NH₂–Fe₃O₄@SiO₂ supported peroxidase catalyzed H₂O₂ for degradation of endocrine disrupter from aqueous solution: Roles of active radicals and NOMs

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Abstract

Horseradish peroxidase (HRP) was successfully loaded on aminated magnetic Fe₃O₄. Estrogens degradation by IM-HRP catalyzed H₂O₂ was fitted well with pseudo-second kinetic equation. Superoxide radical (·O₂⁻) and hydroxyl radicals (·OH) were identified in IM-HRP catalyzed H₂O₂ system. ·O₂⁻ was the dominant active radicals responsible for E₂ removal. Oxidative degradation of E₂ was inhibited in presence of humic substances.

Keywords:
Horseradish peroxidase
NH₂–Fe₃O₄@SiO₂
Immobilization
Catalytic oxidation
Radicals
Natural organic matters

Highlights

- Horseradish peroxidase (HRP) was successfully loaded on aminated magnetic Fe₃O₄.
- Estrogens degradation by IM-HRP catalyzed H₂O₂ was fitted well with pseudo-second kinetic equation.
- Superoxide radical (·O₂⁻) and hydroxyl radicals (·OH) were identified in IM-HRP catalyzed H₂O₂ system.
- ·O₂⁻ was the dominant active radicals responsible for E₂ removal.
- Oxidative degradation of E₂ was inhibited in presence of humic substances.

Graphical Abstract

In this work, magnetic Fe₃O₄ was utilized to immobilize horseradish peroxidase (IM-HRP) in order to improve its stability and reusability by crosslinking method process with glutaraldehyde. The physico-chemical properties of NH₂–Fe₃O₄@SiO₂ and IM-HRP were characterized by powder X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FT-IR), Thermo-gravimetric Analysis (TGA) and Transmission electron microscopy (TEM). The thermal stability of immobilized-HRP was considerably improved in comparison with free counterpart. The catalytic performance of IM-HRP for estrogens removal from aqueous solution was evaluated, it was found that the presence of natural organic matters (NOM) have no significant effects on E₂ removal and the E₂ enzyme-degradation reached around 80% when pH = 7.0 with 0.552 × 10⁻³ ratio of IM-HRP/H₂O₂. In addition, the active radicals responsible for estrogens degradation were identified with electro-spin resonance spectra (ESR). It was found that immobilization process on Fe₃O₄ showed no adverse effects on catalytic performance on HRP, estrogens degradation could be fitted well with pseudo—second kinetic equation. Estrogens degradation efficiency was reduced in the presence of humic substances. Both ·O₂⁻ and ·OH were detected in IM-HRP catalyzed H₂O₂ system and radicals quenching test indicated ·O₂⁻ played a more important role in estrogens removal. IM-HRP exhibited excellent stability and E₂ removal efficiency could reach 45.41% after use.
1. Introduction

It has well been established that endocrine system of human can be disturbed by various substances which were called endocrine disturbing compounds (EDCs). These EDCs may also potentially cause other undesirable effects on human health, they have been widely detected in different water bodies, such as rain water, well water, lakes, and oceans, as well as fresh water marine and terrestrial foods (Auriol et al., 2006a, b). EDCs are of critical concern long-term impacts for human exposure to these medicals, which are mainly released into environment by humans, animals and industry, and mainly through the sewage treatment systems before they are reaching the receiving bodies (soil, rain water and ground water) (Liu et al., 2009). Hence, it’s a critical matter to control the content of EDCs before releasing into environment. Thus, since 1996, the US Environmental Protection Agency’s (USEPA) Office of the Research and Development has considered the EDCs disruption to be one of top six research priorities (Auriol et al., 2006a, b). For such purpose, one of the USEPA’s goals was to control the discharge and improve the removal of endocrine disturbing compounds from aqueous environment in cost-effective way.

