

# Synthesis of Enzyme-Metal-Organic Framework Composites for Fast Detection of Hydrogen Peroxide

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## Abstract

The determination of  $H_2O_2$  is of great significance in clinical analysis, life sciences, pharmaceuticals, food industry, environmental monitoring and many other applications. Among numerous methods for  $H_2O_2$ determination, the electrochemical detection is one of the most important methods because of its simplicity, fast response and low detection limit.

Electrochemical biosensors with high selectivity and sensitivity have been attracting much attention. Moreover, there is a challenge to find the most suitable method of preparing immobilized enzyme electrodes and improving the detection sensitivity for H<sub>2</sub>O<sub>2</sub>. In this paper, the composite made of enzyme and amorphous metal-organic frameworks (aMOFs) was prepared by an aqueous in-situ co-precipitation method. During the preparation, metal nanoparticles (NPs) were introduced and co-embedded in aMOFs together with the enzyme. The enzyme-NP-aMOF composite was the utilized to construct the screen-printed enzyme electrode for H<sub>2</sub>O<sub>2</sub> detection, which was expected to enhance the detection sensitivity. In this study, cytochrome c (Cyt c) was selected as the enzyme for H<sub>2</sub>O<sub>2</sub> detection. The imidazole skeleton compounds were used as the enzyme immobilization carriers.

The composite of Cyt c and amorphous zeolite imidazole framework-8 (Cyt c@aZIF-8) was synthesized by the aqueous in-situ co-precipitation method, and the gold nanoparticles were co-embedded to prepare the Cyt c-AuNP@aZIF-8 composite. The structure and performance of Cyt c@aZIF-8 and Cyt c-AuNP@aZIF-8 were characterized by scanning electron microscopy, transmission electron microscopy, thermogravimetric analysis, X-ray diffraction and catalytic activity assay. The results showed that the apparent activity of the Cyt c@aZIF-8 and Cyt c-AuNP@aZIF-8 was increased to 500%-600% of native enzyme. The Cyt c@aZIF-8 and Cyt c-



AuNP@aZIF-8 were then applied to construct the enzyme electrode, displaying a linear detection range in the  $H_2O_2$  concentration of 5-30 mM. The sensitivity of Cyt *c*@aZIF-8 was increased by 72% compared with that of native enzyme, and the sensitivity of Cyt *c*-AuNP@aZIF-8 was further improved by 23% compared with that of Cyt *c*@aZIF-8. The results indicate that the enzyme-gold nanoparticle-amorphous metal-organic framework composite is very promising in the construction of highly efficient enzyme electrodes for fast detecting of trace amount of  $H_2O_2$ .

**Keywords**: hydrogen peroxide detection; electrochemical biosensor; enzyme electrode; metal-organic frameworks



# **Statement of Originality**

The research process and results of this team are conducted and derived under the guidance of the instructor. Other than the referenced content and the acknowledged sources, this paper does not include any published findings by this group or any other researchers. If there is any inaccuracy, this team is accountable for all liabilities.

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## 1 Background

### **1.1 Introduction**

Hydrogen peroxide is a crucial intermediate or product, which has a direct connection with many bioprocesses. However, high-level of  $H_2O_2$  is poisonous to cells and may cause disorders of morphology and function of cells<sup>[1]</sup>. Meanwhile,  $H_2O_2$  is the most common reactive oxygen species. It may diffuse into other cell organelles, and lead to a variety of harmful biological modification<sup>[2]</sup>. Food and Drug Administration (FDA) has ruled that the remnant of  $H_2O_2$  in the package of food must not exceed 0.5ppm<sup>[3]</sup>. The determination of hydrogen peroxide possesses a significant role in many areas such as clinical medicine, medicine analysis, food industry, and environmental detection<sup>[2-4]</sup>.

Numerous methods of analysis have been used in the area of the determination of  $H_2O_2$  such as titration<sup>[5,6]</sup>, fluorescent methods<sup>[7,8]</sup>, chemiluminescence<sup>[9,10]</sup>, high performance liquid chromatography<sup>[11]</sup> and methods<sup>[1-4,12-15]</sup>. electrochemical Among all these methods, the electrochemical methods have been widely studied because of the advantages of simple operation, fast response and low detection limit<sup>[16,17]</sup>. However, the electrochemical oxidation or reduction of  $H_2O_2$  requires high over potential on conventional bare gold or carbon electrodes, and the reaction is easily disturbed by other electroactive substances<sup>[17-19]</sup>. As a result, it is essential to develop electrochemical sensors with high selectivity and sensitivity for  $H_2O_2$  detection, which leads to the investigation of enzyme electrochemical biosensors.

