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Fe₃O₄ Nanoparticles Loaded on Lignin Nanoparticles Applied as a Peroxidase Mimic for the Sensitively Colorimetric Detection of H₂O₂

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Abstract: Lignin is the second largest naturally renewable resource and is primarily a by-product of the pulp and paper industry; however, its inefficient use presents a challenge. In this work, Fe₃O₄ nanoparticles loaded on lignin nanoparticles (Fe₃O₄@LNPs) were prepared by the self-assembly method and it possessed an enhanced peroxidase-like activity. Fe₃O₄@LNPs catalyzed the oxidation of 3,3′,5,5′-tetramethylbenzidine (TMB) in the presence of H₂O₂ to generate a blue color, was observable by the naked eye. Under the optimal conditions, Fe₃O₄@LNPs showed the ability of sensitive colorimetric detection of H₂O₂ within a range of 5–100 µM and the limit of detection was 2 µM. The high catalytic activity of Fe₃O₄@LNPs allows its prospective use in a wide variety of applications, including clinical diagnosis, food safety, and environmental monitoring.

Keywords: Fe₃O₄ nanoparticles; Lignin nanoparticles; peroxidase mimic; colorimetric; H₂O₂ detection

1. Introduction

Hydrogen peroxide (H₂O₂), as one of the most important reactive oxygen species (ROS), is widely used in the food and chemical industries, clinical medicine, and environmental monitoring [1–3]. Studies have shown that long-term exposure to H₂O₂ can cause serious harm to a person’s health, including poisoning, respiratory inflammation [4], cancer, Alzheimer’s disease, and Parkinson’s disease [5]. Therefore, the substantial research efforts are devoted to develop methods for the detection of H₂O₂ such as high-performance liquid chromatography (HPLC) [6], electrochemistry [1], spectrometry [7], chemiluminescence [8], and colorimetric assays [9]. HPLC, electrochemistry, and chemiluminescence methods are sensitive, but their processes are complex and time-consuming. Spectrometry has the advantage of a simple and low-cost operation but exhibits the disadvantage of poor sensitivity. At present, colorimetric assay methods have been garnering attention due to their high sensitivity and simplicity. Furthermore, colorimetric assay results can be observed unaided and without the use of any sophisticated instruments. Horseradish peroxidase (HRP) is commonly used as a catalyst in conventional colorimetric assay methods to obtain the color signal. As a natural enzyme, HRP is easily affected by acidity, temperature, and inhibitors. In addition, the high cost associated with its preparation, purification and extreme storage conditions also inevitably limits its widespread application [10].
Since the first report of Fe$_3$O$_4$ nanoparticles (Fe$_3$O$_4$ NPs) possessing peroxidase-like activity [11], many other nanomaterials, including CeO$_2$ nanoparticles [12], NiO$_2$ nanoparticles [13] and MnO$_2$ nanosheets [14], have also demonstrated intrinsic peroxidase-like activity. These metal nanomaterials are low-cost, sensitive, and maintain their activities in a relatively wide range of environmental conditions, but they all present the problem of agglomeration [15]. To tackle this problem, researchers use graphene or graphene oxide as a structural support to prevent these metal nanomaterials from aggregating in solution [16,17]. However, the high cost of graphene and graphene oxide seriously restricts the widespread application of metal nanozymes.

Lignin is the second most abundant organic macromolecular compound from biomass after cellulose [18]. The primary structure of lignin is composed of three different phenylpropane monomer units: guaiacyl (G), syringyl (S), and p-hydroxyphenol (H) units [19]. These monomer units are mainly linked by ether bonds (β-4, 4-O-5, α-O-4, α-O-γ, etc.) and carbon-carbon bonds (β-β, β-1, β-5, 5-5, β-6, etc.) [20,21]. A proposed structural diagram of lignin is shown in Figure 1. Similar to graphene oxide, lignin molecules contain many functional groups, such as hydroxyl and carboxyl groups, which offers the possibility of a stable dispersion of lignin-based materials in aqueous solutions. Recent studies showed that lignin-based nanoparticles can disperse stably in aqueous solutions [18]. Therefore, metal nanozymes carried by lignin nanoparticles can be stably dispersed in solution. Nevertheless, the literature contains few reports of lignin nanoparticles being used to enhance the dispersity of metal nanozymes in the colorimetric detection of H$_2$O$_2$.

