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Hemoglobin immobilized with modified "fish-in-net" approach for the catalytic removal of aniline

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ABSTRACT

Blood is a waste product of the slaughter industry, while its main component hemoglobin (Hb) is a pseudo-peroxidase which is able to oxidize polycyclic aromatic hydrocarbons (PAHs) in the presence of H_2O_2. In order to use Hb for wastewater treatment, we encapsulated it in silica-based matrix by modified "fish-in-net" approach. The as-synthesized catalysts were characterized by SEM, TEM, BET and solid-state UV–vis spectroscopy. It was found that Hb was partially homogeneously dispersed in microspheres and showed more stable peroxidase-like activity than free Hb. Moreover, it had substantially increased storage stability as well as pH stability. It was used as biocatalyst to remove aniline in aqueous solution and gave a reduction of 65% aniline removal, while 76% in the presence of additive PEG. No significant activity loss was observed after ten runs. These experimental results suggest that the resultant product was a promising biocatalyst for aromatic wastewater treatment.

1. Introduction

Aniline is widely used as raw material in many industries including the manufacture of dyes and pigments, herbicides and pesticides, pharmaceuticals and explosives, and as a solvent in perfumes, varnish and resins [1]. It is discharged directly in industrial wastewater and indirectly through the degradation of some of the above mentioned organic compounds (herbicides [2], pesticides, dyes [3]). It subsequently accumulates in the environment and is toxic to living forms. The toxic and least biodegradable organic gathered environmental concerns, so many efforts have been made to find efficient ways to remove aniline from wastewater.

Several processes have been described, including physical processes such as adsorption [4–6], pervaporation [7] and reverse osmosis [8]; chemical processes such as oxidation [9,10], photolysis [11], liquid membranes [12,13], and nanofiltration [14]; biological processes such as biodegradation [15,16] and enzyme catalysis [17]. Among these processes, enzyme treatment shows great potential. Enzyme reactions can be carried out under milder conditions than chemical approaches, with shorter contact time than microbial degradation, and often require relatively simple equipment.

Of the enzymes used, peroxidase is often chosen. Peroxidases are heme proteins containing ferriprotoporphyrin IX as the prosthetic group. They are able to utilize hydrogen peroxide (H_2O_2) to oxidize a wide range of PAHs to their corresponding radicals, which are subsequently polymerized.

Polymers are easily removed from the solution through coagulation and precipitation followed by sedimentation or filtration [18]. Researchers used horseradish peroxidase (HRP), lignin peroxidase (LiP) and some other peroxidases for organic wastewater treatment [19]. However, due to the high cost of purification and relatively short living in the environment, a suitable substitute of native peroxidase is needed.

Hb is a member of the large class of heme proteins. It is not a real peroxidase but a heme protein with multiple promiscuous pseudocatalytic functions [20]. In mammals it makes up about 97% of the red blood cells' dry content, and around 35% of the total blood content which leads to its high purity. What's more, blood from slaughtering livestock is a waste product. In China alone about 500 million pigs are slaughtered each year, but only a small part of the slaughter blood is scientific and effectively used. Therefore based on its peroxidase-like activity, using the slaughter blood to treat wastewater can not only make use of the blood, but also help remove the aromatic compounds in wastewater. Making use of a waste product and eliminating other hazardous substrates accords with the concept of sustainable chemistry. As a result, ever since Vazauez-Duhalt observed in 1995 that human hemoglobin was able to oxidize some PAHs through a peroxidase-like pathway [21],

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researchers have been increasingly interested in Hb due to its easy availability in high purity and at low cost.

However, soluble Hb may cause secondary pollution. To avoid this, it can be immobilized by inorganic supports as biocatalysts that can be easily removed and reused. Studies have shown that mesoporous silica is very effective supports to maintain activity of biomolecules [22,23]. Laveille et al. immobilized Hb by simple adsorption in selected large pore mesoporous silica, produced satisfactory results [24]. However, leakage is an inevitable problem of adsorption which may cause contamination. Tang et al. immobilized Hb on magnetic nanoparticles by covalent attachment to degrade bisphenol A [25], but covalent immobilization may severely alter the conformation of biomolecule and lead to the decrease of enzymatic activity [26]. Compared with other methods, encapsulation is more suitable for immobilizing Hb. Lai Truong Phuoc encapsulated Hb in phospholipids-templated silica (NPS) by sol–gel process. The encapsulated Hb showed relative low activity in immobilizing aggregated enzymes because substrates can only react on the surface of the aggregation, and the Hb inside can hardly participate [27].

