Synthesis of Chitosan-Functionalized Fibrous Membrane for Immobilization of Horseradish Peroxidase: Interfacial Property and Application for Catalytic Oxidation of P-Nitrophenol

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ABSTRACT

A hydrophilic fibrous membrane consisting of a network of a non-crystalline hydrophobic polymer and an in situ crosslinked hydrophilic chitosan polymer is studied. This chitosan-functionalized membrane can be employed as a support for immobilization of horseradish peroxidases (HRPs). The immobilized HRPs (mass loading ~110 mg /g) show high stabilities over a wide pH range of 4~10 and temperature range of 25~60^{o}C. In addition, the immobilized HRPs can function as a recyclable biocatalyst for catalytic oxidation of p-nitrophenol (PNP). After 10 cycles, the PNP removal efficiency is 70 percent. The results demonstrate that the chitosan-functionalized fibrous membrane is a promising support for immobilization of enzymes and has applications related to enzyme catalysis.

INTRODUCTION

The growing concern about sustainable development promoted the establishment of the concept of "green chemistry" in the early 1990s [1,2]. Enzymes are regarded as environ-friendly and versatile biocatalysts with high activities and superior chemo-, stereo- and region- selectivities. Besides, enzyme-catalyzed reactions are generally mild with low energy consumption. However, the practical applications of enzymes are still limited by some restrictions, such as short catalytic lifespan and difficulties with reusability [3,4]. Immobilization of enzymes onto solid supports by physisorption and covalent attachment could provide attractive methods to overcome these limitations [5]. However, the attached enzyme often suffers from a decay of activity due to steric hindrance effect and interfacial limitation [6-9]. Thus, it remains still a challenge to provide support for bonding enzymes without sacrificing bioactivity. Enzymes can be bound to commercial materials such as, dextran, agarose, chitosan and functionalized macroporus acrylic polymer resins such as Amberlite FPC3500 (cationic) or FPA54 (anionic), using non-covalent immobilization techniques [10]. These supports can be functionalized with a variety of chemical groups to achieve ionic interaction. Binding is reversible, and although advantageous for re-use of the support, protein leaching is a potential problem. Besides, these supports are expensive. Recently, electrospun membranes have been employed as efficient supports for enzyme immobilization, which improves the stability of enzymes at harsh reaction conditions, such as extreme pH and high temperature. It also facilitates separation and reuse of the enzyme [11]. In addition, the surface activities of the electrospun membrane can be freely designed with the support of selected polymers [12-15]. A number of polymers have been used to link enzymes, such as polyacrylonitrile, [11] polyvinylpyrrolidone, [12] polyvinyl alcohol, [13] and polystyrene/poly (styrene-co-maleic anhydride) [14]. However, the polymers are still limited by the surface density of the covalent linking sites compared to the amount of the immobilized enzyme molecules, which results in the leakage of the enzymes upon cycling. Further, the active site of the enzyme molecule may be distorted by directly linking to the polymers. In 2005, our group demonstrated an improvement in enzyme loading and activity by introducing a biomaterial of feather polypeptide (FP), [16] while maintaining the -NH_2 group density and hydrophilicity of the FP. A promising candidate, chitosan (CTS) possesses distinct chemical and biological properties such as nontoxicity, hydrophilicity, biocompatibility, antibacterial properties, abundant NH_2 groups and remarkable affinity to proteins [17,18]. Therefore, it is worth designing a CTS-based electrospun membrane for practical application of enzymes.

In this work, it is demonstrated that the combination of CTS with poly (methyl acrylate-co-acrylic acid) (PMA-co-PAA) makes the electrospun membrane more biocompatible to immobilize enzyme without sacrificing its activities. *Figure 1* presents a process for immobilizing enzymes. The -COOH groups on the surface of PMA-co-PAA are primarily activated by the EDC/NHS, which is analogous to previous work [19]. After immobilization of the CTS, the -NH_2 groups on the CTS subsequently reacted with glutaraldehyde (GA), thus generating the -CHO groups. The HRP can be anchored onto the membrane by forming covalent bond between the CTS and HRP, and the final membrane is denoted as PMA-co-PAA@CTS-HRP. To achieve a high enzyme loading and stability, a comparative study has been conducted on the immobilization parameters such as CTS, pH or temperature induced changes in enzyme stabilities. The immobilized HRP shows high stability and good reusability in catalytic oxidation of p-nitrophenol, implying that the CTS-functionalized PMA-co-PAA membrane has potential applications in enzyme immobilization and catalysis.