Many studies have demonstrated that the natural estrogens such as 17β-estradiol were one of the major contributors to the estrogenic activity observed in waste water (Auriol et al., 2006a, b), By the fact concentrations as low as 1–10 ng.L⁻¹ of estradiol could induce negative effect on male’s endocrine systems (Aerni et al., 2004), thus it’s necessary to improve the removal of estradiol from waste water. In previous studies, conventional waste water and drinking water treatment processes for EDCs removal could be classified into three categories: physical adsorption, biodegradation and chemical advanced oxidation (Liu et al., 2009). For instance, the hydrophobic interaction was responsible for EDCs removal in growing attention as an effective way for enzyme recovery. Wu et al. (2009) reported horseradish peroxidase was successfully immobilized onto the clay minerals using organic matters for phenol removal in aqueous solution (Kim et al., 2012). Gustafsson et al. immobilized glucose oxidase in combination to horseradish peroxidase on the surface of solid silica supports (Gustafsson et al., 2015). Although the immobilization has many advantages over traditional free enzymes, the fine particles were still very difficult to be separated from aqueous solution. Thus, using magnetic materials as biological enzyme supporters has received growing attention as an effective way for enzyme recovery. Wu et al. (2009) reported lipase was efficiently immobilized onto magnetic Fe₃O₄-chitosan nanoparticles by crosslinking with sodium tripolyphosphate. And the immobilization of laccase onto bimodal carbon-based mesoporous magnetic composites which exhibited a large adsorption capacity (491.7 mg g⁻¹) as reported by Liu et al. (2012).

Since magnetic supporter is a good choice for HRP enzymes immobilization, in this paper, HRP enzymes were immobilized onto Fe₃O₄ particles which were coated by [3-Aminopropyl] triethoxysilane (APTs) modified SiO₂. In order to successfully immobilize HRP on the surface Fe₃O₄ particles, Bilal et al. using cross-linked enzyme aggregates to deal with emerging endocrine-disrupting and investigated the effects of the glutaraldehyde concentration to enzyme activity recovery (Bilal et al., 2017a, b, c, d), hence glutaraldehyde solution was selected as activator agent that could activate the −NH₂ groups on the surface of Fe₃O₄ to bind free HRP enzymes. Then, the IM-HPM particles were characterized by XRD, FT-IR, TGA and TEM. The immobilized HRP (IM-HPM) was utilized for catalytic degradation of 17β-estrogens (E2) from aqueous solution. In addition, the effects of reaction conditions (time, H₂O₂ dosage, HRP dosage, pH and NOMs) on E2 removal were comparatively examined in free HRP or IM-HPM catalyzed H₂O₂ systems. Meanwhile, the active radicals responsible for E2 removal were seven times. Therefore, HRP enzymes immobilized on NH₂–Fe₃O₄@SiO₂ by cross-linking method in glutaraldehyde solutions was an effective way to improve stability and reusability of HRP, and which could avoid potential secondary pollution in water environment caused by free HRP after treatment.

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identified with electro-spin resonance spectra (ESR), and their contribution was evaluated with radical quenching tests.

2. Materials and methods

2.1. Materials

All reagents and materials are described in details in Section 1.1 of Supporting Information (SI).

2.2. Synthesis of NH2–Fe3O4@SiO2 and HRP/NH2–Fe3O4@SiO2

2.2.1. Synthesis of Fe3O4, Fe3O4@SiO2 and APTS-Modified Fe3O4@SiO2 microspheres

Fe3O4 particles were synthesized according to the method reported by Deng et al. (Deng et al., 2010) (see Section 1.2.1 of Supporting Information), the Fe3O4@SiO2 microspheres were prepared through a traditional solution sol-gel method and followed with modification by (3-Aminopropyl) triethoxysilane (APTS) (see Section 1.2.2 of Supporting Information).

2.2.2. Immobilization of HRP on APTS-Modified SiO2@Fe3O4 particles via crosslinking method

Enzyme activity of immobilized and free HRP enzymes was measured with the method reported by Klibanov et al. (Klibanov, 2001; Ai et al., 2016), (see Section 1.2.4 of Supporting Information). The preparation procedure of immobilized HRP on APTS-Modified SiO2@Fe3O4 particles was presented in details in Section 1.2.3 of Supporting Information.