![Figure 1. The proposed structure of lignin. Inset: three primary units of lignin.](image)

As a common mimetic enzyme of horseradish peroxidase, Fe$_3$O$_4$ NPs have high catalytic activity and stability during reactions [22]. However, Fe$_3$O$_4$ NPs also present the urgent problem of agglomeration in solution. In this work, we explored the influence of the preparation conditions on the morphology of Fe$_3$O$_4$@LNPs. Then, Fe$_3$O$_4$ NPs loaded on lignin nanoparticles (Fe$_3$O$_4$@LNPs) were prepared. The prepared Fe$_3$O$_4$@LNPs exhibited excellent dispersion stability in water and showed higher catalytic activity than Fe$_3$O$_4$ NPs. The prepared Fe$_3$O$_4$@LNPs effectively catalyzed H$_2$O$_2$ to generate hydroxyl radicals, followed by the oxidation of 3,3',5,5'-tetramethylbenzidine (TMB) [23]. The oxidized TMB had a characteristic absorbance at 652 nm [24]. Therefore, in the presence of H$_2$O$_2$, TMB, and Fe$_3$O$_4$@LNPs as catalyst, the reaction system changed from colorless to blue that was identified by the naked eye or quantified by UV-vis spectrophotometer. Because of its excellent dispersion stability in aqueous solution and sensitive peroxidase-like catalytic activity, Fe$_3$O$_4$@LNPs can be used in a diverse set of applications.
2. Experimental

2.1. Materials and Reagents

Spruce chips were obtained from a Swedish farm. Tetrahydrofuran (THF), Fe$_3$O$_4$ NPs (average diameters: 20–30 nm), and TMB were purchased from Aladdin Industrial Co., Ltd. (Shanghai, China). The other analytical grade chemicals were purchased from Boyu Chemicals Company (Nanning, Guangxi, China), and were used without further purification.

The chips were cooked in a pressure vessel under the following conditions: the total titratable alkali value was 26%, the sulfidity was 20%, the solid-to-liquid ratio was 1:4, a residence time was 4.5 h, and the temperature was 165 °C. When the cooking was completed, the separated black liquor was adjusted to pH = 2 with 2 M H$_2$SO$_4$ while stirring to precipitate lignin. After standing for 3 h, the supernatant was removed. After centrifugation, washing, and freeze-drying, the crude Kraft lignin was obtained. The crude Kraft lignin was dissolved in THF and stirred for 30 min; the undissolved material was removed by filtering through a 0.45 µm organic filter membrane (Pall Scientific, New York, NY, USA). The lignin/THF solution was stirred under the fume hood until the THF evaporated completely. Then, the powder was dried at 40 °C under vacuum for 48 h to obtain the purified Kraft lignin.

2.2. Statistical Analysis

All the experiments were performed in triplicate, and the averaged values were reported in this study.

2.3. Preparation of Fe$_3$O$_4$@LNPs

The purified lignin was dissolved in THF to prepare solutions of 0.5, 1.0, and 5.0, mg/mL. With vigorous mechanical stirring, 0.1 mg/mL Fe$_3$O$_4$ NPs suspension was added dropwise to the lignin/THF solutions using a microsyringe until the suspension with 20% consistency was obtained. The suspension was stirred for an additional 20 min to evaporate THF. Then, the suspension was transferred into a dialysis bag (MWCO: 6000, Union Carbide, Connecticut, CT, USA) and immersed in an excess of deionized water to remove the residual THF. Little trace of THF was detected after 24 h dialysis. Then, the suspension was centrifuged to achieve the solid part which was freeze-dried to obtain Fe$_3$O$_4$@LNPs. Fe$_3$O$_4$@LNPs prepared from the solution with 0.5, 1.0, and 5.0 mg/mL initial lignin concentration was respectively denoted as L$_1$, L$_2$, and L$_3$.

2.4. Peroxidase-Like Activity of Fe$_3$O$_4$@LNPs

The peroxidase-like activity of Fe$_3$O$_4$@LNPs was investigated by catalytically oxidizing TMB in the presence of H$_2$O$_2$. The concentrations of TMB and H$_2$O$_2$ were respectively 4 mM and 100 mM, 0.1 M Citric acid-disodium hydrogen phosphate solution (CPBS) (pH = 3.0) was applied as the buffer, and the final volume of the mixture was 3 mL. The mixture was incubated at 50 °C for 30 min. As for the blank experiments, Fe$_3$O$_4$@LNPs was replaced by LNPs (lignin nanoparticles) or Fe$_3$O$_4$ NPs to complete the reaction under the same conditions. The oxidized TMB had a maximum absorbance at 652 nm [25]. Therefore, the peroxidase-like activity of Fe$_3$O$_4$@LNPs was evaluated through changes of the solution color and the absorbance at 652 nm.