EXPERIMENTAL

Materials

Bovine serum albumin (BSA) protein and Coomassie Brilliant Blue G-250 were purchased from Sigma-Aldrich. HRP was purchased from Shanghai Yeasen Biological technology Co., Ltd. All other chemicals and reagents were of analytical grade, purchased from Sinopharm Chemical Reagent Co., Ltd., and used without further purification. Deionized water was used throughout.

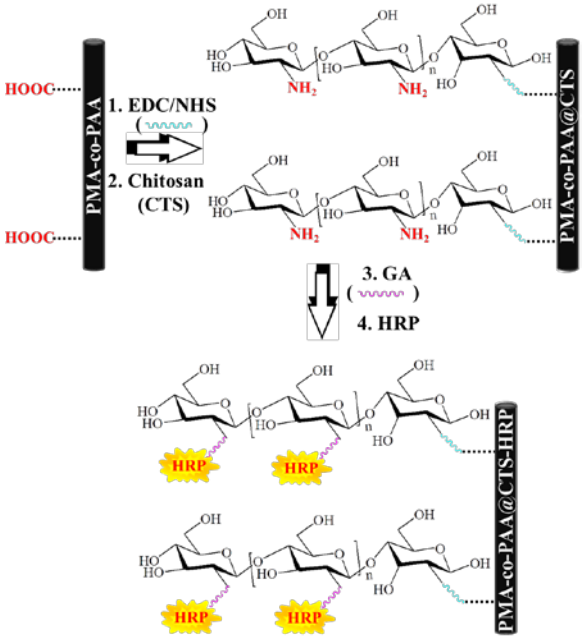

FIGURE 1. Schematic representation of step-by-step immobilization of HRP.

Synthesis of CTS-functionalized fibers

In a typical synthesis, 2.5 g PMA-co-PAA was mixed with 8 mL DMF and added to a 10 ml BD Luer-Lok™ tip plastic syringe fitted with a 0.7 mm internal diameter stainless steel needle. The electrospinning was carried out at 16 kV and the feed rate was fixed at 0.5mL/h maintained by a JZB-1800D microinfusion pump at room temperature and 65% humidity. The PMA-co-PAA membrane was collected on the electrically grounded aluminum foil and the distance between the aluminum foil and the tip of needle was set at 15 cm. The electrospun PMA-co-PAA membranes that attached to the aluminum foil were dried at 25°C under vacuum for 6 h before they were detached. Then, the membranes were placed in 4mL EDC/NHS solution (0.1M EDC, 0.2M NHS, the molar ratio of EDC to NHS =1:2) at 25°C for 1h. After reaction, the membranes were rinsed with water, and then the activated membranes immersed in the CTS solution (0.1~0.4 wt %) for 2 hours at 25°C. The CTS solutions were prepared by the dissolution of CTS in a 0.2 M acetic acid. Finally, the CTS- functionalized membranes (PMA-co-PAA @CTS) were rinsed three times with deionized water and dried under nitrogen.

Immobilization of HRP

Ten mg of the PMA-co-PAA@CTS was immersed in 4 mL GA solution (8 wt%) for 2 hours at 25°C and shaken. The resulting membrane was denoted as PMA-co-PAA@CTS-GA. It was then submerged in 3 mL HRP solution (1 mg/mL) and shaken gently at 25^{o}C for 4 hours. Finally, the PMA-co-PAA@CTS-HRP was rinsed with the PBS until protein was undetectable. The obtained PMA-co-PAA @CTS-HRP was stored at 4^{o}C prior to use. The immobilization efficiency was expressed as the amount of the HRP bound on the fibrous membrane per unit mass. The protein concentration was determined by Bradford's method by using a UV-752 spectrophotometer (Spectru, China). Bovine serum albumin (BSA) was employed as a standard to construct the calibration curve. Bradford protein assays indicated that the HRP loading on PMA-co-PAA@CTS membrane reached levels of about 110 mg of protein per g.