2.3. Characterization of Fe3O4, SiO2@Fe3O4, NH2–SiO2@Fe3O4 and HRP/NH2–SiO2@Fe3O4 microspheres

Fe3O4, SiO2@Fe3O4, NH2–SiO2@Fe3O4 and HRP/NH2–SiO2@Fe3O4 microspheres were characterized by powder X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FT-IR), thermo gravimetric analysis (TGA) and transmission electron microscopy (TEM), all of the analysis method were provided in details in Section 2 of Supporting Information.

2.4. Estrogens (E2), natural organic matters and electron paramagnetic resonance (EPR) analysis

Estrogens concentrations were determined by single liquid chromatography/fluorescence detector (LC-FLD), and the intermediates of E2 enzyme-degradation were identified with negative ion mode in m/z 50–350 by LC/MS/MS (Zhao et al., 2008) (see Section 3.1 of Supporting Information). In addition, the natural organic matters (NOM) of water sample from DongHu Lake in Wuhan city were analyzed by using FP-6500 fluorescence spectrophotometer (Jasco, Japan) (see Section 3.2 of Supporting Information). And the active radicals were identified with electron paramagnetic resonance (JES-FA200 (JEOL Ltd)) instrument at room temperature as described in details in Section 3.3 of Supporting Information.

2.5. Reusability test of IM-HRP

In order to determine the stability of immobilized enzymes, the reusability test of IM-HRP were discussed in the study as described in details in Section 4 of Supporting Information.

3. Results and discussion

3.1. Characterization of IM-HRP

3.1.1. XRD of Fe3O4, SiO2–Fe3O4 and SiO2@NH2–Fe3O4 characterization

The XRD pattern of Fe3O4, SiO2@Fe3O4 and NH2–Fe3O4 microspheres showed the characteristics broad diffraction peaks indexed to the Fe3O4 nanoparticles (Deng et al., 2010). According to the XRD analysis as shown in Fig. 1 (1), the results exhibited the well-crystallized Fe3O4 structure with several different forms, including Fe3O4, SiO2@Fe3O4 and NH2–Fe3O4. For the Fe3O4, the diffraction peaks of 30.20°, 35.54°, 43.12°, 53.52°, 57.11°, 62.78° and 74.40° were observed, and it exhibited a cubic spinel structure with magnetic property (Wang et al., 2010). The same fixed characteristics were still observed for SiO2@Fe3O4 and NH2–Fe3O4, which was in agreement with the work reported by Deng et al. (2010). The results showed that the structure of the Fe3O4 was not destroyed after SiO2 encapsulation and APTS modification, indicating that its magnetic performance was not affected.

3.1.2. FT-IR of Fe3O4, SiO2@Fe3O4 and NH2–Fe3O4 characterization

The Fe3O4, SiO2@Fe3O4 and NH2–Fe3O4 nanoparticles were characterized by using FT-IR methods, as shown in Fig. 1 (2). For the SiO2@Fe3O4, the peak at 590 cm\(^{-1}\) was related to the stretching vibrations of Fe–O bonds, which that confirmed the presence of Fe3O4 composites. This result was agreement with the previous reports (Li et al., 2013; Fang et al., 2016). The absorption bands at 1095 cm\(^{-1}\) indicated the presence of stretching band of Si–O–Si vibrations (Li et al., 2014). These results confirmed successful silica coating on the surface of Fe3O4 particles during the encapsulation process, which was able to prevent Fe3O4 particles from oxidative destruction caused by oxygen. The high transmittance of brands at 1631 cm\(^{-1}\) and 2924 cm\(^{-1}\) were attributed to the stretching vibrations of N–H bonds or H–N–H bonds which were associated with NH\(^2\)-groups of particle surfaces after Fe3O4 was modified with APTS (Wang et al., 2015a, b), indicating the successful occurrence of the amination reaction between SiO2@Fe3O4 and APTS.