2.5. Steady Kinetic Analysis of Fe$_3$O$_4$@LNPs

The steady-state kinetic measurements were carried out to investigate the mechanism of the peroxidase-like activity of Fe$_3$O$_4$@LNPs at room temperature. Approximately 600 µL of Fe$_3$O$_4$@LNPs (264 µg/mL) was mixed with 1.8 mL CPBS (0.1 M, pH = 3.0). As for the first experiment, 300 µL H$_2$O$_2$ (100 mM) was used, while the TMB concentration was varied (0.4, 0.6, 0.8, 1.0, 1.5, 2.0, 3.0, 4.0 and 6.0 mM). As for the second experiment, 300 µL TMB (4.0 mM) was used, while the
concentration of H$_2$O$_2$ was varied (10, 15, 25, 30, 50, 100, 150, and 250 mM). The Michaelis-Menten constant was calculated from the Lineweaver-Burk double reciprocal plot by the following equation:

$$\frac{1}{v} = \frac{K_m}{v_m} \left( \frac{1}{[S]} + \frac{1}{K_m} \right),$$

where $v$ is the initial velocity, $K_m$ is the Michaelis constant, $v_m$ is the maximum reaction rate, and $[S]$ is the concentration of substrate [26].

2.6. Colorimetric Detection of H$_2$O$_2$

For the colorimetric analysis of H$_2$O$_2$, 600 µL Fe$_3$O$_4$@LNPs (264 µg/mL), 300 µL of TMB (4 mM) and 300 µL H$_2$O$_2$ (different concentrations) were mixed with 1.8 mL CPBS (0.1 M, pH = 3.0). The mixed solution was incubated at 50 °C for 30 min. The absorbance of the resulted sample measured by a UV-visible spectrophotometer at 652 nm was used to generate the standard curve for the detection of H$_2$O$_2$.

The limit of detection (LOD) of H$_2$O$_2$ was calculated based on previous literature [27] via the following equation: LOD = 3S/M, where $S$ is the standard deviation of blank samples and $M$ is the slope of the linear curve between the absorbance at 652 nm and H$_2$O$_2$ concentration. Eleven blank experiments were carried out as follows: 600 µL Fe$_3$O$_4$@LNPs (264 µg/mL), 300 µL of TMB (4 mM) and the corresponding volume (300 µL) of H$_2$O replacing H$_2$O$_2$ were mixed with 1.8 mL CPBS (0.1 M, pH = 3.0). The mixtures were incubated at 50 °C for 30 min. The absorbance of 11 blank experiments at 652 nm was collected to calculate $S$ value.

2.7. Characterization

The $^{31}$P nuclear magnetic resonance (NMR) spectrum of lignin was acquired on an NMR spectrometer (AVANCE III 500 MHz, Bruker, Germany). The morphologies and element analysis of Fe$_3$O$_4$@LNPs were recorded by field emission transmission electron microscope (FE-TEM) (Tecnai G2F20, FEI Company, Hillsboro, OR, USA) with an accelerating voltage of 200 KV. UV-vis spectra were recorded with a UV-visible spectrophotometer (Agilent 8453, California, CA, USA). The size and zeta potential of the samples were analyzed with a Nanoparticle Size analyzer (Nano-ZS90X, Malvern, Malvern, UK).

3. Results and Discussion

3.1. Quantification of Hydroxyl Groups of Lignin

The hydroxyl groups of lignin was quantified by $^{31}$P NMR according to the reported method [28]. Approximately 20 mg of lignin was placed in a 1 mL vial. Then, 500 µL of a prepared solution of anhydrous pyridine and CDCl$_3$ (1.6:1, v/v) was added and magnetically stirred. Subsequently, 100 µL of chromium (III) acetylacetonate (5 mg/mL, dissolved in pyridine/CDCl$_3$, 1.6:1, v/v) was added as a relaxation agent and 100 µL of cyclohexanol (30 mg/mL) was added as an internal standard. Finally, 85 µL of phosphitylation reagents was added. After stirring for 10 min, the resulting solution was transferred to a 5 mm NMR tube and tested by NMR spectrometer. The $^{31}$P NMR spectrum of lignin was shown in Figure 2. The result indicated that the hydroxyl group of guaiacyl was the most abundant in the lignin that is 3.76 mmol/g based on lignin, followed by the aliphatic hydroxyl that was 2.82 mmol/g, the condensed phenolic hydroxyl that was 1.90 mmol/g, and the carboxyl group that was 0.63 mmol/g. More hydroxyl groups indicated the lignin nanoparticles dispersed more stably in water [18].
3.2. Optimization of Fe₃O₄@LNPs Formation