The challenge we face is encapsulated Hb with an immobilized approach which avoids leakage, covalent attachment and enzyme aggregation. "Fish-in-net" encapsulation is an approach which uses traditional materials but different synthesis system [28]. It has advantages such as encapsulating enzymes with a wide range of sizes, carried out at neutral pH, in non-denaturing solvent, and under mild temperature and pressure [28]. As a result, the conformation of proteins is retained, so is the enzymatic activity. Thus the inorganic support is formed suitably for the size of the proteins, avoiding leakage. It had been used to encapsulate several enzymes, and the results showed satisfactory activity, stability and feasibility [28,29]. However, enzymes are encapsulated by aggregated form in macroporous cages. Since "fish-in-net" encapsulation is an evaporation-induced self-assembly process, micelles assembled on the glycerol protective protein, aggregation was resulted by nonuniform spray.

With the aim to obtain less aggregated enzymes, we encapsulated Hb solution instead of protein powder; we also increased the initial pH value of the synthesis system, which led to smaller particles [30]. Generally, smaller particle size increases the biocatalytic performance of proteins [31]. After encapsulation, the size and morphology, activity and some other properties of the resultant material were characterized. It was used as the biocatalyst to remove aniline in aqueous solutions. We also conducted a series of experiments to determine some optimal parameters to be used. Feasibility was tested as well.

2. Materials and methods

2.1. Materials

Slaughter blood was obtained from Haoyue Slaughterhouse. TEOS, P123 and 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulphonate) (ABTS) were purchased from Sigma–Aldrich. Aniline was purchased from Aladdin. Hydrogen peroxide (H_2O_2, 3%, v/v) was obtained from Fluka. PEG (average molecular weight 35,000) was bought from Merck. All the other chemicals and solvents were of analytical grade, commercially available and used as received.

2.2. Purification of Hb

The blood was initially centrifuged at 1000 × *g* for 20 min at 4 °C to sediment the red blood cells (RBCs). The supernatant was discarded and the RBCs were washed with an equal weight of isotonic saline solution (0.9%, NaCl). The washing/centrifugation procedure was repeated 3 times. Then mixed the RBCs pellet with 3 equiv. of 3.75 mM PBS buffer (pH 7.2) for 0.5 h to extract Hb and centrifuged at 12,000 × *g* to remove cell debris. After that, Hb was purified by DEAE chromatography and ultrafiltration membrane with 10,000 MWCO and 80,000 MWCO. The final Hb was stored at −80 °C until used [32,33].

2.3. Synthesis of precursors and MSF-Hb

Precursors in this paper were prepared according to "fish-in-net" approach reported previously [28]. The synthesis of "fish-in-net" was realized by using the following reactants in the molar composition; TEOS/P123/ethanol/HCl = 1/0.015/5.3H_2O/18.1/0.2, and pH value was about 5.5–6.0. After removal of ethanol by evacuation for 72 h at 4 °C, preformed precursors were assembled in the glycerol solution of same amount.

10 ml Hb solution (normal saline) was added into the precursors simultaneously with magnetic stirring. Two hours later, the mixture was washed by normal saline with 10% ethanol and collected by centrifugal for five times to remove the templates as well as the residual free Hb until no soret band peak was shown in the supernatant. Subsequently the resulting material was dried in the air.

2.4. Characterization of MSF-Hb

SEM was performed on a JSM-6700 electron microscope (JEOL, Japan) with an acceleration voltage of 150 kV. TEM (TECNAI-G2) was used to examine the morphology inside. N_2 adsorption–desorption isotherms of MSF-Hb were measured using ASAP 2020 at 77 K after degassing at 100 °C for 12 h. The pore size distributions were calculated based on BJH model. Solid-state UV–vis spectrum was measured by UV 2550 (Shimadzu, Japan). The inorganic support ""MSF"" (heated MSF-Hb in a muffle furnace, 450 °C for 5 h) was used as the control in solid-state UV–vis and BET.

2.5. Activity assay of MSF-Hb

Activity was determined according to the method of ABTS assay [33]. The ABTS oxidation reaction is commonly used to characterize the oxidation activity of enzymes in presence of H_2O_2.The reaction mixture (total volume) contains 0.25 g MSF-Hb, including 0.12 mM ABTS, 25 mM H_2O_2, in 0.03 M phosphate buffer at pH 6.0. Oxidation of ABTS was followed at 660 nm ($\varepsilon_{660\,nm}$ = 14.7 mM^{-1} cm^{-1}).

2.6. Aniline removal with MSF-Hb

10 ml batch reactions were carried out in 25 ml glass vials at 25 °C, and the glasses were covered to prevent evaporation. Reagents were added in the following order: 10 ml phosphate buffer (pH 7.0, 0.2 M), 5 mM aniline, MSF-Hb and 7.5 mM H_2O_2. The optimal molar ratio of H_2O_2 to aniline was 1.5 [34] and mass ratio of PEG to aniline was 0.4 [35]. Reactions were initiated by the addition of H_2O_2 and proceed at 25 °C. Blank reactions have been performed with MSF instead of MSF-Hb. Upon completion of the conversion reaction, the products were removed by filtration, and the dialysis solution was measured by UV 2550 to measure the residue aniline concentration [36].