RESULTS AND DISSCUSSION

SEM and FRIR measurements

The microstructures of the membrane are confirmed by scanning electron microscopy (SEM). According to *Figure 2a*, the pristine PMA-co-PAA is relatively slender and homogeneous. As shown in *Figure 2b*, the fibrous structure is well retained. After loading with HRP, the PMA-co-PAA@CTS is covered by film-like coatings (*Figure 2c*). As a result, it may confirm the step-by-step immobilizing of the HRP on membrane. As presented in the insets in *Figure 2 (a, b, c)*, the pore size is gradually decreasing in the enzyme immobilization process. The pore diameters less than 1.5 μm of pristine PMA-co-PAA, PMA-co-PAA@CTS, and PMA-co-PAA @ CTS-HRP accounted for 60%, 72%, and 78%, respectively.

The BET surface area is obtained from the nitrogen adsorption–desorption isotherms shown in *Figure 3*. BET surface area of pristine fiber is 45m^2/g; after modification with CTS, surface area decreases to 29.5 m^2/g, because the diameter of the fibers became larger. Finally, after immobilization of the enzyme, the surface area decreased to 7.3 m^2/g. This suggests lipase is anchored on the fiber. The measured surface area of this sample is therefore consistent with the enzyme immobilization process.

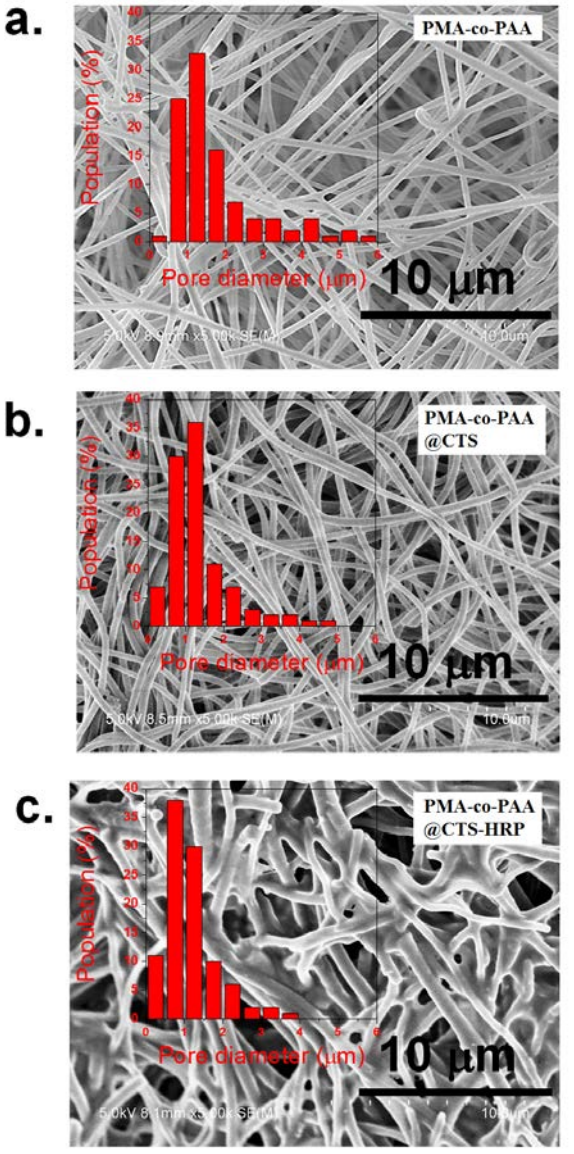

FIGURE 2. SEM images of the pristine PMA-co-PAA (a), PMA-co-PAA@CTS (b), PMA-co-PAA@CTS-HRP (c), respectively. The insets in (a, b, c) show corresponding pore size distributions.

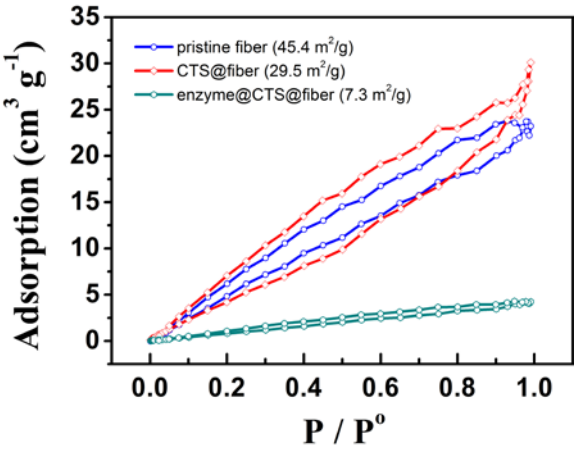

FIGURE 3. Nitrogen adsorption-desorption isotherm of the as-prepared pristine fiber, CTS@fiber and enzyme@CTS@fiber structure and morphology of the fibers, as deduced from our SEM studies.