3.1.3. TGA of Fe3O4, SiO2–Fe3O4 and NH2–Fe3O4 characterization

The APTS-modified coating on the surface of Fe3O4 particles was also confirmed by TGA analysis. As depicted in Fig. 1 (3), it showed the changes of weight loss of samples (Fe3O4, SiO2@Fe3O4 and NH2–Fe3O4) with temperature. At high temperature, the organic materials or magnetite transformed to gaseous products or iron oxide by heating treatment, respectively. As shown in Fig. 1 (3), TGA curve of SiO2@Fe3O4 compared to Fe3O4 particles, there is a significant weight loss stage (14.14%) by increasing temperature to 461 °C, which was attributable to evaporation of water and silicon compounds. While in APTS-modified particles, weight loss started at temperature of 196 and decreased by 20.03% at 800 °C, which could be attributed to both evaporation water and silicon compounds and decomposition of amino. Hence, according to the weight loss, the APTS-modified on the surface of Fe3O4 particles was calculated to be possibility around 5.89% of amino groups.

3.1.4. TEM of Fe3O4 and SiO2–Fe3O4 characterization

TEM was used to observe the changes in morphological properties of Fe3O4 and SiO2@Fe3O4. Fig. 1 (O) and (Os) illustrated the TEM of Fe3O4 and SiO2@Fe3O4 microspheres, respectively. It can be clearly observed that the silicon was fully covered with Fe3O4 microspheres after being treated by TEOs. Since the Fe3O4 spheres were protected by SiO2 layers to maintain its magnetism, and it was advantageous to the modification with other oxides or polymers such as amino groups. This result was in agreement with the
SiO\textsubscript{2}@Fe\textsubscript{3}O\textsubscript{4} particles properties reported by Deng et al. (2010). In addition, through a sol-gel process by hydrolysis of TEOS in ethanol and amino mixture, uniform SiO\textsubscript{2} layers could be formed on each magnetic Fe\textsubscript{3}O\textsubscript{4} seeds, consequently resulting in generation of core shell SiO\textsubscript{2}@Fe\textsubscript{3}O\textsubscript{4} microspheres as showed in Fig. 1 (Os).

3.1.5. Effects of temperature and pH on immobilized/free HRP enzyme activities

The stability of IM-HRP enzymes in comparison with free HRP enzymes at different temperature and pH was evaluated in this paper. As depicted in Fig. 2 (A), the optimal temperature of free/immobilized HRP enzymes was about 35 °C, and the result showed free-HRP almost lost enzyme activity at above temperature range over 70 °C. However, immobilized HRP enzymes exhibited more stability in comparison with free HRP enzymes at temperature in range 25 °C–80 °C. Moreover, the optimal pH for both free and immobilized HRP enzymes was about 7.0, at this time the residual enzyme activity of free-HRP and IM-HRP account for the maximum

**Fig. 1.** Powder X-ray diffraction (XRD) (1) XRD of Fe\textsubscript{3}O\textsubscript{4}, SiO\textsubscript{2}@Fe\textsubscript{3}O\textsubscript{4} and NH\textsubscript{2}—Fe\textsubscript{3}O\textsubscript{4}; (2) Fourier transform infrared spectroscopy (FT-IR) spectra of Fe\textsubscript{3}O\textsubscript{4}, SiO\textsubscript{2}@Fe\textsubscript{3}O\textsubscript{4} and NH\textsubscript{2}—Fe\textsubscript{3}O\textsubscript{4}; (3) Thermo-gravimetric Analysis (TGA) of Fe\textsubscript{3}O\textsubscript{4}, SiO\textsubscript{2}@Fe\textsubscript{3}O\textsubscript{4} and NH\textsubscript{2}—Fe\textsubscript{3}O\textsubscript{4}; (O/OS) Transmission electron microscopy (TEM) of Fe\textsubscript{3}O\textsubscript{4} and SiO\textsubscript{2}@Fe\textsubscript{3}O\textsubscript{4}.
enzyme activity. As described in Fig. 2 (B), the residual enzyme activity of IM-HRP exhibited significantly higher stability in the pH examined range 4.0–10.0 in comparison with the free-HRP. As a result, the IM-HRP enzymes was more stable in different reaction conditions (temperature and pH), it was very likely to be that the stability of HRP enzymes to harsh conditions was improved by loading the HRP enzymes to the surface of the NH$_2$–Fe$_3$O$_4$.