After each batch the MSF-Hb was washed by decined water to avoid the effect of products on the next batch. The reaction prolonged to 12 h to ensure the reaction was sufficiency completed. The removal efficiency each time was recorded. The residual aniline concentration was determined by UV spectra, measuring the absorbance at 251 nm (ε = 151) in 1.0 M HCl [36].

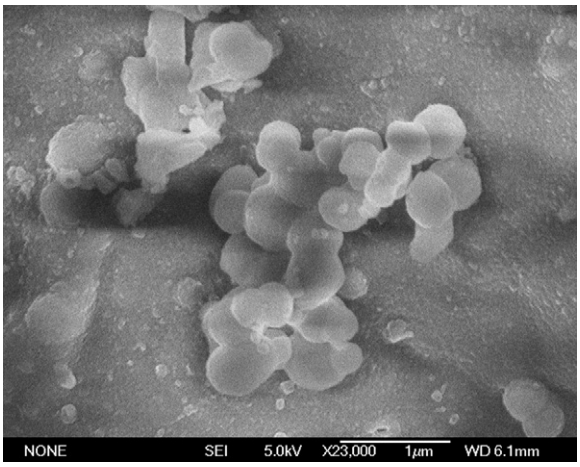


Fig. 1. SEM image of MSF-Hb.

2.7. Parameters

Experiments were carried out at a 25 °C, varying the process parameters to obtain the optimal results. With the aim to assess the optimal contact time required for the aniline removal, the solution was analyzed for the residual aniline concentration every hour; experiments were also carried out at various MSF-Hb amounts ranging from 10–60 U (0.33–2 g) to find out the optimal amount of MSF-Hb required to bring out the maximum removal of aniline. After adding PEG (final concentration of 0.1826 mg ml^{-1}) [35], the residual aniline was measured every 2 h, in order not only to observe the final difference but also change during the process.

3. Results and discussion

3.1. Characterization of MSF-Hb

We named the resultant material "MSF-Hb" for short which means "mesoporous silica, fish-in-net, Hb". After centrifugal separation, washing (10% ethanol) and drying, MSF-Hb was characterized by SEM, TEM, solid-state UV–vis spectrum and nitrogen adsorption–desorption method. The inorganic support "MSF" (heated MSF-Hb in a muffle furnace, 450 °C for 5 h) was used as the control in solid-state UV–vis and BET.

It can be seen from SEM images (Fig. 1) that MSF-Hb was formed by aggregated microspheres with the diameter of less than 500 nm, smaller than the size of traditional "fish-in-net" microspheres. In Fig. 2 (a) nitrogen adsorption–desorption isotherms of MSF-Hb and MSF are shown. Notably, the two samples exhibit typical type-IV isotherms, indicating the presence of mesostructures. Fig. 2 (b) displays the pore size distributions of MSF-Hb and MSF, centered on 3.26 nm and 3.55 nm respectively. The distributions were sharp identified mesoporous with uniform pore size. The specific surface area, accumulative pore volume and average pore size of MSF-Hb and MSF are summarized in Table 1. After heated, MSF shows an increase in both BET surface area and pore volume which indicates that some "organics" occupy the space.

As a heme protein, Hb can be simply measured by UV spectrum, for porphyrin (prosthetic group of Hb) exhibits soret absorption band. Fig. 3 shows solid-state UV–vis spectra of MSF-Hb and MSF (the inset graph shows their differential spectrum). Two peaks at 410 nm and 280 nm of MSF-Hb are observed. They are soret absorption band and the protein absorption band [37], which indicated the existence of Hb. Presumably the organic that took up space was Hb.

Table 1
Pore size, pore volume and BET surface of MSF-Hb and MSF.

Sample	BET surface area ($mg^2\ g^{-1}$)	Pore volume ($cm^3\ g^{-1}$)	Average pore diameter (nm)
Precursor	284.27	0.419	3.55
Immobilized Hb	232.13	0.332	3.26

