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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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 ($\cdot\text{O}_2^-$) and hydroxyl radicals ($\cdot\text{OH}$) were identified in IM-HRP catalyzed H₂O₂ system.
- $\cdot\text{O}_2^-$ was the dominant active radicals responsible for E2 removal.
- Oxidative degradation of E2 was inhibited in presence of humic substances.

GRAPHICAL ABSTRACT

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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 $\cdot\text{O}_2^-$ and $\cdot\text{OH}$ were detected in IM-HRP catalyzed H₂O₂ system and radicals quenching test indicated $\cdot\text{O}_2^-$ played a more important role in estrogens removal. IM-HRP exhibited excellent stability and E2 removal efficiency could reach 45.41% after use

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seven times. Therefore, HRP enzymes immobilized on $\text{NH}_2\text{-Fe}_3\text{O}_4\text{/SiO}_2$ 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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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 concerns long-term impacts for human exposure to these chemicals, 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 $1\text{--}10\text{ ng}\cdot\text{L}^{-1}$ 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 activated carbon adsorption treatment (Ternes et al., 2002) and removal efficiency was considerably reduced due to competitive effects for active sites in the presence of others hydrophobic natural organic matters (NOMs) (Fukuhara et al., 2006), such as humic and protein-like substances (Zhang and Zhou, 2005). Likewise, chemical advanced oxidation processes are efficient in a wide varieties organic contaminants removing such as EDCs and pharmaceuticals (Klavarioti et al., 2009; Pera-Titus et al., 2004) but they are also limited by the competitive radical removal as matrix of the treated water (Zwiener and Frimmel, 2000). In addition, previous study discovered that EDCs can also be removed by wastewater treatment and the majority of EDCs in wastewater treatment is regarded as reduced by biodegradation (Liu et al., 2009). However, since EDCs are very refractory to biodegradation, traditional biological treatment showed a limited effect on an acceptable level with the respect to the estrogens concentration (Auriol et al., 2006a, b).

Enzymatic degradation has been reported to be an environmentally friendly and promising method for removing phenolic compounds and EDCs compounds from water, eg. phenolics or estrogens were effectively oxidized in wastewater by horseradish peroxidase (HRP) (Auriol et al., 2006a, b; Karam and Nicell, 1997; Bilal et al., 2017a, b, c, d). As an effective biocatalyst, HRP has many advantages of substrate specificity, high catalytic efficiency

and good biodegradability. In addition, the estrogens removal could also be achieved by the combination of enzymatic and non-enzymatic process (Auriol et al., 2006a, b). Byproducts of enzymatic treatment were able to polymerize through a non-enzymatic process and converted into higher molecular weight product of low solubility, which could be easily separated and removed from waste-water by using filtration, co-precipitation or sedimentation (Karam and Nicell, 1997).

However, it is well known free HRP enzymes were subject to water quality conditions (Wang et al., 2015a, b). Especially, their original proteins structure might be destroyed and lose its catalytic function in adverse conditions (Kim et al., 2012). In addition, free enzymes might also cause secondary organic pollution since they are difficult to be separated from treated water. Hence, it is necessary to improve its stability and recovery capacity in order to amplify the full-scale application of enzymatic treatment of water and wastewater. Studies on improvement of enzyme stability through mutagenesis is shown to be an effective method which performed a broader pH range and higher stability than native enzymes but it has the disadvantages of poor recovery (Asgher et al., 2016). However immobilization of HRP on insoluble organic or inorganic carriers was a very effective way to achieve these goals (Bilal et al., 2017a, b, c, d). For example, Kim et al. reported that 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 Fe_3O_4 -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 Fe_3O_4 particles which were coated by (3-Aminopropyl) triethoxysilane (APTs) modified SiO_2 . In order to successfully immobilize HRP on the surface Fe_3O_4 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 -NH_2 groups on the surface of Fe_3O_4 to bind free HRP enzymes. Then, the IM-HRP particles were characterized by XRD, FT-IR, TGA and TEM. The immobilized HRP (IM-HRP) was utilized for catalytic degradation of 17β -estrogens (E2) from aqueous solution. In addition, the effects of reaction conditions (time, H_2O_2 dosage, HRP dosage, pH and NOMs) on E2 removal were comparatively examined in free HRP or IM-HRP catalyzed H_2O_2 systems. Meanwhile, the active radicals responsible for E2 removal were