3.2. Effects of reaction conditions on E2 removal using IM-HRP catalyzed H$_2$O$_2$ systems

3.2.1. Effects of H$_2$O$_2$ dose on E2 removal

In this paper, experiments were carried out by choosing different H$_2$O$_2$ (6 g/L, 30%) dosage in a range of 1 mL–9 mL and the initial E2 concentration was adjusted to be 1000 µg/L. As depicted in Fig. 3 (b), the E2 removal efficiency reached the maximum at 80.85% when H$_2$O$_2$ (6 g/L, 30%) dosage was 24 mg/L, and the removal of E2 was decreased with further increase of H$_2$O$_2$. It was clearly observed that addition of excessive H$_2$O$_2$ could lead to deterioration of substrate degradation in Fig. 3 (a). It was very likely that the addition of excessive H$_2$O$_2$ could result in production of intermediate products that might inhibit the catalytic capacity of HRP enzymes (Wang et al., 2015a, b; Duarte-Vázquez et al., 2001). Some previous studies also presented that overdosed H$_2$O$_2$ could inhibit the enzyme catalytic performances as reported by Nicell et al. (1999; Nicell and Harold, 1996), which was in agreement with our study as depicted in Fig. 3 (a) and Fig. 3 (b). Some studies also come up with the opinion that the overdose of H$_2$O$_2$ could inhibit the enzyme catalysis capacity (Wang et al., 2015a, b; Duarte-Vázquez et al., 2001). In addition, H$_2$O$_2$ could act as scavengers of active radicals through reduction reactions. Hence, the optimal concentration of H$_2$O$_2$ of the HRP enzymes catalysis system was 24 mg/L.

3.2.2. Effects of molar ratio of IM-HRP to H$_2$O$_2$ on E2 removal in present of NOM

The removal efficiency of E2 at the different level of experiments involving a range of IM-HRP to H$_2$O$_2$ molar ratio as a function of initial E2 concentration (1000 µg/L (500 mL)) in present of NOM were also studied in Fig. 3 (b). In these experiments, the dose of IM-HRP concentration was fixed at 0.064 g/L in Donghu lake water contained E2. The amount could ensure that the degradation of E2 was mainly limited by the availability of the NOM and H$_2$O$_2$. The NOM effects in E2 removal efficiency could be calculated around 10% for that the maximum E2 removal efficiency was 80.85% without NOM as shown in Fig. 3 (a) and 70.16% in present of NOM as shown in Fig. 3 (b). It was very likely to be the result of the NOM degradation by IM-HRP which has effected the E2 degradation, in addition, the signal of three dimensional fluorescence of humic acid-like was indeed weaken after treated by IM-HRP/free-HRP in Fig. 6.

In addition, the gradual addition of H$_2$O$_2$ could lead to lower possibility of enzymes inactivation as shown in Fig. 3 (a) and Fig. 3 (b) with/not contained NOM, therefore, the experiments calculated that the optimal addition of IM-HRP to H$_2$O$_2$ molar ratio (IM-HRP/H$_2$O$_2$) was 0.552 × 10$^{-3}$ at a reaction time of 90 min until to different designed amount. This phenomenon was likely to be when the molar ratio of HRP/H$_2$O$_2$ was exceed to the optimum reaction ratio of IM-HRP/H$_2$O$_2$, the H$_2$O$_2$ was not enough to run out of the enzymes catalytic capacity, and the E2 removal efficiency could be increased slightly with the increase of H$_2$O$_2$ and the reached the maximum value with 0.552 × 10$^{-3}$ of IM-HRP to H$_2$O$_2$ molar ratio (Cheng et al., 2006). However, when the ratio of IM-HRP/H$_2$O$_2$ was over the optimum reaction ratio, the H$_2$O$_2$ was over dose to the enzyme catalytic reaction systems and the overdose of the H$_2$O$_2$ could inhibit the enzyme catalytic performances as reported by previous study (Wang et al., 2015a, b; Nicole et al., 1999). For that the H$_2$O$_2$ must be limited as the inhibition to the enzymes activity by the excess quantities of H$_2$O$_2$ as reported by Nicell et al. (Nicell and Harold, 1996), which was in agreement with our study as depicted in Fig. 3 (a) and Fig. 3 (b). Some studies also come up with the opinion that the overdose of H$_2$O$_2$ could inhibit the enzyme catalysis performance (Wang et al., 2015a, b; Nicole et al., 1999). Hence high H$_2$O$_2$ dosage could reduce the IM-HRP enzymes catalytic capacity.