This chapter initially summarized the methods for  $H_2O_2$  detection, introduced the enzyme electrochemical biosensors, and analyzed the advantages and the drawbacks of them. Moreover, the chapter introduced



the preparation of enzyme-metal-organic framework composites. At last, the purpose of this study was given.

#### 1.2 Methods for determining hydrogen peroxide

There are several common methods to determine hydrogen peroxide, which are titration<sup>[5,6]</sup>, fluorescent methods<sup>[7,8]</sup>, chemiluminescence<sup>[9,10]</sup>, high performance liquid chromatography <sup>[11]</sup> and electrochemical methods<sup>[1-4,12-15]</sup>.

#### 1.2.1 Titration

The chemical titration method is majorly based on the oxidizing and reducing reactions of  $H_2O_2$ , using chromogenic agents to determine the discoloration by  $H_2O_2$  redox reactions and observing the endpoint of the reaction. In the standard of China's food safety level GB 5009.226-2016, the chemists are recommended to use the Iodometric method and the Titanium Colorimetric method to determine the amount of  $H_2O_2$  in food<sup>[5]</sup>. Both methods use titration, which is easy to be operated. However, if the content of protein and lipid is relatively high in the sample, the accuracy of the method is poor<sup>[6]</sup>. In addition, the titration method is not suitable for the accurate analysis of trace amount of  $H_2O_2$ .

#### **1.2.2 Fluorescent methods**

Some molecules are excited when they are irradiated by ultraviolet light. When the molecules at excited state return to the ground state, they will emit visible or ultraviolet fluorescence that reflects the characteristics of the molecules, thus enabling qualitative or quantitative analysis of molecules. Fluorescence detection is one of the most sensitive and accurate methods for detecting  $H_2O_2^{[7]}$ .

For example, by using the process of biomineralization, fluorescent gold



nanoclusters (NCs) were integrated with enzyme molecules for detecting hydrogen peroxide. Horseradish peroxidase (HRP) was used as a functional and soft template to guide the synthesis of Au NCs under physiological conditions and to form the HRP-Au NCs conjugates (Figure 1.1). The enzyme HRP can maintain its activity towards  $H_2O_2$ , and the fluorescence of HRP-Au NCs can be quantitatively quenched by adding  $H_2O_2$ , therefore enabling the determination of  $H_2O_2$ . At an ideal condition, the detection of  $H_2O_2$  can reach the limit of 30 nM by this method<sup>[8]</sup>. However, in general, fluorescent methods require expensive fluorescent probe compounds with high cost<sup>[7]</sup>.



Figure 1.1 The illustration of HRP-Au NC formation and H<sub>2</sub>O<sub>2</sub> directional quenching

(derived from reference[8])

#### **1.2.3 Chemiluminescence**

Most of the  $H_2O_2$  detection methods based on chemiluminescence principle are using Lumino or oxalic acid ester as the chemiluminescence reagent. For example, by fixing hydroxyethyl cellulose, cobalt chloride and sodium dodecyl sulfate on the cover glass, Tahirovic *et al.* prepared a  $H_2O_2$ 



sensor based on the chemiluminescence. The linear detection range for  $H_2O_2$  of the sensor is in the concentration range of 20-1600 µg/L, with a detection limit of 9 µg/L. This method has gained a success in the detection of  $H_2O_2$  in rainwater<sup>[10]</sup>. Chemiluminescence sensors are simple, sensitive and of fast response. However, they are susceptible to interfere by excessive metal ions<sup>[10]</sup>.

#### 1.2.4 High performance liquid chromatography

Megan *et al.* combined HPLC with fluorescent detection (FD) and electrochemical detection (ED) respectively, and constructed the HPLC/FD and HPLC/ED systems, which were used to detect  $H_2O_2$  in various typical forensic samples in field studies. This study analyzed and confirmed the presence of  $H_2O_2$  residues in various samples, demonstrating the complementary properties of several  $H_2O_2$  detection technologies<sup>[11]</sup>.

#### 1.2.5 Electrochemical methods

Electrochemical methods with the advantages of high sensitivity, longterm stability, low cost, simple operation, fast response and easy of miniaturization have received the most extensive research<sup>[1,2]</sup>. Electrochemical sensors, especially screen-printed sensors, have been widely used for detection of  $H_2O_2^{[1]}$ .

