by 400$cm^3\ g^{-1}$ along the vertical axis for clarity.

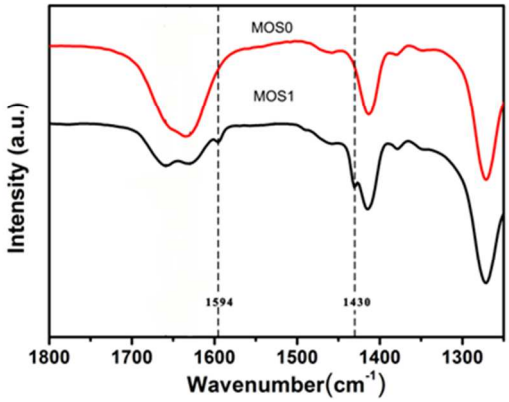


Fig. 3 FTIR spectra of supports from 1800 $cm^{-1}$ to 1250 $cm^{-1}$.

Existence of organic functional groups in these materials was proven by FT-IR spectra and TG plot. As is shown in Fig. 3, the adsorption bands at 1594 and 1430 $cm^{-1}$ are ascribed to the skeletal vibrations of the benzene ring.[39]

TG analysis of materials is conducted from 30°C to 800°C in air. From the profiles displayed in Fig. 4, the samples all exhibit high thermal stability.Between 250°C and 700°C, it indicates

the degradation of organic groups in the mesoporous wall. The different data obtained from TG analysis can be attributed to the different organic content in the samples. Based on the different weight loss, the mass percentage of DPDM/(DPDM+BTEE) in the framework can be calculated (shown in Table 1). The phenyl group content in the samples shows following fact: MOS0< MOS1< MOS2.5< MOS5< MOS10. The calculative result is roughly consistent with the starting materials ratio.

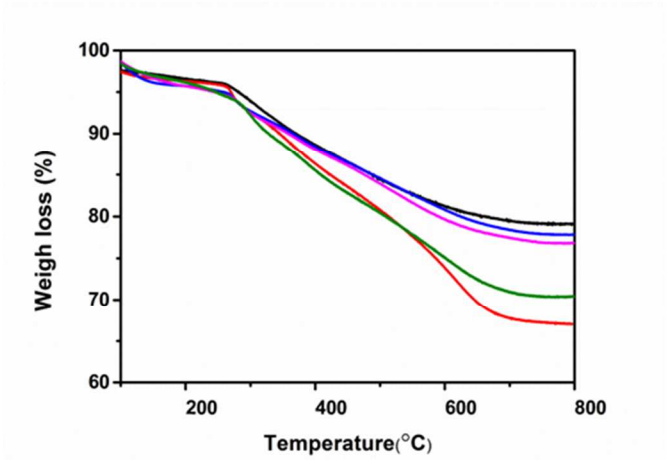


Fig. 4 TG plots of supports MOS0 (black), MOS1 (blue), MOS2.5 (magenta), MOS5 (olive) and MOS10 (red).

In conclusion, we has successfully synthesised five supports with different content of phenyl groups as designed. These mesoporous materials have been used in enzyme immobilization.

**HRP immobilization**

Our exploratory experiment shows that the initial concentration of 2 mg $ml^{-1}$ is adopted for immobilization of enzyme on the supports. Further increasing the initial concentration of enzyme cannot improve the immobilization of enzyme. The result is consistent with the literature.[35]

Fig. 5(a) shows that the support MOS5 adsorbs the largest amount of HRP (35 mg $g^{-1}$). The HRP immobilization capacity of supports shows the fact: MOS10 (16 mg $g^{-1}$) < MOS0 (17mg $g^{-1}$) < MOS1 (21 mg $g^{-1}$) < MOS2.5 (25 mg $g^{-1}$) < MOS5 (35 mg $g^{-1}$). The facts indicate that the existence of phenyl groups in the supports is significant for the immobilization.