3.2.3. Effects of pH on the E2 removal by immobilized HRP

As depicted in Fig. 3 (c), the E2 degradation efficiency reached the maximum at 79.84% when solution pH was around 7 under IM-HRP catalyzed H$_2$O$_2$. Moreover, it was obvious that the catalytic capacity of IM-HRP was more stable under acidic conditions in comparison to alkaline environment. The stability and performance against acidic systems of HRP enzymes was improved by loading HRP on the surface of Fe$_3$O$_4$ via cross-linking immobilization process.

3.2.4. Comparison of E2 removal efficiency in free-HRP and IM-HRP catalyzed systems

The catalytic capacity of IM-HRP and free HRP were comparatively evaluated to get insights into the effects of loading process on enzymatic activity of HRP. Fig. 3 (d) showed the changes of E2 removal efficiency with the reaction time by in free-immobilized HRP. It was found that the reaction reached the equilibrium and no significant improvement was observed with time proceeded further. At this time, the maximum E2 removal was 89.50% and 77.04% when the free HRP and IM-HRP were used as H$_2$O$_2$ catalysts respectively. Free HRP enzymes performed better in E2 removal than IM-HRP enzymes, indicating that loading process resulted in
decrease of enzymatic ability. However, since the immobilized HRP can be recovered with magnetic separation and alleviate the secondary pollution generated by free HRP, it was still more promising in water treatment.

Moreover, the pseudo-second-order kinetic model could better describe the degradation of E2 in IM-HRP catalysis system, while that was fitted well with pseudo-first-order equation free-HRP catalysis system (see Section 5 of Supporting Information). The result revealed that E2 removal was more dependent on substrate rather than H2O2 concentration in free-HRP catalytic system, while concentrations of both substrate and H2O2 affected E2 degradation in IM-HRP enzymes catalytic system. This results might be the reason of the different of reaction process between immobilized enzymes and free enzymes, it was homogenous catalytic reaction process in free-HRP catalytic process as free-HRP was soluble in water and the heterogeneous catalytic reaction process in IM-HRP catalytic system since free-HRP enzymes were successful immobilized to the surfaces of Fe3O4.

3.3. Identification of active oxidants for E2 removal in IM-HRP catalyzed H2O2 system

3.3.1. ESR analysis and enzyme-catalytic reaction performed in different conditions

The ESR spin-trapping technique was utilized to detect the radicals during the reaction between free-HRP/IM-HRP enzymes and H2O2. As depicted in Fig. 4, there is no DMPO-OH or DMPO-O2 spin adducts were detected in H2O2 or CH3OH system as shown in (a) and (b) when free-HRP/IM-HRP was reacted with H2O2 in CH3OH. Meanwhile, when the estrogens presented in the system of free-HRP/IM-HRP and H2O2, both peaks of DMPO-O2 and DMPO-OH were fade away as seen in (a) and (d), indicating radicals was very likely to be responsible for E2 degradation. It was reported that OH and O2 were highly active non-selective radicals and were very effective in removal of a wide variety of organic contaminants in aqueous solution.

The radicals quenching test was performed in order to understand the contribution of OH and O2 to E2 removal. Specifically, the methonal was able to scavenge OH rather than O2 (Amyl et al., 2001), while tertiary butanol could eliminate both OH and O2 (Paulson et al., 1999; Michael et al., 1999). As depicted in Fig. 5(a), the SiO2@Fe3O4 nano-particles had very limited effects on E2 removal. Specifically, the methonal was able to scavenge OH rather than O2 (Amyl et al., 2001), while tertiary butanol could eliminate both OH and O2 (Paulson et al., 1999; Michael et al., 1999). As depicted in Fig. 5(a), the SiO2@Fe3O4 nano-particles had very limited effects on E2 removal. In addition, under H2O system, characteristic peaks of DMPO-OH adducts were obviously observed as shown in (b), though no obvious peaks of DMPO-O2 adducts were detected. This is likely that O2 is unstable in aqueous solution and rapidly easily decomposed into •OH, and the reaction rate of DMPO with •OH was much faster than that of •O2 radicals (Yin et al., 2009). Hence, it was difficult to detect the DMPO-O2 peaks, though it was formed in the reaction process (Yin et al., 2009). In order to further verify whether •O2 is formed during the reaction process between free-HRP/IM-HRP with H2O2, CH3OH instead of H2O was used to slow down the decomposition rate •O2. As shown in Fig. 4, there was DMPO-OH or DMPO-O2 spin adducts was shown in (a) and (b) when free-HRP/IM-HRP was reacted with H2O2 in CH3OH. Meanwhile, when the estrogens presented in the system of free-HRP/IM-HRP and H2O2, both peaks of DMPO-O2 and DMPO-OH were fade away as seen in (a) and (d), indicating radicals was very likely to be responsible for E2 degradation. It was reported that OH and O2 were highly active non-selective radicals and were very effective in removal of a wide variety of organic contaminants in aqueous solution.

