Synthesis of Porous CoFe$_2$O$_4$ and Its Application as a Peroxidase Mimetic for Colorimetric Detection of H$_2$O$_2$ and Organic Pollutant Degradation

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Abstract: Porous CoFe$_2$O$_4$ was prepared via a simple and controllable method to develop a low-cost, high-efficiency, and good-stability nanozyme. The morphology and microstructure of the obtained CoFe$_2$O$_4$ was investigated by X-ray diffraction (XRD), X-ray photoelectron spectroscopy (XPS), transmission electron microscopy (TEM), high-resolution TEM (HRTEM), specific surface area and pore analysis, and Raman spectroscopy. The results show that the annealing temperature has an important effect on the crystallinity, grain size, and specific surface area of CoFe$_2$O$_4$. CoFe$_2$O$_4$ obtained at 300 $^\circ$C (CF300) exhibits the largest surface area (up to 204.1 m$^2$ g$^{-1}$) and the smallest grain size. The peroxidase-like activity of CoFe$_2$O$_4$ was further verified based on the oxidation of peroxidase substrate 3,3$',5,5'$-tetramethylbenzidine (TMB) in the presence of H$_2$O$_2$. The best peroxidase-like activity for CF300 should be ascribed to its largest surface area and smallest grain size. On this basis, an effective method of colorimetric detection H$_2$O$_2$ was established. In addition, the porous CoFe$_2$O$_4$ was also used for the catalytic oxidation of methylene blue (MB), indicating potential applications in pollutant removal and water treatment.

Keywords: CoFe$_2$O$_4$; peroxidase-like activity; artificial enzyme mimetics; hydrogen peroxide

1. Introduction

Peroxidases are a kind of efficient redox enzymes that can catalyze the oxidation of enzyme substrate in the presence of H$_2$O$_2$. Owing to strong selectivity and high catalytic efficiency, they have been widely applied to various fields, such as biotechnology, chemical industry, biosensors, and immunoassays [1–3]. Unfortunately, there still exist some intrinsic drawbacks, such as being easy to denature in extreme environmental conditions, and the high cost of preparation and purification [4]. Therefore, the construction of novel and efficient artificial enzymes is very essential and has become an increasingly important focus. Over the past few decades, a vast variety of artificial enzymes have been explored to mimic the functions of natural enzyme, such as cyclodextrins, nanomaterials, DNA complexes, and Schiff base complex [5–8]. Among them, nanomaterials have attracted intensive attention because of their ease of preparation, low price, and good stability [9–13]. Additionally, the large specific surface and more activation centers of nanomaterials also greatly extend their application range [14].
To date, a large number of nanomaterials have been found to possess unexpected peroxidase-like activity (mimicking the functions of peroxidase), including Pt nanoclusters [15], AgVO$_3$ nanobelts [16], MnO$_2$ nanoparticles (NPs) [17–19], WO$_x$ quantum dots [20], Ag NPs [21], V$_2$O$_5$ nanowires [22], and Fe$_3$O$_4$ magnetic nanoparticles (MNPs) [23–25]. Additionally, a series of hybrid complexes based on metal nanomaterials have been investigated as peroxidase mimetics, such as GQDs/Ag NPs [26], Au nanocomposites [27,28], and FeSe-Pt@SiO$_2$ nanospheres [29]. Furthermore, carbon-based nanomaterials, e.g., single-walled carbon nanotube [30], graphene oxide (GO) [6], C@Fe$_3$O$_4$ NPs [31], GQDs/CuO nanocomposites [32] were also explored to mimic the functions of naturally-occurring enzymes. In particular, magnetic nanomaterials have been widely applied in biology, medicine and environmental fields because of their low cost, better stability, ease of separation, and recyclability. For example, Wei and his co-workers reported that the Fe$_3$O$_4$ MNPs could be a highly-efficient peroxidase-like catalyst towards H$_2$O$_2$ reduction for biosensing applications [23]. Chen’s group found that magnetic carbon nitride nanocomposites possessed the superior peroxidase-like catalytic activity toward H$_2$O$_2$ and glucose and can be used as an enzyme-free biosensor [33]. Feng et al., reported the controllable synthesis of monodisperse CoFe$_2$O$_4$ nanoparticles by the phase transfer method and applied them in the degradation of methylene blue (MB) with H$_2$O$_2$ [34].