Sanford *et al.* monitored the rapid  $H_2O_2$  fluctuations in vitro and brain slices by rapid scanning cyclic voltammetry on uncoated carbon fiber microelectrodes to study the  $H_2O_2$  enzyme degradation<sup>[12]</sup>. In the experiment, the oxidation potential was depended on the scanning rate. When the scanning rate was 400 V/s, the oxidation potential was +1.2 V. The relationship between the oxidation peak current and the  $H_2O_2$ concentration was linear in the physiological range of the test, with a detection limit of 2  $\mu$ M<sup>[12]</sup>.



To improve the electron transfer efficiency between the electrode and the molecule to be measured, conventional metal nanoparticles were introduced to increase the electrochemical active surface area<sup>[20]</sup>. For example, Zhang et al. constructed a highly sensitive H<sub>2</sub>O<sub>2</sub> sensor for the detection of H<sub>2</sub>O<sub>2</sub> released in living cells which was based on grapheneplatinum (RGO-PT) composites (Figure 1.2). A linear detection range of 0.5  $\mu$ M to 3.475 mM H<sub>2</sub>O<sub>2</sub> can be achieved by the sensor, with a detection limit of 0.2  $\mu$ M, which can be applied in detecting H<sub>2</sub>O<sub>2</sub> released from living cells. The graphene-platinum (RGO-PT) composite electrode showed a higher sensitivity for H<sub>2</sub>O<sub>2</sub> detection than Pt nanoparticles or graphene modified electrodes<sup>[2]</sup>.



Figure 1.2 A schematic diagram of RGO-Pt modified GCE for detection of  $\rm H_2O_2$  effluent from cells stimulated with ascorbic acid (AA)

(derived from reference[2])

## 1.3 Enzyme electrochemical biosensor

In traditional electrochemical methods, the oxidation or reduction of  $H_2O_2$  requires a high over potential. That means common electroactive



substances may easily interfere the determination of  $H_2O_2^{[2]}$ .

Compared with the conventional catalysts, enzyme is a highly efficient and specific biocatalyst with high selectivity. And the enzymatic reaction conditions are usually mild. The enzyme has been applied to prepare enzyme electrochemical biosensors, which can significantly improve the selectivity and sensitivity of the analysis compared with conventional electrodes<sup>[21]</sup>. The over potential can be greatly reduced in enzyme biosensors, which avoids the interference of other electroactive substances. Up to date, enzymes used in enzyme electrochemical biosensors for detecting  $H_2O_2$  include horseradish peroxidase, cytochrome, myoglobin, etc<sup>[22,23]</sup>.

Liu *et al.* utilized Au nanoparticle-modified TiO<sub>2</sub> nanotubes as a rigid supporting matrix for the construction of horseradish peroxidase (HRP) enzyme biosensor on glassy carbon electrodes. The presence of Au nanoparticles promoted the electron transfer between the enzyme and the electrode. The enzyme electrode showed a linear response in the H<sub>2</sub>O<sub>2</sub> concentration range of 15-750  $\mu$ M with a detection limit of 2.2  $\mu$ M<sup>[22]</sup>.

A porous carbon electrode with immobilized Cyt c was constructed by Zhang *et al.* to monitor the concentration of H<sub>2</sub>O<sub>2</sub>. A three-dimensional ordered macroporous activated carbon was used as the electrochemical electrode for immobilizing Cyt c. The immobilized Cyt c maintained its biological activity and the electron transfer rate constant (K<sub>S</sub>) was measured to be 17.6 s<sup>-1</sup>. The steady-state current response of the H<sub>2</sub>O<sub>2</sub> concentration ranged from 2 x 10<sup>-5</sup> to 2.4 x 10<sup>-4</sup> mol/L. The detection limit of H<sub>2</sub>O<sub>2</sub> was 1.46 x 10<sup>-5</sup> mol/L<sup>[23]</sup>.

#### **1.4 Enzyme-metal-organic framework composites**

Enzyme electrochemical biosensors can effectively improve the



selectivity and sensitivity of electrochemical detection methods. However, if the free enzyme was directly adsorbed on the electrode surface, it could easily denature the enzyme. Moreover, it could be very difficult for electrons transferring between free enzyme and the electrode. Therefore, the development of methods for immobilizing enzyme on electrodes is of significance for constructing efficient enzyme electrochemical biosensors. Metal-organic frameworks (MOFs) have recently emerged as a new type of materials for enzyme immobilization.