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the E2 was decreased from 80% to 72% and 12% of HRP catalyzed system in methanal and tertiary butanol solutions in Fig. 5(b). The results demonstrated that the \( \cdot \mathrm{O}_2 \) radicals were dominant oxidants for E2 removal since high removal efficiency could still be achieved in methanol solution.

3.3.2. Enzyme-catalytic reaction mechanism of E2 removal by IM-HRP in present of \( \mathrm{H}_2\mathrm{O}_2 \)

The cycle of E2 removal efficiency from aqueous solution by IM-HRP with \( \mathrm{H}_2\mathrm{O}_2 \) could be summerized as follows: At first, estrogens (E2) and \( \mathrm{H}_2\mathrm{O}_2 \) molecules were adsorbed to the surface of the
HRP@Fe3O4, and then the reaction was involved to the enzymatic catalytic process. As previous study, the primary catalytic cycle of HRP enzymes can be summarized into three portions (Wagner and Nicell, 2002; Khan and Nicell, 2007; Wu et al., 1998) as summed in equation 1–3 (see in Section 6 of Supporting Information). Moreover, three of the typical intermediates of E2 enzyme-degradation detected by LC/MS/MS were shown in Table S4 of Supporting Information, m/z of E2 was 271.17 and the retain time (RT) was 6.46, the m/z value for 269.10 corresponds to be that the E2 has lost two H (m/z = 1) and it was in agreement with the previous work reported by Zhao et al. (2008). For the sensitivity of the LC/MS/MS, m/z of standard 288 and 178.98 confirmed the presence of these two new specific intermediates (Table S4, Fig. 5 in supporting information) during the process of E2 enzyme-degradation, which was confirmed to the previous reports (Zhao et al., 2008). According to the study (Wagner and Nicell, 2002), the enzyme degradation reaction may be initiated by the addition of enzymatic substance and H2O2 generated RO/H and /OH to E2 undergoing different enzymatic degradation pathway. When RO/H and /OH radicals attack the aromatic rings such as E2, a number of intermediates may be formed during the enzymatic degradation, and these single aromatic intermediates may be further oxidized through enzymatic degradation. In a conclusion, E2 was degraded into radicals or smaller molecules by immobilized/free HRP

Fig. 6. Three-dimension excitation emission matrix fluorescence spectroscopy (3D-EEM) of water sample; (0) Estrogens only in ultrapure water; (0₁) Untreated lake water from DongHu; (A) Untreated lake water containing estrogens, (A₁) treated lake water containing estrogens by free HRP enzymes; (B) Untreated lake water containing estrogens, (B₁) treated lake water containing estrogens by IM-HRP enzymes.
enzymes with H$_2$O$_2$.

However, in the enzymatic catalytic process, several side reactions can also occur that reduce the catalytic capacity of the enzyme through inactivation (Khan and Nicell, 2007) as summarized in details in Section 6 of Supporting Information.