The lignin hardly solubilized in water but dissolved in some organic solvents (e.g., THF). Therefore, Fe₃O₄NPs suspension used as the anti-solvent and THF used as the solvent were applied to prepare Fe₃O₄@LNPs through the lignin self-assembly. The initial concentration of lignin played a vital role in the morphology of lignin nanoparticles [29]. Figure 3 shows that Fe₃O₄@LNPs exhibited spherical shape. However, the size of Fe₃O₄@LNPs and its distribution varied with the initial concentration of lignin. Compared to L₁ prepared from 0.5 mg/mL lignin solution, L₂ and L₃ respectively prepared from 1.0 mg/mL and 5.0 mg/mL were more homogenous. Furthermore, L₂ had the highest load of Fe₃O₄ NPs.

The effect of lignin concentrations on the size and the size distribution of Fe₃O₄@LNPs were analyzed by dynamic light scattering (DLS). Figure 4 shows L₂ possessed a narrow size distribution with an average size of 152.8 nm. Compared to L₂, L₁ possessed a wide size distribution with an average size of 181.8 nm, and L₃ possessed a wide size distribution with an average size of 764.0 nm. The result indicated that 1.0 mg/mL was the optimum initial concentration of lignin for Fe₃O₄@LNPs preparation.
which comprises the “nucleation growth mechanism” [18]. Figure 3 demonstrates that the morphology of LNPs is affected by the hydrophobic surface of LNPs, resulting in the generation of strong negative zeta potential on the surface of Fe3O4@LNPs. Therefore, Fe3O4 NPs loaded on LNPs can stabilize Fe3O4 NPs and prevent the aggregation of Fe3O4 NPs in solution.

In the process of adding water droplets to the lignin/THF solution, lignin as an amphiphilic polymer, with its hydrophilic and hydrophobic properties, gradually aggregates and forms particles, which comprises the “nucleation growth mechanism” [18]. Figure 3 demonstrates that the morphology of the formed nanoparticle was impacted by the initial concentration of lignin. For example, 0.5 mg/mL lignin was applied, the average diameter of L1 was only 181.8 nm but the size distribution was wide. The amount of lignin hardly meets the demand for the nanoparticles growth, resulting in the heterogeneous Fe3O4@LNPs (Figure 3A). However, when the high initial lignin concentration was applied, the extra lignin continued to aggregate on the surface of the formed nanoparticles, eventually leading to the large size Fe3O4@LNPs (e.g., L3) (Figure 3C). The homogeneous morphology of L2 indicated that 1.0 mg/mL was the optimum concentration for Fe3O4@LNPs preparation (Figure 3B). The result was consistent with the previous report [30].

The surface charge of Fe3O4@LNPs was evaluated by the zeta potential. The results indicated that L1, L2, and L3 possessed comparable negative zeta potentials of −29.2 mV, −32.4 mV and −32.4 mV, respectively, indicating that the initial lignin concentration had little effects on the surface charge of Fe3O4@LNPs. Therefore, L1, L2, and L3 dispersed stably in aqueous solutions for an extended time without aggregation due to the electrical double layer repulsion induced from the protonation of phenolic hydroxyl and carboxyl groups of lignin. The comparable result was reported that LNPs dispersed stably in water for two months [18]. Based on the above results, L2 was applied as the starting material for the further analysis in this study.

3.3. Characterization of Fe3O4@LNPs

Fe3O4@LNPs surface possessed the negative charge which was explained as follows. The abundant hydroxyl and carboxyl groups of LNPs provide Fe3O4@LNPs surface a negative charge. In addition, when the hydrophobic surface of LNPs contacts with water, the hydroxyl ions are absorbed by the hydrophobic surface of LNPs, resulting the generation of strong negative zeta potential on the surface of Fe3O4@LNPs [18]. Therefore, Fe3O4 NPs loaded on LNPs can stabilize Fe3O4 NPs and prevent the aggregation of Fe3O4 NPs in solution.