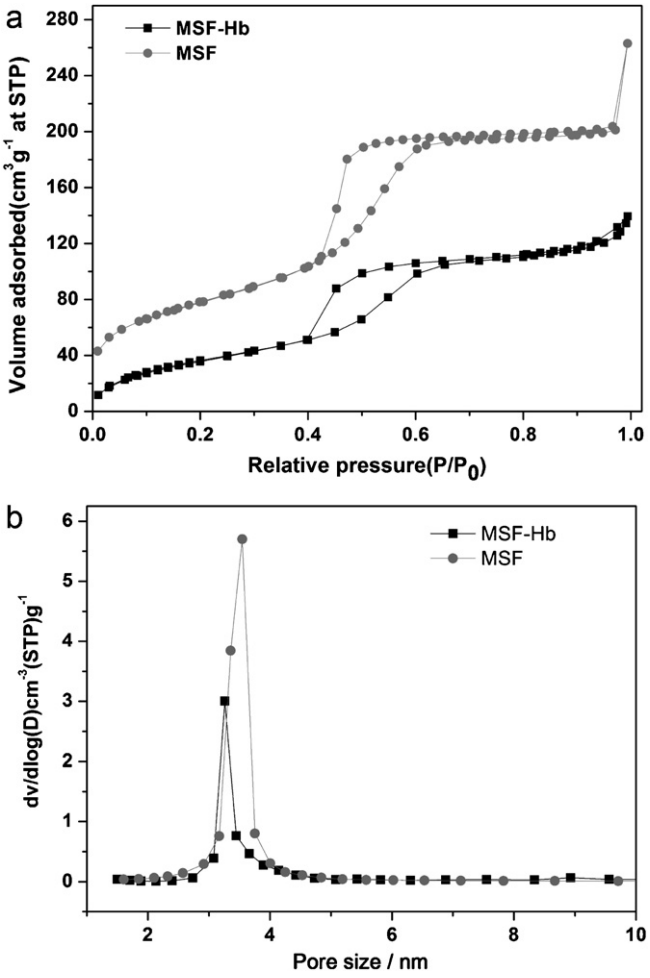


Fig. 2. Nitrogen adsorption–desorption isotherms (a) and BET pore size distribution plots (b) of MSF-Hb and MSF.

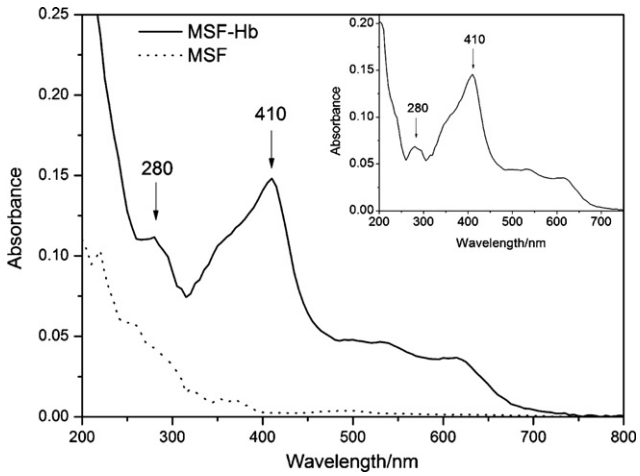
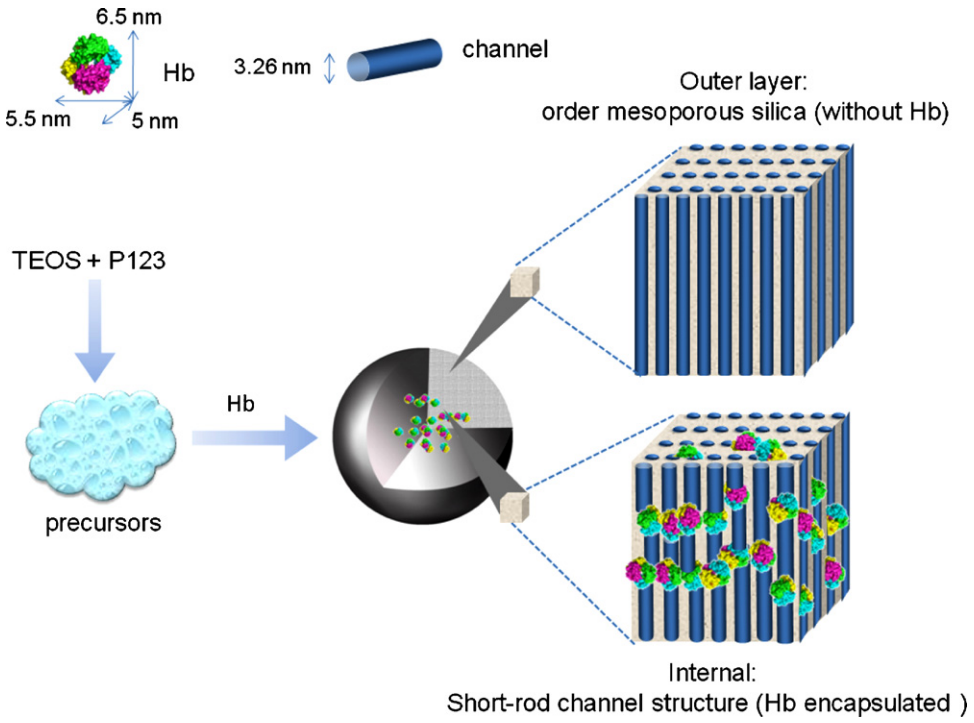


Fig. 3. Solid-state UV–vis spectra of MSF-Hb and MSF (inset graph: differential spectrum – Hb encapsulated).



