One-Step Hydrothermal Synthesis of N, Fe-Codoped Carbon Dots as Mimic Peroxidase and Application on Hydrogen Peroxide and Glucose Detection

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In this paper, N, Fe-codoped carbon dots (N, Fe-CDs) were synthesized from β-cyclodextrin, ethylenediamine, and ferric chloride for the first time using a convenient one-step hydrothermal method. The obtained N, Fe-CDs were characterized by various methods including transmission electron microscopy, X-ray photoelectron spectroscopy, and Fourier-transform infrared spectroscopy. The N, Fe-CDs exhibited better catalytic activity than horseradish peroxidase (HRP) and caused an evident color change for 3,3′,5,5′-tetramethylbenzidine in the presence of H2O2. Kinetic experiments show that the apparent Km value for the N, Fe-CDs with TMB (0.40 mM) or H2O2 (0.35 mM) as the substrate was lower than that of HRP (0.43 and 3.70 mM), suggesting that the N, Fe-CDs have a much higher affinity for TMB and H2O2 than HRP. The Km/Vmax value for the N, Fe-CDs (21.74 × 103 · s for H2O2) is significantly lower than that for HRP (42.53 × 103 · s), suggesting that the N, Fe-CDs have a stronger catalytic efficiency for H2O2 than HRP. Furthermore, a highly efficient and sensitive colorimetric detection method for glucose was developed using the N, Fe-CDs as mimic peroxidase to detect the hydrogen peroxide generated by the oxidation of glucose by glucose oxidase. The limit of detection for H2O2 and glucose was found to be 0.52 and 3.0 μM, respectively. The obtained N, Fe-codoped carbon dots, which possess simulated peroxidase activity, can potentially be used in the field of biotechnology.

1. Introduction

Peroxidase such as horseradish peroxidase (HRP) is a kind of natural enzyme that catalyzes the decomposition of peroxides and prevents biological cells from being damaged by toxic substances [1]. However, the natural enzyme would suffer from degradation, denaturation, and inactivation in practical applications especially under harsh conditions. The extraction, purification, and storage processes of natural peroxidase are also fussy. High price further limits its applications [2–4]. Under this background, various enzyme mimics have been developed in order to address these issues.

Recently, multiple inorganic nanomaterials, such as FeOOH nanorods [5], Co3N nanowires [6], WSe2 nanosheets [7], yolk-shell nanostructured Fe3O4@C magnetic nanoparticles [8], MnO2 nanowires [9], amine-grafted metal-organic frameworks (MOFs) [10], and apoferritin-paired gold clusters [11], have been found to exhibit the properties of oxidase or peroxidase-like enzymes [12]. In addition, carbon-based nanomaterials, such as graphene oxide [13], carbon nanotubes [14], carbon nanodots [15], carbonitrile sheets [16], graphene quantum dots [17], and C3N4 nanosheet-supported Prussian Blue nanoparticles [18], have been found to possess peroxidase-like or superoxide dismutase-like
activity [19]. This nanomaterial has been widely used in biosensor, bioimaging, catalysis, light-emitting diode device, and medical diagnosis due to their low cytotoxicity, good biocompatibility, easy functionalization, and high stability [20–27].

In this paper, a new mimic peroxidase, N, Fe-codoped carbon dots (N, Fe-CDs) were synthesized from β-cyclodextrin, ethylenediamine, and ferric chloride using a convenient one-step hydrothermal method. The obtained N, Fe-CDs exhibited better catalytic activity than HRP. Combining with glucose oxidase (GOx), a good colorimetric method for glucose detection was developed.

2. Experimental Section

2.1. Materials. β-Cyclodextrin was purchased from Shanghai Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China; www.reagent.com.cn). Ethylenediamine, glucose, fructose, maltose, xylose, ascorbic acid, uric acid, α-lactose, sucrose, terephthalic acid (TA), ferric chloride hexahydrate, and
hydrogen peroxide (30%) were purchased from XiLong Chemical Co. Ltd. (Guangdong, China; www.xlhg.com). 3,3′,5,5′-tetramethylbenzidine (TMB), glucose oxidase (180U/mg), and rhodamine B (RhB) were purchased from Aladdin Reagent Co. Ltd. (Shanghai, China; http://www.aladdine.com). Ultrapure water (18.2 Ω cm) was prepared by a Milli-Q system (Millipore, Bedford, MA, USA; http://www.merckmillipore.com) and used in all experiments.

2.2. Analytical Methods. The morphology and size distribution of the nanoparticles were studied using a Tecnai-G2 F20 transmission electron microscope (TEM) with an accelerating voltage of 200 kV (FEI; http://www.fei.com). X-ray photoelectron spectroscopy (XPS) was performed using a Thermo Escalab 250 Xi photoelectron spectrometer (Thermo Fisher; http://www.thermoscientific.com). Fourier transform infrared (FTIR) spectra were recorded using a Magna-IR 750 Fourier transform infrared spectrometer (Nicolet; http://www.thermofisher.com), and absorption spectra were obtained using a SPECORD 200 PLUS UV-vis spectrometer (Analytik Jena; http://www.analytik-jena.com.cn).