Figure 4. Size distribution of Fe3O4@LNPs prepared by (a) L1: 0.5 mg/mL, (b) L2: 1.0 mg/mL, and (c) L3: 5mg/mL lignin.
The high resolution transmission electron microscope (HRTEM) image of individual \( \text{Fe}_3\text{O}_4 \@\text{LNPs} \) confirmed that \( \text{Fe}_3\text{O}_4 \) NPs was loaded on the surface of LNPs (Figure 5A). The high-angle annular dark field (HAADF) image demonstrated the morphology of individual \( \text{Fe}_3\text{O}_4 \@\text{LNPs} \), where C and Fe element respectively stands for LNPs and \( \text{Fe}_3\text{O}_4 \) NPs (Figure 5B, C). Thus, the HAADF and element mapping analysis confirmed the formation of \( \text{Fe}_3\text{O}_4 \@\text{LNPs} \). The correlation of the size distribution of \( \text{Fe}_3\text{O}_4 \@\text{LNPs} \) and pH was investigated by DLS (Figure 6B). The result indicated that \( \text{Fe}_3\text{O}_4 \@\text{LNPs} \) had a narrow size distribution within pH from 3.0 to 10.5, confirming that \( \text{Fe}_3\text{O}_4 \@\text{LNPs} \) can stably disperse in aqueous solution with a wide pH range. This considerable dispersion ability of \( \text{Fe}_3\text{O}_4 \@\text{LNPs} \) was correlated with its zeta potential (approximately \(-30 \text{ mV}\)) in solution within pH 3.0–10.5 (Figure 6C). When pH was below 3.0, the decrease of zeta potential induced the aggregation of \( \text{Fe}_3\text{O}_4 \@\text{LNPs} \). On the other hand, when pH was above 11, the surface charge decreased significantly which caused the aggregation of \( \text{Fe}_3\text{O}_4 \@\text{LNPs} \) enlarging the average diameter of nanoparticles. The surface charge of \( \text{Fe}_3\text{O}_4 \@\text{LNPs} \) decreased with the increased concentration of \( \text{Na}^+ \) counter ions associated with the addition of NaOH for the pH adjustment. The result was consistent to the reported result on the dispersion stability of polymer nanoparticles correlated with the pH change [18,31].

![Figure 5](image_url)

**Figure 5.** (A) HRTEM image of individual \( \text{Fe}_3\text{O}_4 \@\text{LNPs} \); (B) High-angle annular dark field image (HAADF) and (C) element mapping analysis of \( \text{Fe}_3\text{O}_4 \@\text{LNPs} \).
where R was the relative activity; A was the absorbance of the solution at 652 nm; and \( A_{\text{max}} \) was set as 100%, and the relative activities were calculated by the equation: \( R = \left( \frac{A}{A_{\text{max}}} \right) \times 100\% \), where R was the relative activity; A was the absorbance of the solution at 652 nm; and \( A_{\text{max}} \) was the maximum absorbance at 652 nm. The impacts of reaction time, pH, and temperature on the activity of \( \text{Fe}_3\text{O}_4@\text{LNPs} \) were demonstrated in Figure 7. The correlation between the reactivity of \( \text{Fe}_3\text{O}_4@\text{LNPs} \) and time was demonstrated in Figure 7A. It was shown that the activity of \( \text{Fe}_3\text{O}_4@\text{LNPs} \) increased to the maximum within 30 min. Thus, 30 min was set as the optimal reaction time for the following investigations. Within pH = 2.0–4.5, the \( \text{Fe}_3\text{O}_4@\text{LNPs} \) possessed a high and stable activity and achieved the maximum activity at pH = 3.0 (Figure 7B) that was contributed to solubilization of TMB in the acidic medium [32]. The comparable peroxidase-like activity were reported from HRP and other metal nanoenzymes [10,25]. The catalytic activity was significantly decreased when \( \text{Fe}_3\text{O}_4@\text{LNPs} \) was applied without the optimal pH (Figure 7B) because of the inherent defect of Fenton reaction [33]. It was reported that the catalytic activity of \( \text{Fe}_3\text{O}_4@\text{LNPs} \) loaded on multi-welled carbon nanotubes retained from pH 1–10 [33]; however the high cost of multi-welled carbon nanotubes limited its application. The catalytic activity of \( \text{Fe}_3\text{O}_4@\text{LNPs} \) was also impacted by temperature, and the maximum activity was achieved at 50 °C (Figure 7C). Compared to natural enzymes and \( \text{Fe}_3\text{O}_4 \)

Figure 6. Effects of pH on (A) average size of \( \text{Fe}_3\text{O}_4@\text{LNPs} \), (B) size distribution of \( \text{Fe}_3\text{O}_4@\text{LNPs} \), and (C) zeta potential value of \( \text{Fe}_3\text{O}_4@\text{LNPs} \).