Scheme 1. Schematic representation of encapsulation of Hb. (1) TEOS was mixed with P123 to synthesis precursors; (2) after adding Hb solution (pH 7.0 normal saline) and stirring, MSF-Hb was obtained; local amplification: ordered mesoporous silica was observed in the outer layer while the internal with short-rod structure.

Finally, the morphology inside MSF-Hb was checked with TEM. It indicated from Fig. 4 that the microsphere gives the uniform pore size of about 3 nm, in agreement with the result of BET. From local amplifications in the observed area, it can be found that MSF-Hb shows different structures in the outer and inner layers. In the outer layer, ordered hexagonal mesoporous silica structure is observed; on the contrary, internally, the channels seem to be ""cut"" down into short rods and twisted. This morphology, which corresponds to the data above, indicates that the ""object"" between channels would be the organic which led to the decrease in BET and exhibit soret band in UV. It is Hb. Furthermore, the size of Hb is about 1.5 times as that of the channel (Hb: 6.5 nm × 5.5 nm × 5 nm, channel: 3.26 nm), in accordance with the distance between short channels in the internal local amplification. Perhaps more interestingly, it was found that judged by the size, Hb may be partially homogeneously dispersed by single molecule. Based on these, the process of encapsulation is illustrated in Scheme 1: after stirring TEOS and P123 for 72 h, precursors were prepared, added Hb solution at this time and Hb would be assembled along with the precursors. After washing and drying, the resultant material MSF-Hb was obtained. Hb was entrapped inside the microspheres.

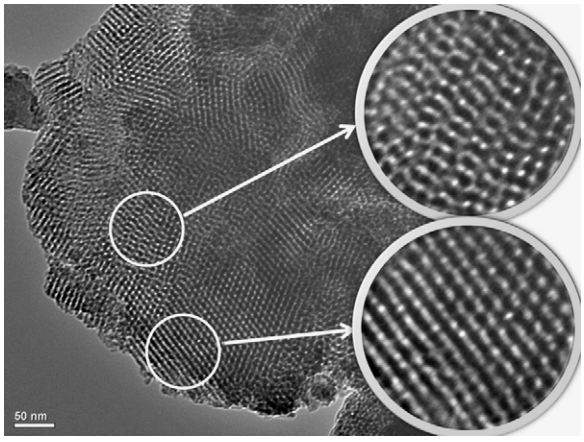


Fig. 4. TEM image of MSF-Hb with local amplifications of internal and outer layer.

Soret band is also an indirect evidence of its peroxidase-like activity because the activity depends on the state of porphyrin. In Fig. 3, the position of soret band demonstrates that Hb retained its essential conformation [37] and encapsulation did not affect its activity much. Activity of MSF-Hb was 30 U g^{-1} which was monitored using the ABTS activity assay [38], lower than free Hb. Its catalytic property is mainly influenced by active sites and diffusion of substrates. Although the porphyrin environment is not very influenced, substrates can only reach the active site and react by going through channels.

3.2. Stability of MSF-Hb

A biocatalyst should be capable of better tolerance toward environmental changes. In order to study the stability of MSF-Hb, pH dependence and storage stability were investigated. Free Hb was used as a control.

The activities of MSF-Hb and free Hb were measured at pH values from 4.0 to 10.0. In Fig. 5 it can be seen that free Hb reached a maximum relative activity at pH 6.0. After encapsulation, the optimal pH for MSF-Hb rose to about 7.0. MSF-Hb kept higher than 60% of activity at all 7 pH values, while free Hb at 5.0–8.0; MSF-Hb kept higher than 80% at 5 pH values (5.0–9.0) while free Hb had only 3 (5.0–7.0). Overall, MSF-Hb maintained higher residue activity than free Hb under neutral and near alkaline conditions. The activity of MSF-Hb is less sensitive to pH compared to that of free Hb. That may be the reason for the better tolerance toward pH changes. The pH stability of Hb is a crucial parameter from an application point of view. Wastewaters to be processed usually feature pH values in the range from 6.5 to 8.5, which makes MSF-Hb suitable for oxidation reactions in wastewater at neutral or near alkaline pH values.