The immobilization process of HRP was further monitored by Fourier transform infrared (FTIR). *Figure 4a* shows the FTIR of the pristine PMA-co-PAA. The $\upsilon_{C=O}$ in acid with a single band at 1708 cm^{-1} and ester with a single band at 1730 cm^{-1} are observed, respectively. After linking with CTS (*Figure 4b*), the intensity of acid at 1703 cm^{-1} decreased due to reaction with CTS and two broad absorption bands at 1638 cm^{-1} and 1564 cm^{-1} indicate the formation of acrylamide ($CONH_2$). After activating by GA (*Figure 4c*), two broad absorption bands at 1651 and 1722 cm^{-1} can be assigned to $\upsilon_{C=N}$ and υ_{CHO}, respectively. The bands at 1703 and 1733 cm^{-1} are ascribed to unreacted acid and ester. After immobilization of HRP (*Figure 4d*), the main difference lies in the great decrease of the υ_{CHO} band at 1721 cm^{-1} and increase of the $\upsilon_{C=N}$ band at 1651 cm^{-1}, indicating successful immobilization of the HRP.

Contact Angle Measurements

The contact angle measurement plays a pivotal role in the characterization of surface hydrophilicity [20]. It is well-known that for enzyme activation at least a monolayer of water per enzyme molecule should be present [21-25]. To evaluate the hydrophilic properties of the membrane, contact angle measurements were performed. As shown in *Figure 5*, the water contact angle on pristine PMA-co-PAA is 100°, which indicates the membrane is hydrophobic. After linking with CTS, the contact angle decreased to 45°, which demonstrates that the chitosan-functionalized membrane is hydrophilic. As for the advantage of hydrophilic CTS, it can prevent the stripping of the essential water from the enzyme microenvironment, thereby prolonging the lifespan of the enzyme.

Assay of Immobilized HRP

The immobilization ability of the membrane was examined by investigating the relative enzymatic activity of HRP before and after immobilization. The catalytic activities of the HRP and PMA-co-PAA@CTS-HRP were evaluated in aqueous media according to a previous report [26}. *Figure 6a* shows the relationship between the CTS concentration and the enzyme loading ability. With an increase of the CTS concentration to 0.2 %, it reaches the highest loading of 110 mg/g. *Figure 6b* shows significant improvement in the activity of immobilized HRP with increasing CTS concentration from 0.1 to 0.2 wt %. However, a further increase in the CTS concentration to 0.4 wt% decreases enzymatic activity and loading ability because the densely connected CTS molecules impede enzyme penetration.