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 $\text{NH}_2\text{-Fe}_3\text{O}_4\text{@SiO}_2$ and $\text{HRP/NH}_2\text{-Fe}_3\text{O}_4\text{@SiO}_2$

2.2.1. Synthesis of Fe_3O_4 , $\text{Fe}_3\text{O}_4\text{@SiO}_2$ and APTS-Modified $\text{Fe}_3\text{O}_4\text{@SiO}_2$ microspheres

Fe_3O_4 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 $\text{Fe}_3\text{O}_4\text{@SiO}_2$ microspheres were prepared through a traditional solution sol-gel method and followed with modified by (3-Aminopropyl) triethoxysilane (APTS) (see [Section 1.2.2 of Supporting Information](#)).

2.2.2. Immobilization of HRP on APTS-Modified $\text{SiO}_2\text{@Fe}_3\text{O}_4$ particles via crosslinking method

Enzyme activity of immobilized and free HRP enzymes was measured with the method reported by Klivanov et al (Klivanov, 2001; Ai et al., 2016). (see [Section 1.2.4 of Supporting Information](#)). The preparation procedure of immobilized HRP on APTS-Modified $\text{SiO}_2\text{@Fe}_3\text{O}_4$ particles was presented in details in [Section 1.2.3 of Supporting Information](#).

2.3. Characterization of Fe_3O_4 , $\text{SiO}_2\text{@Fe}_3\text{O}_4$, $\text{NH}_2\text{-SiO}_2\text{@Fe}_3\text{O}_4$ and $\text{HRP/NH}_2\text{-SiO}_2\text{@Fe}_3\text{O}_4$ microspheres

Fe_3O_4 , $\text{SiO}_2\text{@Fe}_3\text{O}_4$, $\text{NH}_2\text{-SiO}_2\text{@Fe}_3\text{O}_4$ and $\text{HRP/NH}_2\text{-SiO}_2\text{@Fe}_3\text{O}_4$ microspheres were characterized by powder X ray diffraction (XRD), fourier transform infrared spectroscopy (FT-IR), thermo gravimetric analysis (TGA) and transmission electron microscopy (SEM), 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 in [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 an 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 Fe_3O_4 , $\text{SiO}_2\text{-Fe}_3\text{O}_4$ and $\text{SiO}_2\text{@NH}_2\text{-Fe}_3\text{O}_4$ characterization

The XRD pattern of Fe_3O_4 , $\text{SiO}_2\text{@Fe}_3\text{O}_4$ and $\text{NH}_2\text{-Fe}_3\text{O}_4$ microspheres showed the characteristics broad diffraction peaks indexed to the Fe_3O_4 nanoparticles (Deng et al., 2010). According to the XRD analysis as shown in [Fig. 1 \(1\)](#), the results exhibited the well-crystallized Fe_3O_4 structure with several different forms, including Fe_3O_4 , $\text{SiO}_2\text{@Fe}_3\text{O}_4$ and $\text{NH}_2\text{-Fe}_3\text{O}_4$. For the Fe_3O_4 , 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 $\text{SiO}_2\text{@Fe}_3\text{O}_4$ and $\text{NH}_2\text{-Fe}_3\text{O}_4$, which was in agreement with the work reported by Deng et al. (2010). The results showed that the structure of the Fe_3O_4 was not destroyed after SiO_2 encapsulation and APTS modification, indicating that its magnetic performance was not affected.

3.1.2. FT-IR of Fe_3O_4 , $\text{SiO}_2\text{@Fe}_3\text{O}_4$ and $\text{NH}_2\text{-Fe}_3\text{O}_4$ characterization

The Fe_3O_4 , $\text{SiO}_2\text{@Fe}_3\text{O}_4$ and $\text{NH}_2\text{-Fe}_3\text{O}_4$ nanoparticles were characterized by using FT-IR methods, as shown in [Fig. 1 \(2\)](#). For the $\text{SiO}_2\text{@Fe}_3\text{O}_4$, the peak at 590 cm^{-1} was related to the stretching vibrations of Fe–O bonds, which that confirmed the presence of Fe_3O_4 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 Fe_3O_4 particles during the encapsulation process, which was able to prevent Fe_3O_4 particles from oxidative destruction caused by oxygen. The high transmittance of bands 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 Fe_3O_4 was modified with APTS (Wang et al., 2015a, b), indicating the successful occurrence of the amination reaction between $\text{SiO}_2\text{@Fe}_3\text{O}_4$ and APTS.