Because zeta potentials for all supports are negative in pH 6.0 (listed in Table 1) and the isoelectric point of HRP is 7.2, the charge of supports favors the adsorption of HRP via electrostatic interaction. The hydrophobic interaction between the hydrophobic domain of HRP and phenyl group of the supports gradually increases with the increase of phenyl group content. The support with more phenyl groups tends to adsorb the more HRP molecule. The HRP immobilization capacity in our supports is higher than that in the inorganic silica support SBA-15 (12 mg $g^{-1}$, $D_{BJH}$ 9.1 nm)[34] The driving force of HRP adsorbed on SBA-15 is mainly electrostatic interaction. Hence, the increasing of hydrophobic interaction between HRP and the support is significant for the immobilization of more HRP.

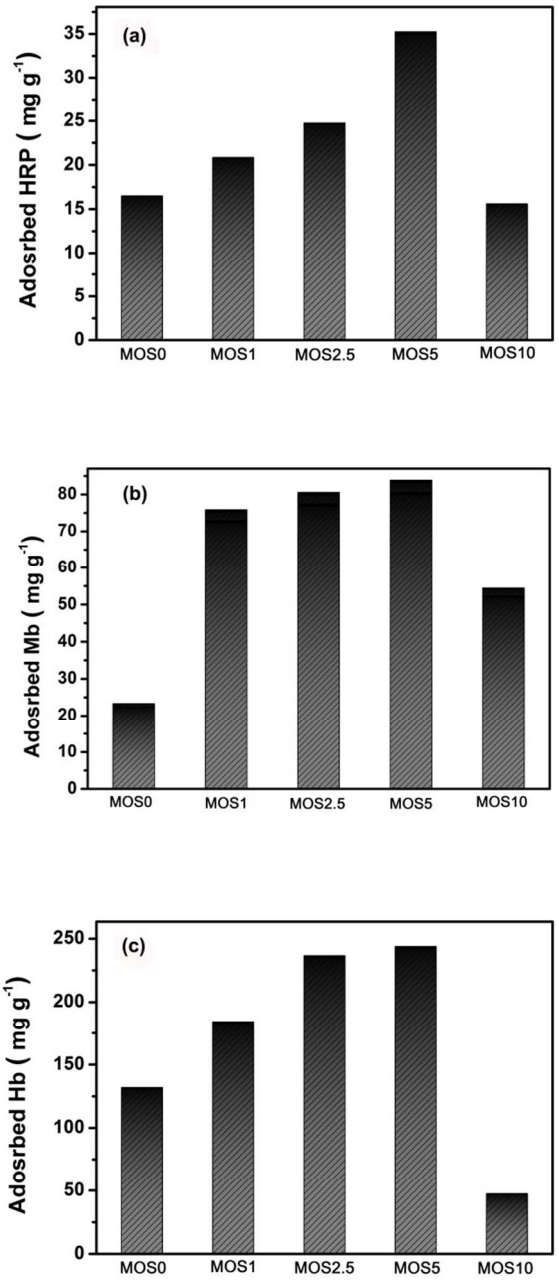


Fig .5 The adsorption capacity of HRP (a), Mb (b) and Hb (c) on different support.

As shown in Fig. 5(a), the HRP immobilization capacity of the support MOS0 is higher than that of the support MOS10, although the support MOS10 has the most phenyl groups. The existence of multilamellar pores (4.7 nm) makes the support MOS10 keep the high BET surface area. The large sized HRP molecules ($M$=40,000, 3.7 nm × 4.3 nm × 6.4nm)[35, 40] are hard to be adsorbed into the small pore. The larger pore for

immobilization of HRP has less contribution for BET surface area. The stronger interactions between support MOS10 and HRP make up for the disadvantages caused by lower useable BET surface area. Thus, the BET surface area, pore size and the organic group synergistically affect the performance of these supports to adsorb the HRP molecule.

To test the potential for our materials as general supports for Heme proteins, two other enzymes with different sizes are selected for the immobilization study. Myoglobin (Mb) ($M$=16,700)[41] has the smaller molecular size and Hemoglobin (Hb) ($M$=64,000)[42] has the larger molecular size.

Fig. 5 (b) shows the adsorbed Mb on MOS1, MOS2.5, and MOS5 is 76mg $g^{-1}$, 81 mg $g^{-1}$, and 84 mg $g^{-1}$, respectively. It is worth mentioning that the immobilization efficiencies of enzymes on these three supports are close to 100%. The influence of phenyl group content in the supports is also important for the immobilization of Mb.