2.3. Synthesis of the N, Fe-CDs. The N, Fe-CDs were prepared using a one-step hydrothermal synthesis method [21, 27]. Briefly, 0.5 g of β-cyclodextrin, 2 mL of ethylenediamine, and 0.5 g of FeCl₃·6H₂O were firstly dissolved in 50 mL of ultrapure water. Then, the solution was transferred to a Teflon-lined, stainless-steel autoclave and heated at 180°C for 0.5 h. The mixture was then cooled to room temperature and filtered through a 0.22 μm filter under the negative pressure by the water pump. The supernatant was dialyzed against ultrapure water for 24 hours, and then freeze-dried for later use. As control, N-CDs were synthesized in the same way but without FeCl₃·6H₂O.

2.4. Mimic Peroxidase Activity of the N, Fe-CDs. The mimic peroxidase activity of N, Fe-CDs was determined using the following procedures. First, 100 μL of N, Fe-CDs (150 μg/mL) was added to an acetic acid solution (0.1 M, pH 2.5) containing 100 μM H₂O₂ and 700 μM TMB. Color changes were observed after the solution had been incubated in a 35°C water bath for 36 min. In order to investigate the catalytic mechanism, the experiment was repeated using different concentrations of TMB and H₂O₂. Then, the key

Figure 2: XPS spectrum of the obtained N, Fe-CDs (a). (b), (c) and (d) are the corresponding C 1s, N 1s, and O 1s spectra, respectively.
The absorption spectrum of the reacted solution was recorded in the range of 200 nm to 800 nm.

2.7. Colorimetric Detection of H$_2$O$_2$ and Glucose. Colorimetric detection of H$_2$O$_2$ was performed as follows. A solution containing 200 $\mu$L of TMB (700 $\mu$M), 100 $\mu$L of N, Fe-CDs (150 $\mu$g/mL), and 600 $\mu$L of H$_2$O$_2$ at different concentrations, and 0.5 mL of acetate buffer (0.1 M, pH 2.5) was incubated in a 35°C water bath for 36 min. After that, the absorption spectra of the reacted solution were recorded in the range of 500 nm to 800 nm.

Glucose detection was carried out as follows: first, 20 $\mu$L of GOx (1 mg/mL), 20 $\mu$L of glucose at different concentrations, and 60 $\mu$L of phosphate-buffered saline (PBS, 0.1 M, pH 7.0) were mixed and incubated at a 37°C water bath for 60 min. Then, 200 $\mu$L of TMB (700 $\mu$M), 100 $\mu$L of N, Fe-CDs (150 $\mu$g/mL), and 600 $\mu$L of acetate buffer (0.1 M, pH 2.5) were successively added to the glucose reaction solution and incubated in a water bath at 35°C for 36 min. Finally, the UV-vis spectrum of the mixed solution was recorded using a UV-vis spectrometer. Control experiments, in which 0.5 mM glucose was replaced by 5 mM fructose, ascorbic acid, maltose, xylose, uric acid, $\alpha$-lactose, and sucrose, were also performed. Each point was measured three times. The standard errors, defined as the average squared deviation of each number from its mean, were calculated using origin software.

3. Results and Discussion

3.1. Characterizations of the N, Fe-CDs. The TEM image in Figure 1(a) shows that the N, Fe-CDs had good dispersibility, and the size was in the range of 0.9–2.5 nm. FTIR was used to analyze the functional groups in the material. The FTIR spectrum (Figure 1(b)) shows a broad peak at 3328 cm$^{-1}$, which was ascribed to the stretching vibrations of the O-H and N-H bonds. The intense band at 2920 cm$^{-1}$ arose from symmetric C-H stretching vibrations, and the characteristic band at 1030 cm$^{-1}$ was assigned to the vibration of the C-N bond.

XPS was performed to analyze the composition of the N, Fe-CDs, and the results are shown in Figure 2(a). Four elements (C, N, O, and Fe) were detected in the XPS spectrum of the N, Fe-CDs. The oxygen content was found to be as high as 31.78%, which further suggests that the N, Fe-CDs contained abundant functional groups. The main peak at 286.25 eV, attributed to C 1s, was deconvoluted into four contributing peaks at 284.5, 285.0, 286.2, and 287.7 eV (Figure 2(b)), which indicates the presence of four carbon environments: sp$^2$ C=C or sp$^3$ C-C, C-N, sp$^3$ N=C=N, and C=O [28]. The peak at 399.51 eV, attributed to N 1s, was deconvoluted into three contributing peaks at 399.2, 399.8, and 401.5 eV (Figure 2(c)), which indicates the presence of three types of nitrogen environments: C-N, C=N, and C=N-H, respectively [29]. The peak at 532.61 eV was ascribed to O 1s, which can be resolved into 532.0, 532.6, and 533.2 eV, corresponding to the C-O, C=O, and O-H bonds, respectively [30]. The weak peak at 716.57 eV was ascribed to Fe 2p. The content of Fe was 0.56%, which is 315 nm, and the emission wavelength range is from 350 to 600 nm.
indicating that Fe has been successfully incorporated into the nanoparticles.