3.1.3. TGA of Fe_3O_4 , $\text{SiO}_2\text{-Fe}_3\text{O}_4$ and $\text{NH}_2\text{-Fe}_3\text{O}_4$ characterization

The APTS-modified coating on the surface of Fe_3O_4 particles was also confirmed by TGA analysis. As depicted in [Fig. 1 \(3\)](#), it showed the changes of weight loss of samples (Fe_3O_4 , $\text{SiO}_2\text{@Fe}_3\text{O}_4$ and $\text{NH}_2\text{-Fe}_3\text{O}_4$) 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 $\text{SiO}_2\text{@Fe}_3\text{O}_4$ compared to Fe_3O_4 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 of both evaporation water and silicon compounds and decomposition of amino. Hence, according to the weight loss, the APTS-modified on the surface of Fe_3O_4 particles was calculated to be possibility around 5.89% of amino groups.

3.1.4. TEM of Fe_3O_4 and $\text{SiO}_2\text{-Fe}_3\text{O}_4$ characterization

TEM was used to observe the changes in morphological properties of Fe_3O_4 and $\text{SiO}_2\text{@Fe}_3\text{O}_4$. [Fig. 1 \(O\)](#) and [\(Os\)](#) illustrated the TEM of Fe_3O_4 and $\text{SiO}_2\text{@Fe}_3\text{O}_4$ microspheres, respectively. It can be clearly observed that the silicon was fully covered with Fe_3O_4 microspheres after being treated by TEOs. Since the Fe_3O_4 spheres were protected by SiO_2 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

Fig. 1. Powder X-ray diffraction (XRD) (1) XRD of Fe_3O_4 , $\text{SiO}_2@\text{Fe}_3\text{O}_4$ and $\text{NH}_2-\text{Fe}_3\text{O}_4$; (2) Fourier transform infrared spectroscopy (FT-IR) spectra of Fe_3O_4 , $\text{SiO}_2@\text{Fe}_3\text{O}_4$ and $\text{NH}_2-\text{Fe}_3\text{O}_4$; (3) Thermo-gravimetric Analysis (TGA) of Fe_3O_4 , $\text{SiO}_2@\text{Fe}_3\text{O}_4$ and $\text{NH}_2-\text{Fe}_3\text{O}_4$; (O/Os) Transmission electron microscopy (TEM) of Fe_3O_4 and $\text{SiO}_2@\text{Fe}_3\text{O}_4$.

$\text{SiO}_2@\text{Fe}_3\text{O}_4$ 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_2 layers could be formed on each magnetic Fe_3O_4 seeds, consequently resulting in generation of core shell $\text{SiO}_2@\text{Fe}_3\text{O}_4$ 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. 2. Effects of temperature and pH on immobilized/free HRP enzymes activity; (A) Effects temperature on enzymes activity; (B) Effects of pH on enzymes activity; IM-HRP enzymes concentration: 0.064 g/L (net content HRP to supporter: 12.5×103 mg/kg, 0.8 mg/L); free HRP enzymes concentration: 2×10^{-3} g/L.

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 $\text{NH}_2\text{-Fe}_3\text{O}_4$.

3.2. Effects of reaction conditions on E2 removal using IM-HRP catalyzed H_2O_2 systems

3.2.1. Effects of H_2O_2 dose on E2 removal

In this paper, experiments were carried out by choosing different H_2O_2 (6 g/L, 30%) dosage in a range of 1 mL–9 mL and the initial E2 concentration was adjusted to be 1000 $\mu\text{g/L}$. As depicted in Fig. 3 (b), the E2 removal efficiency reached the maximum at 80.85% when H_2O_2 (6 g/L, 30%) dosage was 24 mg/L, and the removal of E2 was decreased with further increase of H_2O_2 . It was clearly observed that addition of excessive H_2O_2 could lead to deterioration of substrate degradation in Fig. 3 (a). It was very likely that the addition of excessive of H_2O_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_2O_2 could prohibit the enzyme catalysis capacity (Karam and Nicell, 1997; Nicole et al., 1999; Nicell et al., 1995). In addition, H_2O_2 could act as scavengers of active radicals through reduction reactions. Hence, the optimal concentration of H_2O_2 of the HRP enzymes catalysis system was 24 mg/L.