The Mb immobilization capacity of MOS10 (84 mg $g^{-1}$) is higher than which of MOS0 (23 mg $g^{-1}$). This phenomenon is different from the one shown in Fig 5 (a), because the smaller sized Mb (ca. 17.6 $nm^3$)[43] molecule could be adsorbed into both multilamellar pores (4.7 nm) and the cage-like pore under the hydrophobic interaction driving.

Fig 5 (c) shows the Hb loading on these supports can reach up to 244 mg $g^{-1}$. The phenyl group is clearly beneficial to the Hb immobilization. The immobilization behaviour of Hb has the similar tendency as that of HRP. The support MOS10 adsorbs the least amount of Hb, because the largest Hb molecule (5.3 nm × 5.4 nm × 6.5 nm)[43] is more difficult to enter into the small pore. The fact further conforms to our inferences.

The stability of immobilized Heme protein is estimated by leaching test. As shown in Fig 6, the leach ratios of Heme protein are all lower than 6.8 %. For immobilized HRP and Mb, the leach ratios are lower than 6.8 % and 3.6 % and the larger pore size of MOS5 and MOS10 make more enzyme molecules leach from the support. On the other hand, Mb with smaller molecule size can be adsorbed into smaller pores in the walls of MOS5 and MOS10. Hence the leach ratios (lower than 1.5 %) of Mb from MOS5 and MOS10 are relative lower. Due to the strength of interactions between Heme protein and the support, the leach ratios of different enzymes are different.

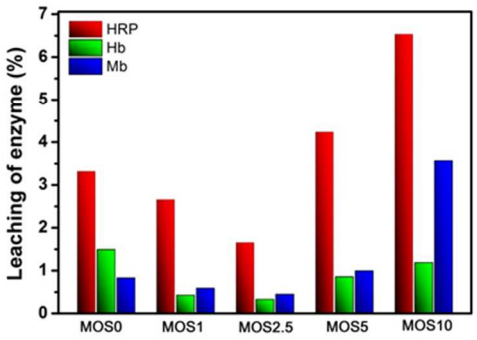


Fig 6 The leaching of Heme proteins on different supports.

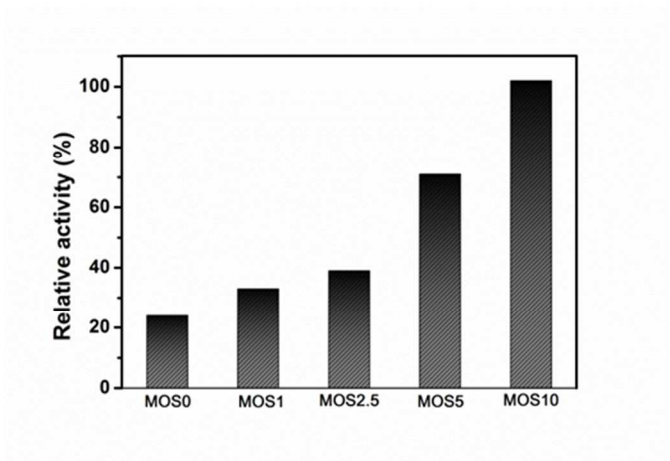


Fig. 7 The relative activity of immobilized HRP on different support. The specific activity of free HRP is set to 100%. Relative activity = (Specific activity of immobilized HRP / specific activity of free) × 100%.

**The activity of immobilized HRP**

Immobilized HRP was chosen to investigate the activity of immobilized proteins. The activities of immobilized HRP on different supports were shown in Fig. 7. HRP-MOS10 has the highest relative activity which is slightly higher than activity of native HRP under the same amount of HRP in the solution. The relative activities of immobilized HRP gradually increase with the increasing the content of phenyl groups.

Increase of hydrophobic group content affects positively the activity of immobilized HRP. The activity of HRP-MOS10 is almost five times than which of HRP-MOS0 though both of them loading the similar amount of enzyme molecules. HRP-MOS5 contains the largest amount of HRP molecules, the average activity of immobilized enzyme molecules in MOS5 is lower than that in MOS10. All the results show that the phenyl moiety in the support is important for the relative activity of the immobilized enzyme.