3.4. Effects of natural organic matters (NOM) on E2 removal

Three-dimension excitation emission matrix (3D-EEM) fluorescence spectroscopy was widely utilized for the characterization of the natural organic matters (NOM) in recycled water system with the advantages of its high sensitivity and selectivity (Henderson et al., 2009). The three-dimensional fluorescence spectrum could be divided into five primary regions, which are mainly for aromatic protein I, aromatic protein II, fulvic acid-like, tryptophan-like substances and humic acid-like as reported by previous study (see Table S2 of Supporting Information) (Chen et al., 2003). As depicted in Fig. 6 (O), peak I (Ex/Em = 285/320) appeared when E2 was dissolved into water, indicating E2 contained the similar fluorescent groups to tryptophan-like compounds. Thus, the presence of tryptophan-like substances originated from microbial metabolism might interfere the detection of E2 with fluorescence spectroscopy. In addition, peak II (Ex/Em = 340/420) was observed in water sample of Donghu lake, which were related to humic substances. Moreover, it was obvious that fluorescent intensities of both E2 and humic substances weakened after the treatment with HRP catalyzed H$_2$O$_2$ as shown in Fig. 6 (see Section 7 of Supporting Information). That is to say, the presence of high concentration of NOMs in water might cause increase of consumption of oxidants and consequently operating like costs. (Auriol et al., 2006a, b).

3.5. Reusability of IM-HRP

The primary purpose of HRP enzymes immobilization was to increase the frequency of use, which could effectively cut down the operating cost of HRP catalyzed system in full-scale application. Hence the reusability of IM-HRP treating estrogens (E2) was investigated in the present study. As depicted in Fig. 7, the removal efficiency of E2 by IM-HRP enzymes was about 77.42% in the first treatment cycle. After seven recycled test, the E2 removal efficiency of IM-HRP was decreased to 45.41%. It was likely to be the reason that part of loss of enzymes from Fe$_3$O$_4$ or lower of the IM-HRP catalytic capacity after the application. In addition, in the enzymatic catalytic process by IM-HRP, the intermediated enzymes could be transferred to irreversible or reversible compound through side reaction process (Khan and Nicell, 2007; Zhang and Nicell, 2000) thereby the catalytic capacity of enzymes was decreased. However, since it has the advantages in reducing the second pollution generated by HRP enzymes and increasing the reusability of HRP enzymes, the technology of immobilization still has the potential value in catalytic application field in environmental.

4. Conclusion

In this study, the feasibility of using a crosslinking immobilization method process to immobilize free HRP enzymes on aminated SiO$_2$@Fe$_3$O$_4$ nano-particles, and its catalytic capacity were evaluated by considering different reaction conditions. The experiment results revealed the immobilization process has no adverse effects on catalytic performance of HRP enzymes and the immobilization process could improve stability HRP enzymes against harsh condition (pH 3.7–7.5). The immobilized HRP enzymes (IM–HRP) could be easily recovered through magnetic separation and decrease the potential secondary pollution generated by free HRP enzymes since free enzymes are soluble in water. The result was also demonstrated that the removal of E2 was significantly dependent on reaction conditions (pH and dosages of IM–HRP/H$_2$O$_2$), 77% of E2 was removed under the optimal reaction system. In addition, ESR analysis demonstrated that active radicals (•O$_2$ and •OH) were responsible for E2 removal in IM-HRP enzymes catalyzed H$_2$O$_2$ system, •O$_2$ played a more important role in E2 removal than •OH. Humic substances could act as radicals scavengers and resulted in decline of E2 removal. Meanwhile, the E2 was initially absorbed on the surface of IM-HRP enzymes and then oxidized by •O$_2$ and •OH radicals which were the intermediate radical products generated from process reaction between IM-HRP enzymes and H$_2$O$_2$, and eventually degraded into other byproducts. In conclusions, this work has developed a novel enzyme immobilization method which could effectively decrease the potential second pollution in the environment and increase the recovery of HRP enzymes further induce the cost of the industry applications. It is a very promising supporter to immobilize enzymes and for stable abatement of emerging organic contaminants from wastewater or drinking water.

Acknowledgement

This study was financially supported by National Natural Science Foundation of China (NO. 51478445, 21277130, 21477118, 51338010, 51678546 and 41630318), National Natural Science Foundation of Hubei province in China (ZRMS2016000811 and 2014CFA530) Chinese Universities Scientific Fund (CUG160824) and China Postdoctoral Science Foundation (2016MS90733).

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.chemosphere.2017.08.039.

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