3.2.2. Effects of molar ratio of IM-HRP to H_2O_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_2O_2 molar ratio as a function of initial E2 concentration (1000 $\mu\text{g/L}$ (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_2O_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 weakened after treated by IM-HRP/free-HRP in Fig. 6.

In addition, the gradual addition of H_2O_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_2O_2 molar ratio (IM-HRP/ H_2O_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_2O_2 was exceed to the optimum reaction ratio of IM-HRP/ H_2O_2 , the H_2O_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_2O_2 and the reached the maximum value with 0.552×10^{-3} of IM-HRP to H_2O_2 molar ratio (Cheng et al., 2006). However, when the ratio of IM-HRP/ H_2O_2 was over the optimum reaction ratio, the H_2O_2 was over dose to the enzyme catalytic reaction systems and the overdose of the H_2O_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_2O_2 must be limited as the inhibition to the enzymes activity by the excess quantities of H_2O_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_2O_2 could inhibit the enzyme catalysis performance (Wang et al., 2015a, b; Nicole et al., 1999). Hence high H_2O_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_2O_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_3O_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_2O_2 catalysts respectively. Free HRP enzymes performed better in E2 removal than IM-HRP enzymes, indicating that loading process resulted in

Fig. 3. (a) Estrogens (E2) removal efficiency as function of H_2O_2 (6 g/L, 30%) dosage; E2 concentration was 1000 μ g/L (500 mL); Reaction time was 90 min; IM-HRP (0.064 g/L (net content of HRP to the supporter: 12.5×103 mg/kg, 0.8 mg/L)) enzymes dosage was 5 mL; (b) Effect of NOM on estrogens removal efficiency as function of IM-HRP/ H_2O_2 molar ratio; E2 concentration was 1000 μ g/L (500 mL); IM-HRP (0.064 g/L (12.5×103 mg/kg, 0.8 mg/L)) enzymes; (c) Estrogens removal as a function of pH; E2 concentration was 1000 μ g/L (500 mL); Catalytic of reaction time was 120 min; IM-HRP (0.064 g/L (net content of HRP to the supporter: 12.5×103 mg/kg, 0.8 mg/L)) enzymes dosage was 5 mL; H_2O_2 (6 g/L, 30%) dosage was 2 mL; (d) Estrogens removal efficiency as a function of reaction time; E2 concentration was 1000 μ g/L (500 mL); IM-HRP (0.064 g/L (net content HRP to supporter: 12.5×103 mg/kg, 0.8 mg/L)) enzymes dosage was 5 mL; free-HRP concentration: 2×10^{-3} g/L; H_2O_2 (6 g/L, 30%) dosage was 2 mL.

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 H_2O_2 concentration in free-HRP catalytic system, while concentrations of both substrate and H_2O_2 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 Fe_3O_4 .

3.3. Identification of active oxidants for E2 removal in IM-HRP catalyzed H_2O_2 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 H_2O_2 . As depicted in Fig. 4, there is no DMPO-OH or DMPO- O_2^- spin adducts were detected in H_2O_2 or CH_3OH system as shown in (a), (b), (c) and (d). While free-HRP/IM-HRP with H_2O_2 reacted

under H_2O system, characteristic peaks of DMPO-OH adducts were obviously observed as shown in (b), though no obvious peaks of DMPO- O_2^- adducts were detected. This is likely that $\cdot O_2^-$ is unstable in aqueous solution and rapidly easily decomposed into $\cdot OH$, and the reaction rate of DMPO with $\cdot OH$ was much faster than that of $\cdot O_2^-$ radicals (Yin et al., 2009). Hence, it was difficult to detect the DMPO- O_2^- peaks, though it was formed in the reaction process (Yin et al., 2009). In order to further verify whether $\cdot O_2^-$ is formed during the reaction process between free-HRP/IM-HRP with H_2O_2 , CH_3OH instead of H_2O was used to slow down the decomposition rate $\cdot O_2^-$. As shown in Fig. 4, there was DMPO-OH or DMPO- O_2^- spin adducts was shown in (a) and (b) when free-HRP/IM-HRP was reacted with H_2O_2 in CH_3OH . Meanwhile, when the estrogens presented in the system of free-HRP/IM-HRP and H_2O_2 , both peaks of DMPO- O_2^- 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 $\cdot O_2^-$ 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 $\cdot OH$ and $\cdot O_2^-$ to E2 removal. Specifically, the methonal was able to scavenge $\cdot OH$ rather than $\cdot O_2^-$ (Amyl et al., 2001), while tertiary butanol could eliminate both $\cdot OH$ and $\cdot O_2^-$ (Paulson et al., 1999; Michael et al., 1999). As depicted in Fig. 5 (a), the $SiO_2@Fe_3O_4$ nano-particles had very limited effects on E2 removal, indicating E2 adsorption on particles could be neglected. There was no obvious change in E2 concentration in H_2O_2 system, confirmed the radicals produced from enzyme catalyzed H_2O_2 rather than H_2O_2 played crucial roles in E2 degradation. In addition,