3.2. Evaluation of the Peroxidase-Like Catalytic Activity of the N, Fe-CDs.

To investigate the peroxidase-like activity of N, Fe-CDs, colorimetry was carried out using TMB as a substrate in the absence and presence of H$_2$O$_2$ and N, Fe-CDs. As shown in Figure 3, a significant absorption peak appeared at 658 nm due to oxidation of TMB in the presence of H$_2$O$_2$ and the N, Fe-CDs (Figure 3(d)). This peak was significantly smaller in the absence of the N, Fe-CDs, which shows that the N, Fe-CDs did show great catalytic performance. No significant absorption peaks were observed in the control experiments, including the absence of the N, Fe-CDs (Figures 3(a) and 3(c)) or H$_2$O$_2$ (Figure 3(f)) and the replacement of N, Fe-CDs with N-CDs (Figures 3(b) and 3(e)).

The influence of the N, Fe-CDs and TMB concentrations, the pH value, the reaction temperature, and duration on the catalytic performance were further evaluated, and the results are shown in Figure 4. The absorbance is shown as a function of the N, Fe-CDs concentration in Figure 4(a), interestingly, low concentrations of N, Fe-CDs showed high activity towards TMB oxidation, but the absorbance changed little when the N, Fe-CDs concentration exceeded 150 μg/mL. Therefore, a N, Fe-CDs concentration of 150 μg/mL was used in the subsequent experiment. The absorbance increased continuously as the TMB concentration and reaction duration increased. The optimal TMB concentration and reaction duration were 400 μM and 40 min, respectively.
duration were determined to be 700 μM (Figure 4(b)) and 36 min (Figure 4(e)), respectively. Figure 4(c) shows the change in absorbance caused by varying the pH from 2.0 to 7.0. The results indicate that catalytic activity of the N, Fe-CDs was highest at a pH of 2.5. The catalytic activity of the N, Fe-CDs was also increased when the temperature increased from 25 to 35 °C, but it decreased temperatures higher than 35 °C (Figure 4(d)).

In order to better understand the mechanism by which N, Fe-CDs catalyze TMB and H₂O₂ oxidation, the apparent steady-state kinetics were measured. As shown in Figures 5(a) and 5(b), a range of TMB and H₂O₂ concentrations were used in the catalytic reactions so that the kinetic parameters could be obtained using Lineweaver–Burk plots (Figures 5(C) and 5(D)). Table 1 compares the parameters for this system with those of HRP. A smaller Km value indicates a stronger affinity between the enzyme and the substrate and a higher catalyst efficiency. The Km value for N, Fe-CDs with TMB or H₂O₂ as substrate was lower than that of HRP, which suggests that the N, Fe-CDs have a higher affinity for TMB and H₂O₂ than HRP does. On the other hand, although the V max values of the N, Fe-CDs were smaller than those of HRP, the Km/V max value of the N, Fe-CDs with H₂O₂ as substrate was smaller than those of HRP. The lower the value of Km/V max is, the higher the catalytic efficiency is. Considering their peroxidase mimetic activity, the N, Fe-CDs could be employed as a potential substitute for HRP.

The fitted Lineweaver–Burk lines were nearly parallel at different concentrations of TMB and H₂O₂. That means the

Table 1: Comparison of the apparent Michaelis–Menten constant (Km), maximum reaction rate (V max), and enzyme efficiency (Km/V max) between N, Fe-CDs and HRP.

<table>
<thead>
<tr>
<th>Catalyst</th>
<th>Substrate</th>
<th>Km (mM)</th>
<th>V max (10⁻⁸ M·s⁻¹)</th>
<th>Km/V max (10⁻³ s⁻¹)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>N, Fe-CDs</td>
<td>TMB</td>
<td>0.40</td>
<td>1.19</td>
<td>33.61</td>
<td>This work</td>
</tr>
<tr>
<td></td>
<td>H₂O₂</td>
<td>0.35</td>
<td>1.61</td>
<td>21.74</td>
<td></td>
</tr>
<tr>
<td>HRP</td>
<td>TMB</td>
<td>0.43</td>
<td>10.0</td>
<td>4.30</td>
<td>[10]</td>
</tr>
<tr>
<td></td>
<td>H₂O₂</td>
<td>3.70</td>
<td>8.7</td>
<td>42.53</td>
<td></td>
</tr>
</tbody>
</table>
N, Fe-CDs bind to and react with the first substrate, release the product, then recombine with the second substrate, react and release the second product in a manner similar to the ping-pong mechanism of HRP [31, 32].