As a kind of high stability ferromagnetism nanomaterials, CoFe$_2$O$_4$ have been widely applied in lithium batteries, immunoassays, environmental science, and food chemistry [35–37]. Owing to the magnetic property for easy recyclability and high catalytic performance, CoFe$_2$O$_4$ is expected to be a good candidate for mimicking peroxidase [38,39]. In this study, we established a simple method to prepare porous CoFe$_2$O$_4$ with the controllable size of nanoparticles and specific surface area. The porous CoFe$_2$O$_4$ exhibited the excellent peroxidase-like catalytic activity and could be used to detect H$_2$O$_2$ and degrade MB. Benefiting from simple operation and high efficiency, CoFe$_2$O$_4$ materials have great potential applications in environmental protection and biotechnology.

2. Materials and Methods

2.1. Reagents

3,3',5,5'-Tetramethylbenzidine (TMB) was purchased from Macklin (Shanghai, China). CoCl$_2$·6H$_2$O, FeSO$_4$·7H$_2$O, oxalic acid, methylene blue (MB), ethanol, H$_2$O$_2$ (30 wt %), isopropanol alcohol (IPA, a scavenger of hydroxyl radicals •OH) and other chemicals were all of analytical reagent grade and were obtained from Guangzhou Chemical Reagent factory (Guangzhou, China). All aqueous solutions were prepared with Milli-Q water (18.2 MΩ cm).

2.2. Preparation of CoFe$_2$O$_4$

CoFe$_2$O$_4$ samples were synthesized via a facile method. In a typical procedure, 2.5 g of CoCl$_2$·6H$_2$O and 5.6 g of FeSO$_4$·7H$_2$O were dissolved in 80 mL of deionized water and then transferred into oil bath heating at 80 °C, under vigorous stirring for 1 h. Subsequently, 30 mL 1 M oxalic acid solution was heated to boiling with magnetic stirring, and then added into the above solution slowly under constant stirring to form a final black precipitation, and then cooled by ice-water mixture. The black precipitates were collected by centrifugation and washed several times with water and ethanol, and further dried at 60 °C under vacuum for 12 h. Then, the precipitates were transferred to a tube furnace, heated to 300 °C, and maintained for 1 h. The temperature was raised at a heating rate of 1 °C min$^{-1}$ [40]. The final product was denoted as CF300. Similarly, other CoFe$_2$O$_4$ samples prepared at 400 °C, 500 °C, 600 °C, and 700 °C were denoted as CF400, CF500, CF600, and CF700, respectively.

2.3. Characterization of CoFe$_2$O$_4$

The X-ray diffraction (XRD) patterns were collected on a Bruker D8 Advance X-ray diffractometer (Bruker Inc., Karlsruhe, Germany) with Cu Kα radiation ($\lambda = 0.154056$ nm). Scanning electron microscopy (SEM) images were observed by used a Hitachi S-4800N microscope (Hitachi Inc.,
Tokyo, Japan). The transmission electron microscopy (TEM) and high-resolution TEM (HRTEM) images were taken on a JEOL JEM-2100 microscope instrument (JEOL Inc., Tokyo, Japan) at an acceleration voltage of 200 kV. The chemical composition and elemental maps of the as-prepared samples were analyzed by energy-dispersive X-ray (EDX) spectroscopy using an EDX attachment to the SEM instrument. The X-ray photoelectron spectroscopy (XPS) data were acquired using AXIS SUPRA (Shimadzu Inc., Kyoto, Japan). The UV-vis absorbance spectra were recorded using a Lambda 750 s UV-vis-NIR absorption spectrophotometer (PerkinElmer Inc., Waltham, MA, USA). The hysteresis loop of the samples was obtained using the vibrating sample magnetometer (VSM, Lakeshore 7400, Westerville, OH, USA). The specific surface areas of the CoFe$_2$O$_4$ were recorded based on Brunauer-Emmett-Teller (BET) equation by an automatic nitrogen adsorption pore size distribution and specific surface analyzer (ASAP 2460, Atlanta, GA, USA). Raman spectroscopy was performed on a Renishaw inVia Reflex Raman microscope (Renishaw Inc., Gloucestershire, UK) using 532 nm green laser excitation.