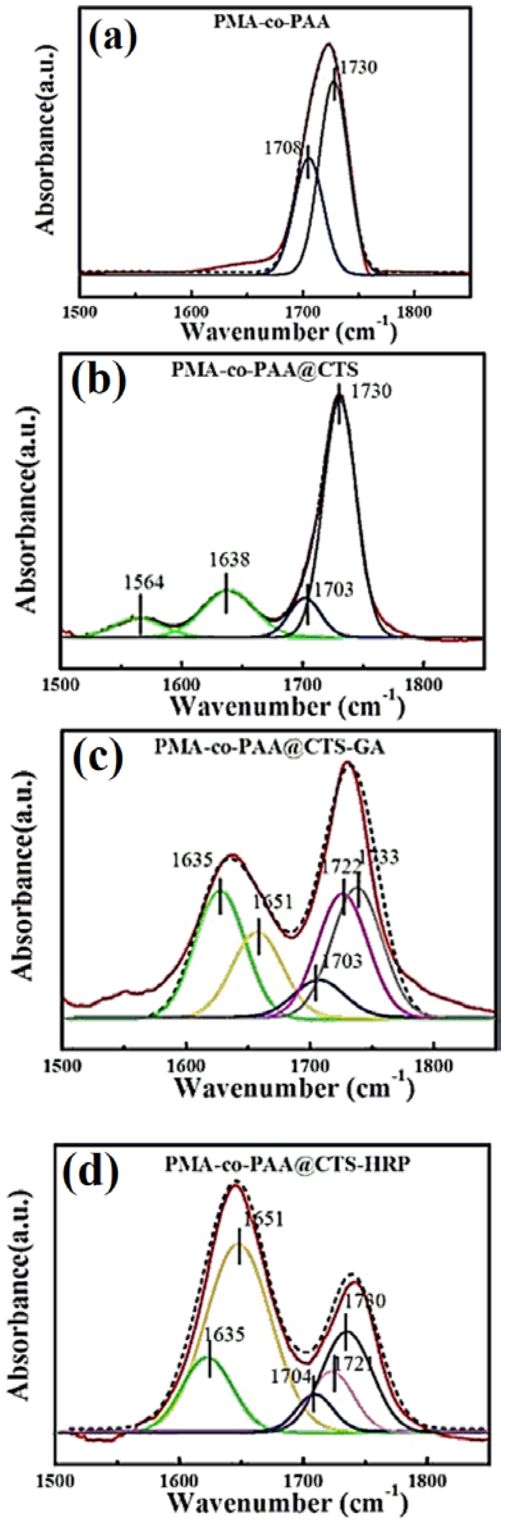

FIGURE 4. FTIR spectra of the pristine PMA-co-PAA (a), PMA-co-PAA@CTS (b), PMA-co-PAA@CTS-GA(c), PMA-co-PAA @ CTS-HRP (d), respectively.

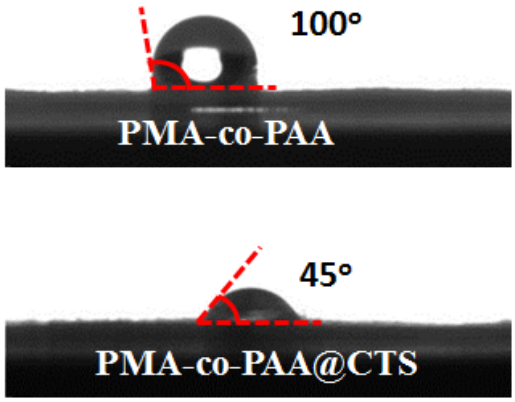

FIGURE 5. Water contact angle on PMA-co-PAA and PMA-co-PAA@CTS.

Figure 7a shows that the optimum temperature for free and immobilized HRP is 30°C in each case. Once the temperature rises to 60°C, the free HRP loses 70% of its initial activity after 5 h of incubation, while the immobilized HRP lost 40% of its activity after this period of time. The enhanced thermal stability of the immobilized HRP can be attributed to the formation of covalent bonds between the HRP and CTS. In addition, the pH stability of HRP can also be optimized through immobilization. *Figure 7b* shows the immobilized and free HRP both reach the activity at a pH of 6, while the immobilized HRP exhibits a higher residual activity than that of the free HRP at other pH values. After 35 days of storage (30°C, pH=6), free HRP maintains only 20% of its initial activity, while the immobilized HRP maintained as much as 80% of its initial activity (*Figure 7c).*

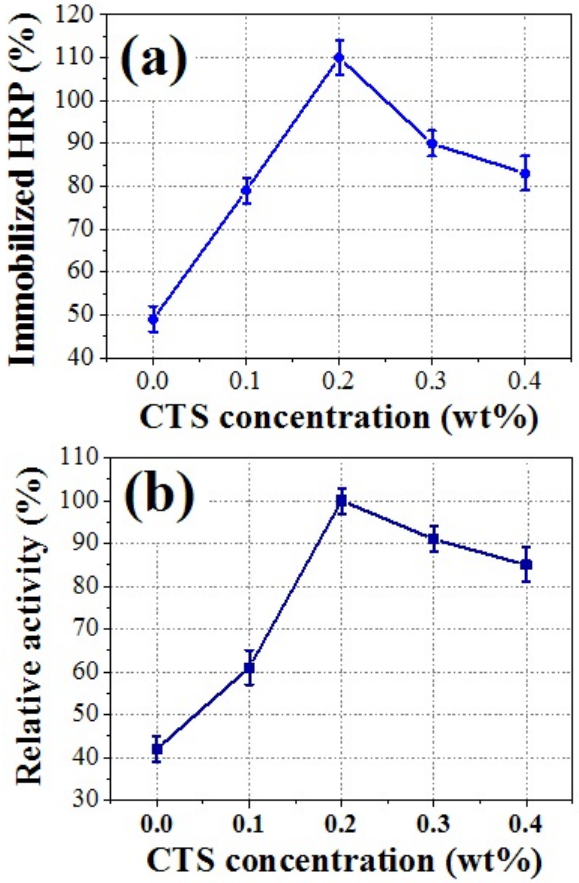

FIGURE 6. The effect of the CTS concentration on HRP loading (a) and activity (b), respectively.