Fig. 4. DMPO spin-trapping electron paramagnetic resonance (EPR) spectrum and computer simulation obtained from the reaction of HRP with H_2O_2 . (a) An EPR spectrum obtained from a mixture reaction containing DMPO (110.46 mM), free HRP (1.5 mg/L) and H_2O_2 in the system of H_2O /Estrogens/methyl-alcohol; (b) An EPR spectrum obtained from a mixture reaction containing DMPO (110.46 mM), free-HRP (4.5 mg/L) or IM-HRP (32 mg/L) and H_2O_2 (0.15 g/L) under H_2O ; (c) An EPR spectrum obtained from a mixture reaction containing DMPO (110.46 mM), free-HRP (4.5 mg/L) or IM-HRP (32 mg/L) and H_2O_2 (0.15 g/L) in the system of methyl-alcohol; (d) An EPR spectrum obtained from a mixture reaction containing DMPO (110.46 mM), free-HRP (4.5 mg/L) or IM-HRP (32 mg/L) and H_2O_2 (0.15 g/L) in the system of estrogens (0.25 mg/L).

Fig. 5. E2 removal in different systems (A) Enzyme-catalytic reaction performed in H_2O system; (B) Enzyme-catalytic reaction performed in methanol solution; (C) E2 degradation efficiency by $\text{SiO}_2@\text{Fe}_3\text{O}_4$ particles; (D) Enzyme-catalytic reaction performed in tertiary butanol solution; the concentration of E2 concentration was 1000 $\mu\text{g/L}$ (500 mL).

the E2 was decreased from 80% to 72% and 12% of HRP catalyzed system in methanol and tertiary butanol solutions in Fig. 5 (b). The results demonstrated that the $\cdot\text{O}_2^-$ 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 H_2O_2

The cycle of E2 removal efficiency from aqueous solution by IM-HRP with H_2O_2 could be summarized as follows: At first, estrogens (E2) and H_2O_2 molecules were adsorbed to the surface of the

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.

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 summarized 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. S5 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• and •OH to E2 undergoing different enzymatic degradation pathway. When RO• 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

enzymes with H_2O_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 ($\lambda_{\text{Ex/Em}} = 285/320$) 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 ($\lambda_{\text{Ex/Em}} = 340/420$) 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_2O_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_3O_4 or lower of the IM-HRP catalytic capacity after the application. In addition, in the

Fig. 7. Reusability of IM-HRP enzymes; E2 concentration was 1000 $\mu\text{g/L}$ (500 mL); Catalytic of reaction time was 180 min; IM-HRP (0.064 g/L (12.5 $\times$ 103 mg/kg, 0.8 mg/L)) enzymes dosage was 5 mL; H_2O_2 (6 g/L, 30%) dosage was 2 mL.

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 $\text{SiO}_2/\text{Fe}_3\text{O}_4$ nano-particles, and its catalytic capacity were evaluated by considering different reaction conditions. The experiment results revealed the immobilize 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_2O_2), 77% of E2 was removed under the optimal reaction system. In addition, ESR analysis demonstrated that active radicals ($\cdot\text{O}_2^-$ and $\cdot\text{OH}$) were responsible for E2 removal in IM-HRP enzymes catalyzed H_2O_2 system, $\cdot\text{O}_2^-$ played a more important role in E2 removal than $\cdot\text{OH}$. 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 $\cdot\text{O}_2^-$ and $\cdot\text{OH}$ radicals which were the intermediate radical products generated from process reaction between IM-HRP enzymes and H_2O_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.

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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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