2.4. Peroxidase-Like Activity Assay

To investigate the peroxidase-like activity of the prepared CoFe$_2$O$_4$ materials, a typical catalytic oxidation experiment was performed at room temperature with 100 µg/mL CF400 in 3 mL of phosphate-buffered saline (PBS, 20.0 mM, pH 3.5), using 0.8 mM TMB, and 4.0 mM H$_2$O$_2$ as the substrate. All the reactions were monitored in time scan mode at 652 nm, right after all of the reagents were added and mixed. Control groups included CF400 (100 µg/mL) with TMB (0.8 mM), CF400 (100 µg/mL) with H$_2$O$_2$ (4.0 mM), and TMB (0.8 mM) with H$_2$O$_2$ (4.0 mM) in 20.0 mM PBS (pH 3.0), respectively.

2.5. Optimal Conditions for H$_2$O$_2$ Detection

The effect of substrate H$_2$O$_2$ concentration (0–0.1 M), CF300 concentration (0–200 µg/mL), temperature (20–40 °C), and pH (2–8) on the peroxidase-like activity of CF300 were also investigated to ascertain the optimal conditions.

2.6. Analysis of Active Species

The active species generated in the reaction were detected by adding scavengers (IPA) into the reaction solutions [16]. The specific procedure was the same as the CF300 peroxidase-like activity assay experiments.

2.7. Steady-State Kinetic Study

A steady-state kinetic experiment was performed in a 3.0 mL reaction solution (20.0 mM PBS, pH = 3.0, 25 °C) with 20 µg/mL CF300. TMB and H$_2$O$_2$ were involved in the reaction as substrates. The steady-state kinetic data were collected by varying the concentration of one substrate while keeping the other substrate concentration constant, and the kinetic value was measured in time course mode at 652 nm [32,41]. The Michaelis-Menten constant was calculated by using the Lineweaver-Burk double reciprocal according to the equation: $1/v = (K_m/V_{max}) (1/[S]) + 1/V_{max}$ [42,43], where $v$ is the initial velocity, $K_m$ is the Michaelis constant, $V_{max}$ is the maximal reaction velocity, and $[S]$ represents the concentration of the substrate.

2.8. Methylene Blue Degradation

The catalytic activity of CoFe$_2$O$_4$ samples was demonstrated by degrading MB in aqueous solution. In a typical reaction, 0.015 mg of CF300 was added to 50 mL MB aqueous solution (10 mg/L), then 10 mL of aqueous H$_2$O$_2$ (30 wt %) was added in the reaction mixture. At various time intervals, a small quantity of the mixture solution was separated quickly by centrifugation. Finally, the supernatants
were pipetted into a quartz cell and analyzed with UV-vis spectrophotometer to evaluate the catalytic degradation MB [41,44].

3. Results

3.1. Characterization of the CoFe₃O₄

The crystal structure and purity of the products were measured by XRD. Figure 1A displays XRD patterns of CoFe₂O₄ prepared at different temperatures. All characteristic peaks of samples match very well with the inverse spinel structure with the lattice parameters of \( a = 8.377 \) Å and \( c = 8.377 \) Å, which is consistent with the reported data (JCPDS file no. 03-0864). The synthesized temperature has a significant effect on the crystallinity of CoFe₂O₄. With the increase of the prepared temperature, the crystallization of samples increases obviously. Moreover, no other characteristic peaks of impurities are detected, indicating a pure phase of CoFe₂O₄ can be obtained using the present method. Figure 1B shows the Raman spectra of the as-synthesized samples. The low-frequency vibrations (below 600 cm⁻¹) are belonged to the motion of oxygen around the octahedral lattice site whereas the higher frequencies can be attributed to oxygen around tetrahedral sites [45,46]. In this work, the mode at 682 cm⁻¹ is a characteristic of the tetrahedral site. The bands at 470 cm⁻¹ and 300 cm⁻¹ correspond to Co²⁺ at octahedral sites [47].

![Figure 1](image-url)  
**Figure 1.** (A) XRD patterns and (B) Raman spectra of CoFe₂O₄ prepared at different temperatures.

The morphology of as-prepared CoFe₂O₄ is presented by SEM image. As shown in Figure 2A, CF300 show the rhombus-like polyhedron morphology with some fragments and cracks. They are several micrometers in size. Other CoFe₂O₄ samples obtained at different temperature also exhibit similar structure and morphology in Figure S1 (Supplementary Materials). The EDX elemental mappings (Figure 2B) and EDX spectrum (Figure S2) reveal the existence Co, O, and Fe elements. All three elements can maintain a uniform rhombus-like morphology, further demonstrating that Co, O, and Fe are homogeneously distributed in the sample.
Figure 2. (A) SEM image of CF300 and (B) EDX elemental mappings.