3.4. Optimizing the Reaction Conditions of \( \text{H}_2\text{O}_2 \) and TMB Catalyzed by \( \text{Fe}_3\text{O}_4@\text{LNPs} \)

To improve the catalytic activity of \( \text{Fe}_3\text{O}_4@\text{LNPs} \), the main parameters, e.g., time, pH, and temperature, were optimized. The experiment was performed as following 600 µL \( \text{Fe}_3\text{O}_4@\text{LNPs} \) (264 µg/mL), 300 µL TMB (4 mM) and 300 µL \( \text{H}_2\text{O}_2 \) (100 mM) were applied as reagents in 1.8 mL CPBS buffer solution (0.1 M). The relative activity of \( \text{Fe}_3\text{O}_4@\text{LNPs} \) with different pH, time and temperature were defined by follows: the maximum absorbance of the solution after reaction at 652 nm was set as 100%, and the relative activities were calculated by the equation: \( R = \left( \frac{A}{A_{\text{max}}} \right) \times 100\% \), where R was the relative activity; A was the absorbance of the solution at 652 nm; and \( A_{\text{max}} \) was the maximum absorbance at 652 nm. The impacts of reaction time, pH, and temperature on the activity of \( \text{Fe}_3\text{O}_4@\text{LNPs} \) were demonstrated in Figure 7. The correlation between the reactivity of \( \text{Fe}_3\text{O}_4@\text{LNPs} \) and time was demonstrated in Figure 7A. It was shown that the activity of \( \text{Fe}_3\text{O}_4@\text{LNPs} \) increased to the maximum within 30 min. Thus, 30 min was set as the optimal reaction time for the following investigations. Within pH = 2.0–4.5, the \( \text{Fe}_3\text{O}_4@\text{LNPs} \) possessed a high and stable activity and achieved the maximum activity at pH = 3.0 (Figure 7B) that was contributed to solubilization of TMB in the acidic medium [32]. The comparable peroxidase-like activity were reported from HRP and other metal nanoenzymes [10,25]. The catalytic activity was significantly decreased when \( \text{Fe}_3\text{O}_4@\text{LNPs} \) was applied without the optimal pH (Figure 7B) because of the inherent defect of Fenton reaction [33]. It was reported that the catalytic activity of \( \text{Fe}_3\text{O}_4@\text{LNPs} \) loaded on multi-welled carbon nanotubes retained from pH 1–10 [33]; however the high cost of multi-welled carbon nanotubes limited its application. The catalytic activity of \( \text{Fe}_3\text{O}_4@\text{LNPs} \) was also impacted by temperature, and the maximum activity was achieved at 50 °C (Figure 7C). Compared to natural enzymes and \( \text{Fe}_3\text{O}_4 \)
nanozymes [11], Fe$_3$O$_4$@LNPs retained the catalytic activity within a wide temperature range from 30 °C to 60 °C because of its considerable dispersion ability in the aqueous solution.

![Figure 7](image-url)

**Figure 7.** The catalytic activity of Fe$_3$O$_4$@LNPscorrelated with time (A), pH (B), and temperature (C).

3.5. Peroxidase-liked Activity of Fe$_3$O$_4$@LNPs

The peroxidase-liked activity of Fe$_3$O$_4$@LNPs was confirmed and evaluated by the reaction between TMB and H$_2$O$_2$ under optimal conditions. Little color changes and absorbance peaks at 652 nm were observed from the reaction of LNPs + TMB and LNPs + TMB + H$_2$O$_2$ (Figure 8 curve a and b), which suggested that LNPs possessed little catalytic activity. Little color change and absorbance peak at 652 nm were observed from the reaction of Fe$_3$O$_4$ NPs + TMB, confirming that TMB could not be oxidized without the presence of H$_2$O$_2$. The color change and absorbance peak at 652 nm were respectively observed from the reaction of Fe$_3$O$_4$ NPs + TMB + H$_2$O$_2$ and Fe$_3$O$_4$@LNPs + TMB + H$_2$O$_2$, which indicated TMB was oxidized during the reaction. However, the absorbance peak of Fe$_3$O$_4$@LNPs + TMB + H$_2$O$_2$ at 652 nm was significantly higher than that of Fe$_3$O$_4$ + TMB + H$_2$O$_2$, indicating Fe$_3$O$_4$@LNPs possessed stronger peroxidase-liked catalytic activity than Fe$_3$O$_4$ NPs due to its considerable dispersion ability. It was consistent with the reported result that Fe$_3$O$_4$ NPs stabilized by chitosan exhibited strong catalytic activity than Fe$_3$O$_4$ NPs [34].
3.6. Steady-State Kinetic Study of Fe$_3$O$_4$@LNPs