When HRP is immobilized onto the supports, many parameters affect tis activity.[33] Firstly, the enlarged pores of the supports reduce the resistance of substance mass-transfer to the enzyme active site.[33, 44] At the same time, the vesicular multishell structure of MOS10 is beneficial to promote the mass-transfer and then improve the activity of immobilized HRP. Secondly, the numerous organic groups in these five supports provide suitable host-guest interactions, enabling the HRP to be adsorbed sufficiently strongly avoiding the enzyme becoming rigid after immobilization.[34] The pore size distributions ($D_{KJS}$) of HRP-MOS0, HRP-MOS1 and HRP-MOS2.5 matrixes are very close. The hydrophobic interaction plays a key role for increasing the activities of HRP-MOS0, HRP-MOS1 and HRP-MOS2.5 matrixes. Thirdly, the dispersion of the HRP molecules in the pore structure is also important to impact the activity of immobilized HRP.[45] Too much enzyme molecules may cause the overlap of the activity site of enzyme molecule. Although HRP-MOS5 contains the

most HRP molecules, the activity of HRP-MOS5 is 71% of free HRP due to the overlap of HRP molecules.

In conclusion, the activity of immobilized HRP molecule is enhanced with the increasing amount of phenyl groups in the supports materials. HRP-MOS10 gives a little improved activity than the free HRP. The presence of both organic groups and vesicular multishell structure are favourable to improve the activity of immobilized HRP.

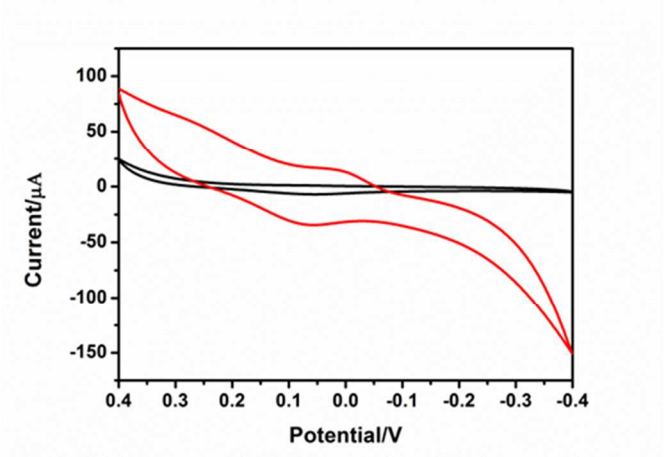


Fig. 8 CVs of HRP-MOS10/GCE (red line) and MOS10/GCE (black line) in the presence H2O2 containing 9 mM catechol at scan rate of 0.1v/s

**Amperometric response of the HRP-MOS10/GCE to catechol**

To test the use of HRP in electrochemical sensor, we easily used our HRP-MOS10 immobilization matrix to get a simple sensor. The response to catechol means the possibility of using the HRP-MOS10 immobilization matrix as the electrochemical sensor for the detection of phenolic compounds.

Fig. 8 shows the cyclic voltammogram curves of MOS10/GCE and HRP-MOS10/GCE in pH 6.0. When the HRP-MOS10/GCE electrode is in the PBS containing 9mM of catechol and 20 mM $H_2O_2$, it shows the redox peaks. Comparing with these two electrodes, the presence of HRP increases the reduced current to an order of magnitude. Meanwhile, the obvious oxidized peak can be observed for the HRP-MOS10/GCE electrodes and it cannot be observed for the MOS10/GCE. In conclusion, the HRP-MOS10 immobilization matrix modified electrode has the potential capacity for detection of phenolic compounds as an amperometric sensor.

## Conclusion

The mesoporous organosilica supports with the phenyl groups show an excellent ability to immobilise Heme proteins. The hydrophobic interaction between the hydrophobic domain of proteins and phenyl groups plays a key factor for the immobilization of proteins. The immobilized HRP on MOS10 show the higher enzymatic activity than free HRP. Meanwhile, a simple sensor based on the HRP-MOS10 immobilization matrix exhibits an electrochemical response for catechol. This type of supports is meaningful for improving the possibility of practical application of the immobilized enzymes.

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## Notes and references

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