The microstructure and morphology of the synthesized samples at different reaction temperature were further investigated by TEM. As observed from the TEM images in Figure 3A–E, all the samples exhibit a homogeneous mesoporous structure. When the synthesized temperature is increased from 300 °C to 700 °C, the pore size gradually enlarged and the pore density also decreased owing to the further crystallization and crystal growth of CoFe$_2$O$_4$ particles. In this work, the pores are located among the particles and mainly derived from a large amount of gases slowly release from oxalate particles during thermal decompositions [48]. Figure 3F displays the HRTEM image of CF300. The image highlights the crystalline lattice fringes with a distance of about 0.296 nm, which can be ascribed to the (220) plane of CoFe$_2$O$_4$. The inset in Figure 3F is the corresponding selected area electron diffraction (SAED) pattern, confirming that the sample has a monocrystalline structure. The diffraction spots can be indexed as inverse spinel-structured CoFe$_2$O$_4$, which is in accordance with the XRD result.

Figure 3. Typical images of (A) CF300; (B) CF400; (C) CF500; (D) CF600; and (E); CF700 (F); HRTEM image of CF300, and the inset in (F) is the SAED pattern.

The chemical composition and surface electronic state of the as-prepared CoFe$_2$O$_4$ samples were further studied by XPS. It can be seen in the XPS survey spectrum (Figure 4A) that the sample mainly contains three elements: Co, Fe, and O. The C 1s peak at around 284.8 eV could correspond to the signal from carbon contained in the instrument as calibration. In the Co 2p spectrum (Figure 4B), the peak located at about 780.58 eV corresponds to Co 2p$_{3/2}$ with a satellite peak at 786.58 eV and the peak at 795.78 eV is ascribed to the Co 2p$_{1/2}$. Figure 4C presents the Fe 2p orbital displaying the splitting peaks at binding energies of around 724.58 eV and 711.28 eV, which can be assigned to the...
Fe 2p$_{1/2}$ orbital and Fe 2p$_{3/2}$ orbital, respectively. These results are well in agreement with many other reports [49,50]. Figure 4D shows the high-resolution XPS spectra of the O 1s. Based on the previous reports, the signal peak located at 530.94 eV could be assigned to typical lattice oxygen in CoFe$_2$O$_4$. The peak located at 531.4 eV is assigned to C–O bonds. The XPS spectrum is in good agreement with the literature [51].

**Figure 4.** XPS spectra of CF300: (A) survey spectrum, high resolution XPS spectra of (B) Co 2p, (C) Fe 2p, and (D) O 1s.

N$_2$ adsorption/desorption isotherms of the samples are depicted in Figure 5A. Typical type IV adsorption isotherms with an H3-type hysteresis loop are observed for all samples except CF700, indicating the mesoporous feature of the CoFe$_2$O$_4$ nanostructures. Figure 5B shows the relevant Barrett-Joyner-Halenda (BJH) pore size distribution of the porous CoFe$_2$O$_4$, in which there exists a sharp maximum at 3.5–20.6 nm, further indicating the material has a mesoporous structure. The porous trait will greatly enhance the surface area and active sites, and thus improve the catalytic performance [52]. The specific surface, pore diameter, and pore volume of these samples are shown in Table 1. We can see that, as the preparing temperature increases, the obvious decrease is found for the pore volume (0.228 to 0.019 cm$^3$/g) and specific surface (204 to 18 m$^2$/g). The magnetism of CF300 sample was determined by the hysteresis loop on VSM. It can be seen in Figure S3 that CF300 displays typical ferromagnetism at room temperature. The coercivity (Hc), saturation magnetization (Ms) and residual magnetization (Mr) are about 12 Oe, 8.372 emu/g and 0.064 emu/g respectively.