The steady-state kinetic mechanism of Fe$_3$O$_4$@LNPsNPs reacted with H$_2$O$_2$ and TMB was investigated. The concentration of TMB-derived oxidation products was calculated by $A = \varepsilon \times b \times C$ (Beer-Lambert Law), where $A$ is the absorbance value at 652 nm at room temperature and the molar absorption coefficient ($\varepsilon$) is 39,000 M$^{-1}$cm$^{-1}$ [35]. The Michaelis-Menten curves were obtained by following steps: the reaction rates of Fe$_3$O$_4$@LNPsNPs in different concentration of TMB or H$_2$O$_2$ were obtained by calculated the slope of the reaction system at 652 nm in the initial 5 min. Then, the reaction rates were plotted on the ordinate and the concentrations of TMB or H$_2$O$_2$ were plotted on the abscissa. The Lineweaver-Burk plots were obtained by taking the reciprocal of the reaction rate as the ordinate and the reciprocal of the concentration of TMB or H$_2$O$_2$. The reaction rate correlated with TMB concentration and H$_2$O$_2$ concentration was respectively demonstrated in Figure 9A,B. The Michaelis-Menten constant ($K_m$) is considered to be a key criterion for evaluating the enzyme’s affinity to a substrate [32]. The maximum velocity ($V_m$) and $K_m$ were obtained from the Lineweaver-Burk plots (Figure 9C,D) and summarized in Table 1. The $K_m$ values of Fe$_3$O$_4$@LNPs towards TMB and H$_2$O$_2$ was comparable to that of HRP which indicated the Fe$_3$O$_4$@LNPsNPs could be used as an effective substitute for HRP. The $K_m$ value of Fe$_3$O$_4$@LNPsNPs with TMB as the substrate was higher than that of Fe$_3$O$_4$ NPs with TMB, indicating that the Fe$_3$O$_4$ NPs have a higher affinity to TMB. The $K_m$ value of the Fe$_3$O$_4$@LNPs with H$_2$O$_2$ was 29 times lower than that of the Fe$_3$O$_4$NPs with H$_2$O$_2$, suggesting that the Fe$_3$O$_4$@LNPs possessed significantly stronger affinity to H$_2$O$_2$ than the Fe$_3$O$_4$ NPs. However, the low $K_m$ value of Fe$_3$O$_4$@LNPs with H$_2$O$_2$ is potentially due to the continuous release of Fe$_3$O$_4$NPs from the Fe$_3$O$_4$@LNPsNPs, thus avoiding the aggregation of Fe$_3$O$_4$ NPs and providing more reactive sites which enhanced the peroxidase-like activity of Fe$_3$O$_4$@LNPs during the reaction. The higher $K_m$ value of Fe$_3$O$_4$NPs with H$_2$O$_2$ was consistent with the reported result that Fe$_3$O$_4$NPs needs a higher H$_2$O$_2$ concentration than Fe$_3$O$_4$@LNPs to obtain the comparable $V_m$ [11]. Table 1 shows Fe$_3$O$_4$@LNPs possessed a comparable or stronger affinity to TMB or H$_2$O$_2$, compared to Fe$_3$O$_4$ NPs prepared by other methods [36–39]. Conclusively, the metal nanoparticles loaded on lignin nanoparticles improved its dispersion stability in solution and eventually increased its catalytic capacity.

Figure 8. The UV-vis spectra of LNPs + TMB (a); LNPs + TMB + H$_2$O$_2$ (b); Fe$_3$O$_4$ + TMB (c); Fe$_3$O$_4$ + TMB + H$_2$O$_2$ (d); Fe$_3$O$_4$@LNPs + TMB (e) and Fe$_3$O$_4$@LNPs + TMB+ H$_2$O$_2$ (f); Insert is the photograph of the corresponding solution.
3.7. The Colorimetric Detection of \( \text{H}_2\text{O}_2 \)

Because the inherent peroxidase-like activity of Fe\(_3\)O\(_4@\)LNPs was \( \text{H}_2\text{O}_2 \) concentration dependent, a colorimetric method was designed to detect \( \text{H}_2\text{O}_2 \). Under the optimal conditions, in a total reaction volume of 3 mL, 264 µg/mL Fe\(_3\)O\(_4@\)LNPs and 4 mM TMB mixed with various concentrations of \( \text{H}_2\text{O}_2 \) in a CPBS buffer (pH = 3.0), the reaction was completed at 50 °C for 30 min. It was demonstrated in Figure 10A the solution color and the absorbance were correlated with \( \text{H}_2\text{O}_2 \) concentration.