This is further indication that the CTS provides a biocompatible microenvironment favoured by HRP, and thus increases the stability of HRP.

Catalytic Oxidation of P-Nitrophenol

Increasingly stringent health-based water quality standards require better removal of organic pollutants from sewage and industrial effluents. p-Nitrophenol (PNP) has been listed as a priority pollutant by the U.S. EPA due to its toxicity to organisms. Many efforts have been devoted to removal of the PNP, whereas the addition of chemicals brings extra pollution [27-31]. Besides, the cost of techniques such as HRP encapsulated into poly (d,l-lactide-co-glycolide) (PLGA) / PEO–PPO–PEO (F108) electrospun fibrous membranes (EFMs) by emulsion electrospinning [26], ultrasonic degradation of PNP in the presence of additives [27], heterogeneous fenton-like reactions on nano-magnetite (Fe_3O_4) were used for the degradation of PNP) [28] are high in all cases.

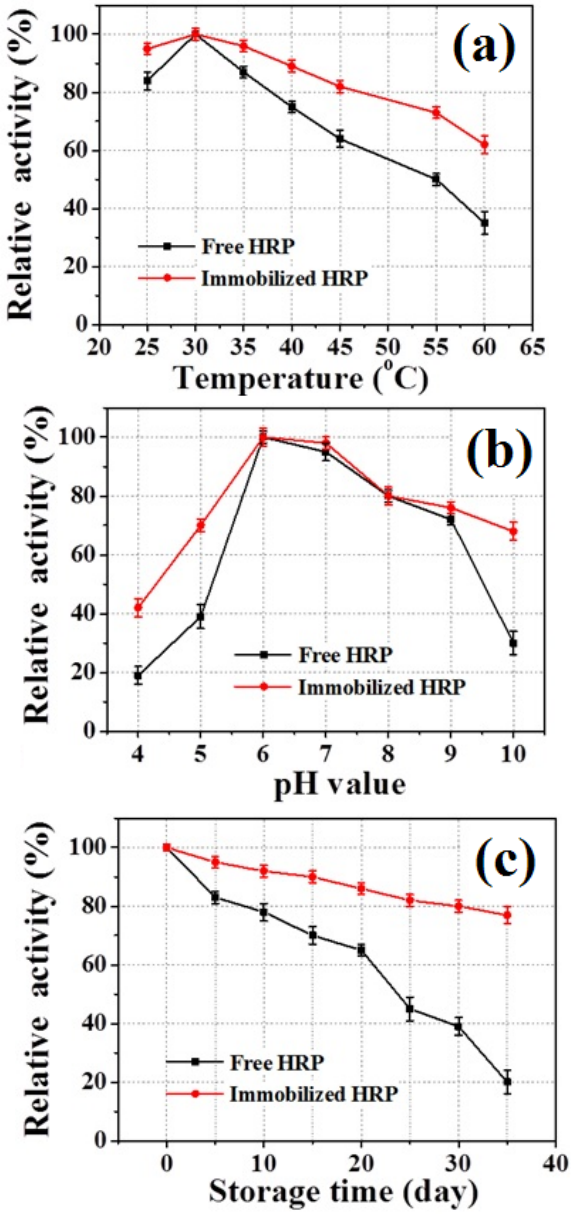

FIGURE 7. The effect of temperature (a), pH value (b) and storage duration (c) on activity of free and immobilized HRP.