**Table 1.** Specific surface, pore diameter, and pore volume of synthesized CoFe$_2$O$_4$ samples.

| Sample | Specific Surface (m$^2$/g) | Pore Diameter (nm) | Pore Volume (cm$^3$/g) |
|--------|---------------------------|--------------------|------------------------|
| CF300  | 204.10                    | 4.47               | 0.228                  |
| CF400  | 112.17                    | 8.84               | 0.248                  |
| CF500  | 58.44                     | 14.29              | 0.209                  |
| CF600  | 21.78                     | 5.56               | 0.030                  |
| CF700  | 18.25                     | 4.18               | 0.019                  |
3.2. Peroxidase-Like Activity of CoFe$_2$O$_4$

To examine the peroxidase-like activity of the synthesized CoFe$_2$O$_4$, the catalytic oxidation of the typical peroxidase substrate TMB in the presence or absence of H$_2$O$_2$ was carried out (Figure 6A). Color changes of different systems can be observed from Figure 6B. The H$_2$O$_2$ + CF400 system and the H$_2$O$_2$ + TMB system show no absorption, while the TMB + H$_2$O$_2$ + CF400 system shows an obvious absorption peak at 652 nm, which indicates CF400 could act as a peroxidase to oxidize TMB to produce a blue color in the presence of H$_2$O$_2$. Interestingly, the TMB + CF400 system shows a weak absorption, indicating that CF400 could catalyze the oxidation of TMB in the absence of H$_2$O$_2$ to produce a blue dye. The oxidase-like activity for TMB oxidation may originate from their catalytic ability to reduce dissolved oxygen [4]. Additionally, the peroxidase mimetic catalytic activities of CoFe$_2$O$_4$ at the different synthesized temperatures were tested and compared through the catalytic oxidation of TMB in the presence of H$_2$O$_2$. It can be seen from Figure 6C that CF300 exhibits the best peroxidase mimetic catalytic activity among the synthesized CoFe$_2$O$_4$ samples. The color changes of TMB with different CoFe$_2$O$_4$ samples are shown in Figure 6D. It can be found that all the CoFe$_2$O$_4$ samples prepared under different temperature possesses distinct peroxidase mimetic catalytic activities. CF300 system shows the most obvious blue color in comparison with that of CF400, CF500, CF600, and CF700. The most superior peroxidase mimetic activity of CF300 could be mainly owing to the rich catalytic activity sites provided by the largest specific surface area. As shown in Scheme 1, H$_2$O$_2$ reduction first takes place in the presence of chromogenic electron donor TMB, and then accelerates by partial electron transferring to the surface of CF300. Subsequently, the TMB is oxidized through one electron transfer and the color of the solution changes to blue.
concentrations of H$_2$O$_2$ that the catalytic activities of horseradish peroxidase (HRP) could be inhibited at excess concentrations. For the convenience of operation, the concentration of CF300, 20 µg/mL, is used in the following experiments. Previous studies showed that the catalytic activities of horseradish peroxidase (HRP) could be inhibited at excess concentrations of H$_2$O$_2$ [53]. Figure 7B depicts the time course-dependent absorbance change at 652 nm for different concentrations of H$_2$O$_2$. The catalytic reaction rates increase with the increase of H$_2$O$_2$ concentration, and no inhibition is found in the catalytic reaction at high H$_2$O$_2$ concentrations. Figure 7A shows that the peroxidase-like catalytic reaction rate increases with the elevating concentrations of CF300.

3.3. Optimal Condition for H$_2$O$_2$ Detection

In order to investigate the intrinsic catalytic activities of CF300, further experiments were carried out with various CF300 and H$_2$O$_2$ concentrations. Figure 7A shows that the peroxidase-like catalytic reaction rate increases with the elevating concentrations of CF300. For the convenience of operation, the concentration of CF300, 20 µg/mL, is used in the following experiments. Previous studies showed that the catalytic activities of horseradish peroxidase (HRP) could be inhibited at excess concentrations of H$_2$O$_2$ [53]. Figure 7B depicts the time course-dependent absorbance change at 652 nm for different concentrations of H$_2$O$_2$. The catalytic reaction rates increase with the increase of H$_2$O$_2$ concentration,

Figure 6. (A) UV-vis absorption spectra and (B) color changes in different reaction systems (a. TMB + CF400; b. H$_2$O$_2$ + CF400; c. H$_2$O$_2$ + TMB; d. TMB + H$_2$O$_2$ + CF400); (C) UV-vis absorption spectra and (D) color changes in the presence of different CoFe$_2$O$_4$ samples (a. CF300; b. CF400; c. CF500; d. CF600; e. CF700).

Scheme 1. Schematic illustration of peroxidase-like activity for CF300.
and no inhibition is found in the catalytic reaction at high H$_2$O$_2$ concentrations, which suggests a more stable catalytic activity for CF300 than that of HRP at high H$_2$O$_2$ concentration. The 4.0 mM is chosen as the optimal H$_2$O$_2$ concentration in the following experiments.