For example, the solution color became darker and the absorbance increased, as the concentration of \( \text{H}_2\text{O}_2 \) increased. The LOD of \( \text{H}_2\text{O}_2 \) concentration corresponding to the color variation was as low as

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**Table 1. Comparisons of \( K_m \) and \( V_m \) among Fe\(_3\)O\(_4@\)LNPs, Fe\(_3\)O\(_4\) NPs, and HRP.**

| Catalyst       | Substrate | \( K_m \)/mM | \( V_m \)/10\(^{-8}\) Ms\(^{-1}\) | Reference |
|----------------|-----------|--------------|-------------------------------|-----------|
| Fe\(_3\)O\(_4@\)LNPs | TMB       | 0.51         | 1.03                          | This work |
| Fe\(_3\)O\(_4@\)LNPs | \( \text{H}_2\text{O}_2 \) | 5.30         | 0.96                          | This work |
| Fe\(_3\)O\(_4\) NPs  | TMB       | 0.01         | 3.44                          | [11]      |
| Fe\(_3\)O\(_4\) NPs  | \( \text{H}_2\text{O}_2 \) | 154          | 9.78                          | [11]      |
| HRP            | TMB       | 0.43         | 10.00                         | [11]      |
| HRP            | \( \text{H}_2\text{O}_2 \) | 3.70         | 8.71                          | [11]      |
| His-Fe\(_3\)O\(_4\) | \( \text{H}_2\text{O}_2 \) | 37.99        | -                             | [37]      |
| Ala-Fe\(_3\)O\(_4\) | \( \text{H}_2\text{O}_2 \) | 226.60       | -                             | [37]      |
| P-Fe\(_3\)O\(_4\) | TMB       | 0.44         | -                             | [36]      |
| CDs-Fe\(_3\)O\(_4\) | \( \text{H}_2\text{O}_2 \) | 56.97        | -                             | [38]      |
| GO-Fe\(_3\)O\(_4\) | \( \text{H}_2\text{O}_2 \) | 305.00       | 1.01                          | [40]      |

Note: His-Fe\(_3\)O\(_4\): Histidine modified Fe\(_3\)O\(_4\); Ala-Fe\(_3\)O\(_4\): Alanine modified Fe\(_3\)O\(_4\); Not given; P-Fe\(_3\)O\(_4\): Porphyrin modified Fe\(_3\)O\(_4\); CDs-Fe\(_3\)O\(_4\): Carbon dots modified Fe\(_3\)O\(_4\); GO-Fe\(_3\)O\(_4\): Graphene oxide-based Fe\(_2\)O\(_3\) hybrid.

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2 μM (Figure 10A, inset). When the concentration of H$_2$O$_2$ was less than 100 μM, there was a linear relationship between the absorbance at 652 nm and the H$_2$O$_2$ concentration. Conclusively, there was the possibility of the rapid detection of H$_2$O$_2$ by the naked eye or a UV-visible spectrophotometer when the Fe$_3$O$_4$@LNPs was applied.

The linear relationship between the absorbance at 652 nm and the H$_2$O$_2$ concentration was identified when the concentration of H$_2$O$_2$ ranged from 5 to 100 μM (Figure 10B). The linear regression equation was $A_{652\text{nm}} = 0.09993 + 0.00186C$ (where $C$ is the concentration of H$_2$O$_2$), and the correlation coefficient ($R^2$) is 0.99077 ($n = 3$). The LOD was 2 × 10$^{-6}$ M, which is lower than the counterpart of Fe$_3$O$_4$ NPs ($3 \times 10^{-6}$ M) [39].

4. Conclusion

Fe$_3$O$_4$@LNPs were prepared from the purified Spruce lignin using the self-assembly method. TEM and DLS analysis indicated that when the initial concentration of lignin solution was 1.0 mg/mL, the homogenous Fe$_3$O$_4$@LNPs was obtained, and the average size was 152.8 nm. The catalytic ability of Fe$_3$O$_4$@LNPs was optimized with the reaction time, pH, and temperature. Fe$_3$O$_4$@LNPs exhibited better peroxidase mimic activity than Fe$_3$O$_4$ NPs due to its more stable dispersion in the reaction system. The catalytic activity of Fe$_3$O$_4$@LNPs allows its colorimetric detection of H$_2$O$_2$ at 2 μM limitation of concentration.

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