3.3. Mechanism of the Peroxidase-like Activity of N, Fe-CDs. Generally, the catalytic pathway for peroxidase-like activity can be divided into two types, reactive oxygen species generation and an electron transfer process [33–35]. To evaluate the possible active intermediates in the present system, various fluorescent and colorimetric probes were employed. RhB was used to detect \\( \cdot \)OH as the presence of \\( \cdot \)OH would cause a decrease in the absorbance intensity of RhB. Figure 6(a) shows the change in absorbance of a solution containing RhB, \( \cdot \)OH, and N, Fe-CDs. After reacting for 12 h, a negligible decrease in the absorbance was observed compared with the control experiments, which indicates the absence of \\( \cdot \)OH in the peroxidase-like catalytic process. TA was used as a fluorescence probe for tracking \( \cdot \)OH, because it can capture \( \cdot \)OH and generate 2-hydroxyterephthalic acid, thereby emitting a unique fluorescence signal at around 425 nm. After reacting for 12 h, the fluorescence of a solution containing Na\(_2\)TA, \( \cdot \)OH, and different concentrations of N, Fe-CDs was lower than that of the mixture of Na\(_2\)TA and \( \cdot \)OH (Figure 6(b)). These results show that \\( \cdot \)OH is not generated as a reaction intermediate during the peroxidase-like catalysis. The reaction route may be described by following reaction equations.

\[
\text{TMB} - e^- \rightarrow ax - TMB \\
\text{H}_2\text{O}_2 + 2H^+ + 2e^- \rightarrow 2\text{H}_2\text{O}
\]

3.4. Detection of \( \text{H}_2\text{O}_2 \) and Glucose. On the basis of the peroxidase mimetic activity of the N, Fe-CDs, an efficient and fast colorimetric method for the detection of \( \text{H}_2\text{O}_2 \) was created. A typical \( \text{H}_2\text{O}_2 \) concentration–absorbance profile is shown in Figure 7(a). The magnitude of the peak at
658 nm increased with the increased concentration of H₂O₂. A linear relationship between the absorbance and the H₂O₂ concentration from 0 to 60 μM was obtained ($R^2 = 0.9932$), and the limit of detection (LOD) was calculated to be 0.52 μM based on three times the standard deviation rule (LOD = 3Sd/k). Also, the color of the mixture changed (inset of Figure 7(a)), which indicates that H₂O₂ can be detected with the naked eye.

As glucose would be oxidized to H₂O₂ and gluconic acid in the presence of glucose oxidase, this method can be used to detect glucose indirectly when combined with the catalytic properties of GOx and N, Fe-CDs. Figure 8(a) shows that the absorbance at the peak of 658 nm increased as the concentration of glucose increased. There exist two parts of linear range between the absorbance and the concentration of glucose in the range of 0 to 60 μM and 60 to 100 μM (Figures 8(b)-8(d)). The LOD was calculated to be 3.0 μM. As shown in Table 2, this method was comparable or more sensitive than other reported systems based on different nanoparticles as peroxidase mimics.

To further investigate the specificity of this method, five other sugars (fructose, maltose, xylose, α-lactose, and sucrose), ascorbic acid, and uric acid were selected as interfering substances for detection. Figure 9 shows the absorbance at 658 nm of solutions containing the other sugars, ascorbic acid, and uric acid at concentrations that were ten times higher than that of glucose (0.5 mM). The absorbance of the other substances differs little from that of the blank (glucose-free) sample, which shows that this method has good glucose selectivity.

### 4. Conclusions

As a conclusion, N, Fe-CDs were synthesized from β-cyclodextrin, ethylenediamine, and FeCl₃·6H₂O using a one-step hydrothermal method for the first time. The raw materials are easily available, and the preparation process is
convenient. The obtained N, Fe-CDs exhibited good activity of mimic peroxidase. In the presence of H₂O₂, the catalytic oxidation of TMB by N, Fe-CDs can produce green water-soluble products. This catalytic oxidation process conforms to the Michaelis–Menten model, and the N, Fe-CDs demonstrated better activity than HRP. On this basis, a highly sensitive and selective colorimetric method was developed for the detection of H₂O₂ and glucose. The LODs of H₂O₂ and glucose were 0.52 and 3.0 μM, respectively. This study presents an artificial enzyme that may replace natural peroxidases in biomedical and environmental testing.

Data Availability
Some or all data, models, or code generated or used during the study are available in a repository or online in accordance with funder data retention policies.

Conflicts of Interest
The authors have declared no conflict of interest.

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