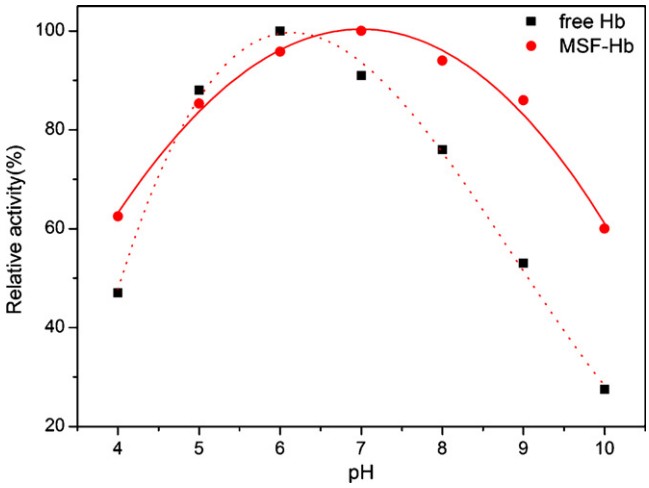


Fig. 5. Relative pseudo-peroxidase activity of MSF-Hb and free Hb versus pH.

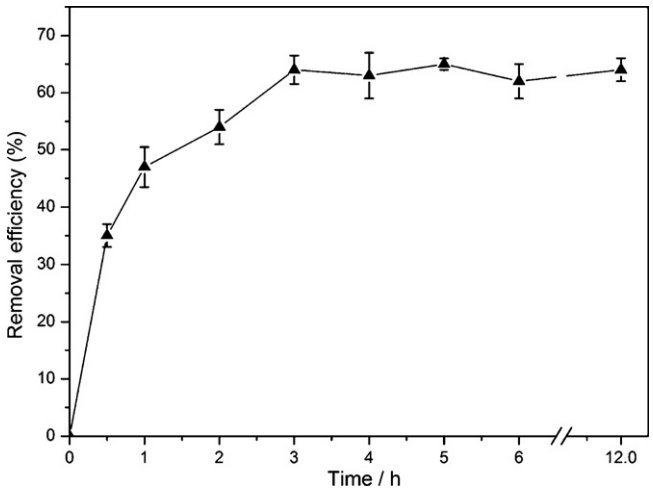


Fig. 6. Optimal contact time of MSF-Hb and substrates, [aniline] = 5 mM, [H_2O_2] = 7.5 mM, MSF-Hb 40 U, phosphate buffer (pH 7.0, 0.2 M), 25 °C.

What is more, after being stored under the same condition at 4 °C for 30 days, the activities of free Hb and MSF-Hb are both reduced. However, MSF-Hb remained at about 80% of its initial activity while the soluble Hb was almost inactive (only about 20%). The activity of MSF-Hb was preserved, even after long term storage, indicating it could be reused for many times.

It can be concluded that the inorganic supports act as a protective layer to improve the stability of MSF-Hb. Because the stability of encapsulated enzyme strongly depends on its immobilization method [26], modified "fish-in-net" approach was proved to be a suitable way to immobilized Hb. From TEM images it can be seen that Hb was ""fixed"", so the conformation of Hb molecules would not be easily changed. This may be the reason for more stable activity and long time storage.

3.3. Parameters

3.3.1. Optimal contact time

Polymerization is a non-enzyme progress. Moreover, Hb is inactivated by free radicals as well as the polymeric products which are attached to the active site [34]. Therefore separating MSF-Hb at an optimal time can not only alleviate the inactivation but also extend the useful life time of MSF-Hb.

From Fig. S1, we know that the reaction takes 8 h to reach the maximal removal efficiency, so the test time should be longer than 8 h. To investigate the optimal contact time, the concentrations of the residual aniline with different contact times with MSF-Hb but the same test time (12 h) were measured. The different removal efficiency is illustrated in Fig. 6. It shows that when the contact time increased from 0.5 h to 3 h, the removal efficiency increased as well. After 3 h, negligible aniline removal was noticed. Three hours are enough to release most radicals in this batch. After MSF-Hb was centrifuged and removed, free radicals continued polymerization.

3.3.2. Optimal amount of MSF-Hb

The enzyme dose was found to have significant influence on the removal reaction. From Fig. 7, it is obvious that 40 U was enough for the maximum aniline degradation at the experimental conditions. The MSF-Hb increasing from 10 U to 50 U resulted in a gradual decrease in residual aniline. However, subsequent increase in MSF-Hb amount up to 60 U yielded significantly low impact on aniline removal. It can be presumed that 40 U should be the optimal dose for maximum aniline removal at specified experimental conditions.

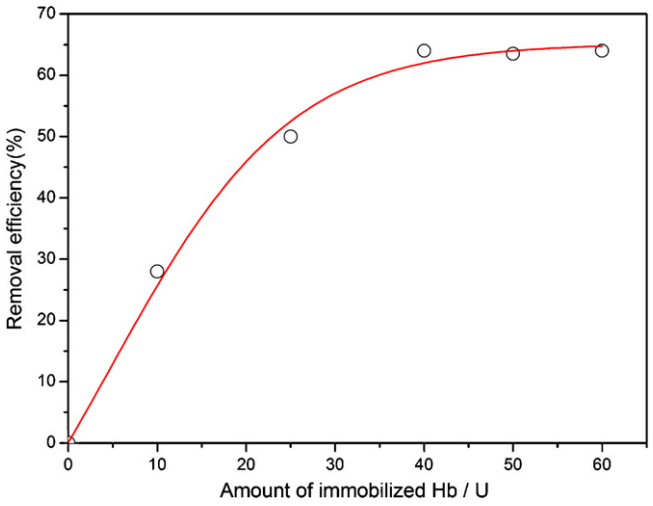


Fig. 7. Optimal amount of MSF-Hb for the removal.