#### 1.4.1 Metal-organic frameworks

Metal-organic frameworks (MOFs) are porous crystalline materials with high porosity and large internal surface area<sup>[24]</sup>. Through the selfassembly of metal ions and organic ligands, a coordination polymer with periodic network structure can be constructed. MOFs are the perfect combination of inorganic and organic components. And due to their large surface area, adjustable structure, high porosity, excellent chemical stability, simple preparation process, MOFs play an important role in gas storage, catalysis, chemical separation, drug delivery, storage and other fields<sup>[25,26]</sup>.

MOFs have regular repeated network structures and regular pore structures with long-range orders<sup>[27]</sup>. The pore structure of most MOFs is microporous, and the pore size is usually less than 2 nm. Most of the protein molecules usually have much larger size than the pore size of the majority of MOFs<sup>[28]</sup>. MOFs have been frequently utilized as carriers to encapsulate enzymes. However, MOFs as the carriers for enzyme immobilization, can easily limit both the conformational change of internal protein molecules and the transportation of large substrate molecules. Therefore, although the stability of encapsulated enzyme can be significantly improved after immobilization on MOFs, the enzymatic activity is also significantly



reduced at the same time. Therefore, it is still a big challenge to prepare the enzyme-MOF composites with both high activity and high stability.

Amorphous metal-organic frameworks (aMOFs) still retain the basic coordination structure and connectivity of the crystalline materials, but they lack long-range ordered regular pore structures (Figure 1.3), causing the formation of more mesopores with pore sizes of 2-50 nm<sup>[27]</sup>. Compared with the crystalline MOFs, the thermal stability and mechanical strength of aMOFs have a certain degree of improvement. Having rich mesopore and micropore structures, aMOFs as enzyme immobilization carriers may provide a greater possibility in achieving higher enzymatic activity of the immobilized enzyme.



Figure 1.3 X-ray diffraction patterns and unit cell structures of amorphous and crystalline ZIF materials

(derived from reference[27])

#### 1.4.2 Method of synthesizing enzyme-MOF composites

Much attention has been made to synthesizing enzyme-MOF composties, which can be generally categorized into three strategies, including physical

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absorption, bioconjugation and co-precipitation<sup>[29,30]</sup>.

#### 1.4.2.1 Physical absorption

For the physical adsorption method, MOFs with specially designed pore structures are synthesized first. Then, on basis of the abundant pore structures, enzyme molecules can be physically adsorbed into MOFs. It is a very straightforward and common strategy to prepare immobilized enzymes. It is usually simple in operation and has high enzyme loading yield. Meanwhile, the method effectively improves the stability of enzyme<sup>[31]</sup>. However, in some cases, the binding of enzyme molecules on MOFs is weak, since no stable chemical bonds are formed. Therefore, it can cause the leakage of enzyme during repeated utilization of the immobilized enzyme. In addition, this method requires the use of MOFs with large pore sizes which can only be synthesized by laborious steps.

#### 1.4.2.2 Bioconjugation

Bioconjugation refers to the formation of covalent bonds through chemical reactions between MOFs and enzyme molecules, resulting in formation of a stable enzyme-MOF composites. Compared with physical adsorption method, the bioconjugation method makes the binding of enzyme on MOFs more firmly, thus effectively avoids the leakage of the enzyme during long-term use. However, the preparation process of bioconjugation is usually complicated, the types of MOFs are limited, and the chemical reactions sometimes cause serious deactivation of enzyme.

#### 1.4.2.3 Co-precipitation

Co-precipitation is the method which directly encapsulate enzyme in MOFs during the formation of MOF crystals in solution. Lyu *et al.* first introduced this co-precipitation method<sup>[31]</sup>. In their study, for example, Cyt



c, Candida Antarctica lipase B (CALB) and horseradish peroxidase (HRP) were incorporated in ZIF-8 crystals via this co-precipitation method (Figure 1.4). Co-precipitation is a new method of synthesizing enzyme-MOF composites, which has been demonstrated to be general, easy of operation and effective in increasing enzyme stability. However, the activity of enzyme in MOFs in this method is usually relatively low, which could be majorly caused by the small pores of ZIF-8 limiting of the transportation of substrate molecules inside MOFs.