In this work, an enzyme membrane (PMA-co-PAA@CTS-HRP) was employed as a "green" and recyclable catalyst for catalytic oxidation of PNP. The removal efficiency of the PNP was determined spectrophotometrically by measuring the decrease in absorbance at 317 nm as a consequence of the PNP consumption over time. In a typical test, the reaction mixture was stirred at 25°C for 30 min to achieve adsorption-desorption equilibrium, and the reaction was initiated with the addition of H_2O_2 (0.015 mol/L). The mechanism is proposed as follows:

$$HRP\ (Fe^{3+}) + H_2O_2 \rightarrow \text{Intermediate-I} \quad (1)$$

$$\text{Intermediate-I} + PNP_{red} \rightarrow \text{Intermediate-II} + PNP_{ox} \quad (2)$$

$$\text{Intermediate-II} + PNP_{red} \rightarrow HRP\ (Fe^{3+}) + PNP_{ox} + H_2O \quad (3)$$

The oxy-ferryl HRP compound (Intermediate-I), comprising a ferryl species and a porphyrin radical cation is produced by the reaction of H_2O_2 and HRP in the iron (III) peroxidase state Eq. (1). In the following step, the oxidation state (Intermediate-II) is formed by the first reduction reaction between the porphyrin radical cation and PNP compounds Eq. (2). In the last reaction Eq. (3), the PNP compounds are mainly converted into quinones or free radical products, regenerating the HRP compound.

As illustrated in *Figure 8a*, the PNP removal efficiency with the pristine PMA-co-PAA membrane is less than 30 % at every PNP concentration tested (20~140 mg/L). This is mainly ascribed to physical absorption. It was observed that the conversion ratios of the PNP by employing the free and immobilized HRP were about 42 % and 45 % at low concentration of 20 mg/L, respectively. With the concentration of PNP increased to 100 mg/L, the conversion capability by both free and immobilized HRP both increased and reached maximum removal efficiencies of about 72 % and 86 %, respectively. In addition, the PMA-co-PAA@CTS- HRP still maintained a high conversion ratio of over 80% with continuously increasing PNP concentration to 140 mg/L.

Finally, the PMA-co-PAA@ CTS-HRP can be easily recycled without significant loss of activity. For ease of presentation, the initial removal efficiency is set as 100 percent. After 10 cycles, the PNP removal efficiency with the PMA-co-PAA @CTS-HRP was still ~70% (*Figure 8b*), which is better than achieved in previous reports, [15, 32]. This suggests that CTS-functionalized PMA-co-PAA is a promising support to prevent enzyme leakage upon cycling.

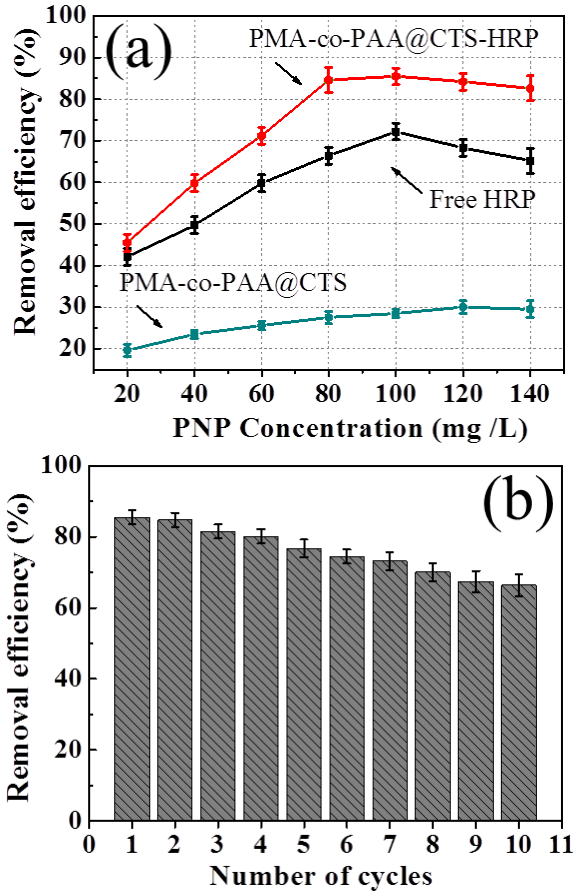

FIGURE 8. (a) PNP removal efficiency as a function of the concentration; (b) reusability of the immobilized HRP for PNP removal, continuously increasing the PNP concentration to 140 mg/L.