Similar to HRP and other mimetic enzymes, the catalytic relative activity of CF300 is dependent on the pH and temperature. Hence, we investigated the peroxidase-like activity of CF300 through varying the pH from 2 to 8 and the temperature from 20 °C to 40 °C. As shown in Figure 7C, the peroxidase-like activity of CF300 is much higher in weakly acidic solutions than in alkaline and neutral solutions. The maximum absorption for CF300 catalytic system appears at pH 3.0. In addition, the catalytic activity of CF300 is significantly affected by the temperature (Figure 7D). The catalytic activity offers an upgrade, firstly, with the increasing temperature. Subsequently, it starts to decline when the temperature is higher than 25 °C. Thus, for the convenience of operation, the 25 °C is selected as the experimental temperature.

![Figure 7](image_url)

**Figure 7.** (A) Time-dependence absorbance at 652 nm of the 0.08 mM TMB reaction solution in the absence or presence of different doses of CF300 in 20 mM PBS (pH = 3.0) with 0.8 mM H$_2$O$_2$ at room temperature. (B) Time-dependence absorbance at 652 nm of the 0.4 mM TMB reaction solution in the absence or presence of different concentrations of H$_2$O$_2$ in 20 mM PBS (pH = 3.0) with 20 μg/mL CF300 at room temperature. Dependence of peroxidase-like activity of CF300 on pH (C) and temperature (D).

### 3.4. Catalytic Mechanism Study

In the presence of H$_2$O$_2$, CF300 could catalyze H$_2$O$_2$ to form the oxidized intermediate •OH radicals and subsequently oxidize TMB to produce blue dye. In order to confirm the catalytic mechanism, the •OH radical scavenger IPA was used in the CF300-TMB-H$_2$O$_2$ system, where IPA would easily react with •OH, leading to the decrease of the absorption at 652 nm and color fading of the system [2]. The corresponding chemical equation was:

\[
\text{CH}_3\text{CH(OH)}\text{CH}_3 + •OH \rightarrow \text{CH}_3\text{CH(O)}\text{CH}_3 + \text{H}_2\text{O}
\]
Figure 8 shows an apparent color fading and absorption decrease at 652 nm for the system with IPA, confirming that CF300 could catalytically activate H$_2$O$_2$ to generate •OH radicals, and then oxidize TMB to produce a TMB oxide with blue color. Obviously, these results indicate that •OH radicals play a key role in the catalytic reaction.

![Figure 8](image_url)

**Figure 8.** Time-dependent absorbance at 652 nm of the 0.4 mM TMB reaction solutions in the absence or presence of scavengers (IPA) in 20 mM PBS (pH = 3) with 20 µg/mL CF300 and 0.8 mM H$_2$O$_2$.

### 3.5. Steady-State Kinetic Assay

The catalytic steady-state kinetic of CF300 was evaluated by using TMB and H$_2$O$_2$ as substrates (Figure 9). The kinetic experiments were performed by only varying the concentration of TMB or H$_2$O$_2$ while keeping the other substrate concentration constant. As illustrated in Figure 9A,B, the Michaelis–Menten curves were obtained for both TMB and H$_2$O$_2$ in a certain concentration range. Accordingly, a series of initial reaction rates were obtained, and the double reciprocal plots (the reciprocal initial velocity vs. the reciprocal substrate concentration) were drawn as demonstrated in Figure 9C,D. The Michaelis–Menten constant ($K_m$) and maximal reaction velocity ($V_{max}$) for this system were calculated using Lineweaver–Burk plots and the Michaelis-Menten equation. The $K_m$ (TMB) of the CF300 is 0.387 mM, which is lower than the reported value of HRP (0.434 mM), suggesting that CF300 has higher affinity for TMB than HRP (Table 2). On the other hand, the $K_m$ (H$_2$O$_2$) of the CF300 is 8.89 mM, significantly higher than that of 3.7 mM for HRP [25], indicating that CF300 has lower affinity for H$_2$O$_2$. Additionally, the peroxidase-like catalytic reaction based on CF300 followed the typical Michaelis-Menten behavior [2,13] towards both substrates: TMB and H$_2$O$_2$. Additionally, the double-reciprocal plots (Figure 9C,D) exhibited the characteristic parallel lines of a ping-pong mechanism, indicating that CF300 bound and reacted with the first substrate and then released the first product before reacting with the second substrate, which is similar to that of HRP [2,25].