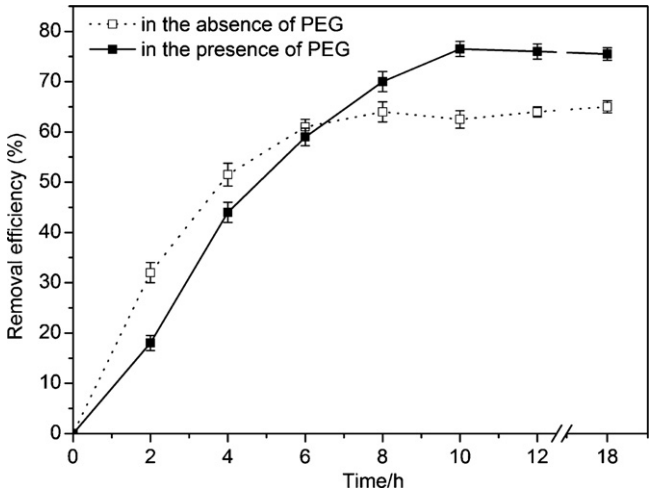
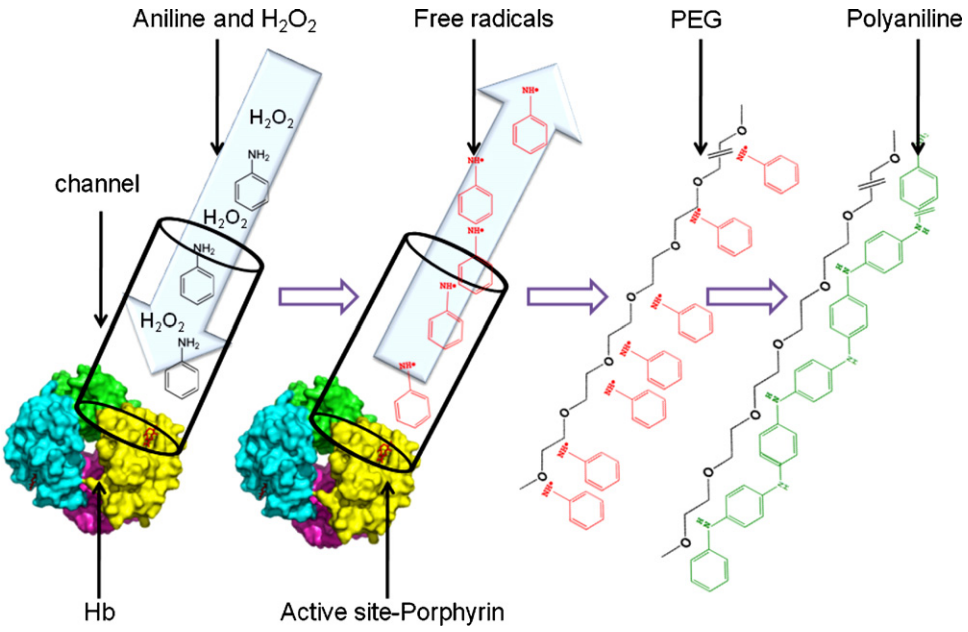


Fig. 8. Removal efficiency as a function of PEG added, [aniline] = 5 mM, [H_2O_2] = 7.5 mM, MSF-Hb 40 U, [PEG] = 0.1826 mg ml^{-1}, phosphate buffer (pH 7.0, 0.2 M), 25 °C.



Scheme 2. Schematic representation of the removal. (1) Aniline and H_2O_2 diffused into the channels and contacted the active site; (2) Hb catalyzed aniline into its free radicals in the presence of H_2O_2; (3) free radicals concentrated in the presence of PEG; (4) polyaniline was formed.

3.3.3. The role of PEG

During the removal process, enzyme was inactivated by free radicals and polymer products [40,41]. So additives as chitosan, gelatin and PEG were used to reduce the effects. Among them, PEG was most widely used and showed satisfactory results [42,43].

PEG is a polyether compound with protective effects increased with the molecular weight [39,41], non-toxic and environmentally friendly. It increased the removal efficiency for different PAHs for both free and immobilized enzymes. According to the previous literature, explanations for this kind of phenomena were that PEG acted as a protective layer for the active site, and it had better affinity with free radicals and polymer products itself, which inhibited inactivation [42,44].