CONCLUSION

In this paper, a two-step approach is presented to prepare a novel type of bio-friendly composite membrane of PMA-co- PAA@CTS, which was employed as a support for immobilization of HRP. This CTS- functionalized membrane enhanced the HRP loading amount without sacrificing the catalytic activity. As a result, the thermal, pH, and storage stabilities of the PMA-co-PAA @CTS-immobilized HRP were significantly higher than that of free HRP. Moreover, the PMA-co-PAA@CTS-HRP can function as an active and recyclable biocatalyst for catalytic oxidation of PNP over 10 cycles. The improved performance of the immobilized HRP is attributed to the CTS, which provides not only a hydrophilic environment but also multiple binding sites for the HRP enzyme. Thereby, it increases the HRP loading amount and stability. This new type of CTS-functionalized membrane provides a step toward developing bio-friendly support for practical application of enzymes.

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REFERENCES
[1] Clark J H. *Green Chem* 1999, 1, 1-8.
[2] Anastas P T and Kirchhoff M M. *Acc Chem Res* 2002, 35, 686-694.
[3] Sheldon R A. *Chem Soc Rev* 2012, 41, 1437-1451.
[4] Sheldon R A. *Adv Synth Catal* 2007, 349, 1289-1307.
[5] Hanefeld U, Gardossi L, Magner E. *Chem Soc Rev* 2009, 38, 453-468.
[6] Shieh F K, Wang S C, Yen C I, et al. *J Am Chem Soc* 2015, 137, 4276-4279.
[7] Kalantari M, Kazemeini M, Tabandeh F, et al. *J Mater Chem* 2012, 22, 8385-8393.
[8] Chen Z, Xu W, Jin L, et al. *J Mater Chem A* 2014, 2, 18339-18344.
[9] Butler J E. *Methods* 2000, 22, 4-23.
[10] Brady D, Jordaan J. *Biotechnology letters*, 2009, 31, 1639-1650.
[11] Wang Z G, Wan L S, Liu Z M, et al. *J Mol Catal B: Enzym* 2009, 56, 189-195.
[12] Selloum D, Chaaya A A, Bechelany M, et al. *J Mater Chem A* 2014, 2, 2794-2800.
[13] Quan J, Liu Z, Branford-White C, et al. *Colloids Surf B: Biointerfaces* 2014, 121, 417-424.
[14] Feng Q, Zhao Y, Wei A, et al. *Environ Sci Technol* 2014, 48, 10390-10397.
[15] Hwang E T, Tatavarty R, Lee H, et al. *J Mater Chem.* 2011, 21, 5215-5218.
[16] Li C, Zhou L, Wang C, et al. *RSC Adv* 2015, 5, 41994-41998.
[17] Younes I and Rinaudo M. *Mar Drugs* 2015, 13, 1133-1174.
[18] El-Sherbiny I M and El-Baz N M. *Eco-friendly Polymer Nanocomposites. Springer India* 2015, 173-208.
[19] Wang C, Yan Q, Liu H B, et al. *Langmuir* 2011, 27, 12058-12068.
[20] Moffa M, Polini A, Sciancalepore A G, et al. *Soft Matter* 2013, 9, 5529-5539.
[21] Karim F, Fakhruddin A N M. *Rev Environ Sci & Bio/Tech* 2012, 11, 261-274.
[22] Rios G M, Belleville M P, Paolucci D, et al. *J Membr Sci* 2004, 242, 189-196.
[23] Ansari S A and Husain Q. *Biotechnol Adv* 2012, 30, 512-523.
[24] Kim J, Grate J W, Wang P. *Trends Biotechnol* 2008, 26, 639-646.
[25] Hartmann M and Kostrov X. *Chem Soc Rev* 2013, 42, 6277-6289.
[26] Zhai R, Zhang B, Wan Y, et al. *Chem Eng J* 2013, 214, 304-309.
[27] Niu J, Xu J, Dai Y, et al. *J Hazard Mater* 2013, 246, 119-125.
[28] Mishra K P and Gogate P R. *Ind Eng Chem Res* 2012, 51, 1166-1172.
[29] Sun S P and Lemley A T. *J Mol Catal A: Chem* 2011, 349, 71-79.
[30] Tasseroul L, Pirard S L, Lambert S D, et al. *Chemical engineering journal* 2012, 191, 441-450.
[31] Wang F, Song S, Li K, et al. *Adv Mater* 2016, doi: 10.1002/adma.201603608.
[32] Feng Q, Wu D, Huan S, et al. *J Eng Fiber Fabr* 2016, 11, 45-54.

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