| Catalysts | Substances | $K_m$ (mM) | $V_{max}$ (M/s) | References |
|-----------|------------|------------|-----------------|------------|
| CoFe$_2$O$_4$ | H$_2$O$_2$ | 8.89       | $1.93 \times 10^{-8}$ | This work  |
| CoFe$_2$O$_4$ | TMB        | 0.387      | $2.90 \times 10^{-8}$ | This work  |
| HRP       | H$_2$O$_2$ | 3.7        | $8.71 \times 10^{-8}$ | [10]       |
| HRP       | TMB        | 0.434      | $10.00 \times 10^{-8}$ | [10]       |

Table 2. Comparison of $K_m$ and $V_{max}$ between CF300 and HRP for H$_2$O$_2$ and TMB.
Figure 9. Steady-state kinetic assays of CF300, the reaction velocity \( (v) \) was measured using 20 \( \mu g/mL \) of CF300 in 20 mM PBS (pH = 3) at room temperature. The TMB concentration was varied and the concentration of \( H_2O_2 \) was 4 mM (A); the \( H_2O_2 \) concentration was varied and the concentration of TMB was 0.4 mM (B); and the double-reciprocal plots of peroxidase-like activity of CF300 with a fixed concentration of one substrate relative to varying concentration of the other substrate (C,D).

3.6. Detection of \( H_2O_2 \) and Oxidative Degradation of Methylene Blue

On the basis that peroxidase mimics the activity of the CoFe\(_2\)O\(_4\) materials, we developed a facile and label-free colorimetric approach to detect \( H_2O_2 \). As shown in Figure 10, a typical \( H_2O_2 \) concentration response curve under the optical conditions is measured. The detection range for \( H_2O_2 \) is from 0.02 to 6 mM. The linear regression equation is \( A_{652nm} = 0.18388 + 0.0049C \) (mM), with a correlation coefficient of 0.9945. The detection limit for \( H_2O_2 \) is 0.02 mM, suggesting that the optical biosensing system built in this study can be applicable to the \( H_2O_2 \) determination.

Some studies confirmed that nanozymes could be used to remove pollutants from waste water [54–57]. Based on the excellent catalytic activity of CF300, we further investigated its MB degradation ability. Similar to other enzyme mimic reactions, the maximum UV-vis absorption \( (A_{max}) \) of MB aqueous solution is located at 665 nm [44]. Figure 11A shows the UV-vis absorption spectra of MB aqueous solution in the presence of CF300 and \( H_2O_2 \) at different reaction time. A sharp decrease for the absorption intensity of MB is observed with the increasing reaction time, the band at 665 nm become very weak and broad, and the removal rate of MB can reach 91.2% at 10 h (Figure 11B), suggesting that the MB has nearly completed degradation.
In summary, we developed a simple and controllable method for porous CoFe$_2$O$_4$ preparation. The specific surface area of CoFe$_2$O$_4$ materials was dependent on the annealing temperature and the CF300 exhibited a much higher specific surface area (204.1 m$^2$ g$^{-1}$) than other CoFe$_2$O$_4$ samples. The large specific surface area of CF300 endowed them more actives which resulted in the enhanced intrinsic peroxidase mimics activity. Additionally, based on the peculiarity, CF300 had excellent catalytic activity for the degradation of MB. Owing to the facile preparation, low cost, large specific surface area, and magnetic properties, the present CoFe$_2$O$_4$ showed great potential for applications in environmental protection, biomedical analysis, and other related areas.

4. Conclusions

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Author Contributions: G.W. (Guizhen Wang) conceived and designed the experiments; L.W., G.W. (Gengping Wan), and S.S. performed the experiments; L.W., K.W., Z.H., X.X., Y.T., N.H., and Y.S. analyzed the data; G.W. (Guizhen Wang) contributed reagents, materials, and analysis tools; and G.W. (Guizhen Wang) and L.W. wrote the paper.

Conflicts of Interest: The authors declare no conflict of interest.

**Figure 10.** A concentration response curve H$_2$O$_2$ detection. Inset: the linear calibration plot.

**Figure 11.** (A) UV-vis spectra obtained during the MB oxidative degradation over CF300; and (B) the MB degradation rate at various reaction time.
Conflicts of Interest: The authors declare no conflict of interest.

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