During the removal, the difference between samples in the presence/absence of PEG is shown in Fig. 8. It can be seen that the sample in the presence of PEG shows a lower removal rate, but higher final removal efficiency. PEG increased the viscosity of the solution, which influenced the diffusion of the mobile phase as well as the substrates, enhanced mass-transfer resistance and resulted in relative low speed.

PEG has both hydroponic and hydrophobic groups, aniline and its free radicals are relatively hydrophobic, and they will show a higher solubility after adding PEG. Free radicals concentrate around PEG and lead to a higher local concentration, making it easier to polymerize. During the polymerization, PEG served as a template and promoted a higher molecular weight polymerization [45]. The removal process is shown in Scheme 2. After 12 h reaction, the UV spectra of the resultant mixtures (with and without PEG) are shown in Fig. 9. The spectrum of the solution with PEG showed sharper absorption peak than that without PEG, and shifted to longer wavelengths. The increased conjugated system resulted in the red shift absorption, and contributed to the higher final removal efficiency. Moreover, a higher molecular weight product is easier to separate and remove.

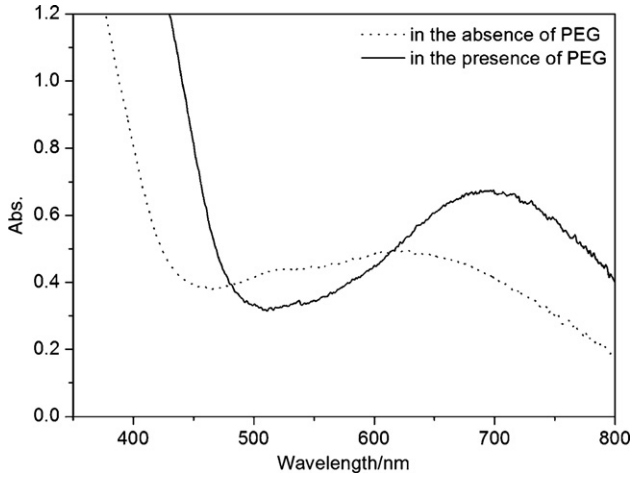


Fig. 9. Spectra of resultant mixtures in the presence/absence of PEG.

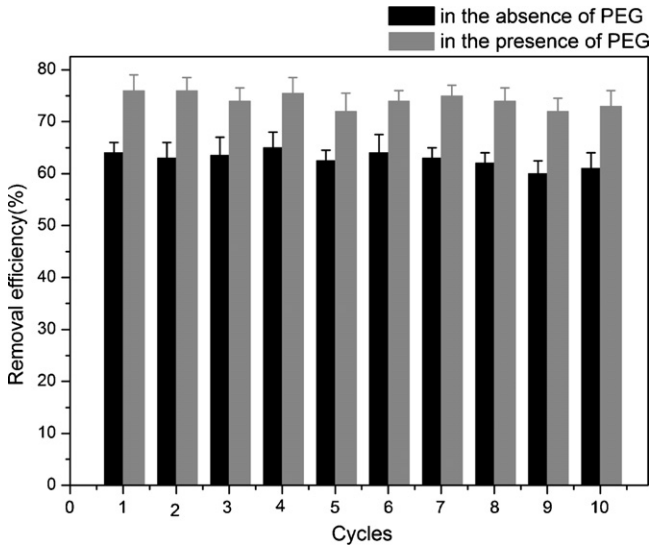


Fig. 10. Reusability in the absence and presence of PEG.

3.4. Reusability

Unlike free enzymes, immobilized enzymes can be separated from the solution and reused easily [26,46]. MSF-Hb was recovered by centrifugation after each batch and rinsed with deionized water before next batch. It can be seen in Fig. 10 that MSF-Hb kept the removal efficiency at about 65% (76% in the presence of PEG). After ten runs, no significant activity loss was observed, in good agreement with its stability data, indicating that the matrix kept the conformation well.

4. Conclusions

Modified "fish-in-net" approach was used to prepare biocatalyst MSF-Hb consisting of microspheres with narrow size distributions and partially well dispersed Hb inside. Characterizations showed structure with relatively uniform dispersed Hb inside. This kind of structure kept the conformation of Hb, improved the stability of MSF-Hb, and increased the utilization ratio.

The MSF-Hb removal experiments resulted in the reduction of aniline concentration (64%). The optimal dose is 40 U and optimal contact time is 3 h for this system. PEG was shown to be an effective additive, which increased the removal efficiency to 76%, although it prolonged the reacting time. The activity of MSF-Hb remained almost the same after 10 runs. It can be extend to degrade other PAHs. Studies on these are the focus of our current research.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jhazmat.2012.03.008.

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