Figure 1.4 Preparation of enzyme-MOF composites by co-precipitation method (derived from reference[28])

### 1.5 Purpose of the research

Among all  $H_2O_2$  detection methods, the electrochemical detection which has the advantages of low detection limit, simple operation, fast response and easily realized miniaturization, has been the most widely studied<sup>[1,2]</sup>. In order to reduce the over potential of electrochemical reaction and reduce the interference of other electroactive substances, enzyme electrochemical biosensors have been developed extensively. However, in the study of



enzymatic electrochemical detection of  $H_2O_2$ , there are still some challenges such as poor immobilized enzyme activity, low electron transfer efficiency and low sensitivity of detection. Our purpose of this study is aiming at improving the immobilized enzyme activity and the electron transfer efficiency between enzyme and the electrode, therefore improving the sensitivity of detection. In this study, Au nanoparticles and Cyt *c* were encapsulated together in amorphous MOFs (aMOFs) by the co-precipitation method (Scheme 1), expecting to increase enzyme activity and enhance the electron transfer between the enzyme and the screen-printed electrode, leading to an increased sensitivity for the fast detection of  $H_2O_2$ .



Scheme 1. Synthesis of Cyt c-AuNP@aZIF-8 and the detection of  $H_2O_2$  by Cyt c-AuNP@aZIF-8/screen-printed electrode



# 2 Materials and methods

## 2.1 Materials

Material	Grade	Manufacturer
2-methyl imidazole	99%	Sigma-Aldrich
Zinc acetate	99%	Sigma-Aldrich
2,2'-azino-bis(3-ethylbenzothiazoline- 6-sulfonic acid) (ABTS)	98%	Sigma-Aldrich
hydrogen peroxide	30%	Xilong Scientific Co., Ltd.
cytochrome c	95%	Sigma-Aldrich
2-quinoline formic acid protein concentration test kit	-	Beyotime Institute of Biotechnology
Nafion	5%	Alfa Aesar

Table 2.1 Chemical reagents used in the experiment

## 2.2 Instruments

Table 2.2 I	Equipment	used in	the	experiment
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Instrument	Model	Manufacturer
Analyzing halance	BSA124S	Sartorius
Thiaryzing balance	D0/11240	Sartorias
pH meter	SeverEASY	Mettler Toledo
Ultraviolet spectrophotometer	UV-2450	Shimadzu



Instrument	Model	Manufacturer
Ultrasonic cleaner	KQ-100	KunShan Ultrasonic Instruments Co., Ltd.
Freeze drier	SCIENTZ-10N	NingBo Scientz Biotechnology Co.,Ltd.
Vacuum drying equipment	ZK	Chengdu Tianyu Experimental Equipment Co., Ltd.
Scanning electron microscope	Sirion 200	Sirion
Scanning electron microscope	JSM 7401F	JEOL
Transmission electron microscope	JEM 2010	JEOL
X-ray diffractometer	D8	Bruker
Thermogravimetric analyzer	TGA 2050	TA Instruments
Electrochemical workstation	CHI852D	CH Instruments, Inc.

Table 2.2 Equipment used in the experiment

## 2.3 Methods

### 2.3.1 Preparation of Cyt c@aZIF-8

Cytochrome c (Cyt c) was dissolved in deionized water at a concentration of 5 mg/mL. 80 mmol/L of 2-methylimidazole in 4 mL water solution and 20 mmol/L of zinc acetate in 4 mL water solution were prepared respectively and subjected to ultrasonic treatment for 10 minutes. Then, 4 mL of 2methylimidazole in water solution, 4 mL of zinc acetate in water solution and 0.333 mL Cyt c solution were mixed and stirred at room temperature (25 °C) for 2 hours. The turbid liquid after reaction was centrifuged and the 13



red-color precipitate was obtained. After washing the precipitate 3 times with deionized water, the product was obtained after lyophilization.



Figure 2.1 Preparation of Cyt c@aZIF-8

### 2.3.2 Preparation of Cyt c-AuNP@aZIF-8

Gold nanoparticles (NPs) were diluted with deionized water to a mass fraction of 0.005%. 4 mL of 2-methylimidazole water solution with a concentration of 80 mmol/L and 4 mL of zinc acetate water solution with a concentration of 20 mmol/L were prepared and subjected to ultrasonic treatment for 10 minutes. Then 4 mL of 2-methylimidazole water solution, 4 mL of zinc acetate water solution, 0.333 mL of Cyt *c* water solution and 0.5 mL of gold NPs solution were mixed and stirred at room temperature (25 °C) reaction for 2 hours. The turbid liquid after reaction was centrifuged and the red-color precipitate was obtained. After washing the precipitate 3 times with deionized water, the product was obtained after lyophilization.





Figure 2.2 Lyophilization

### 2.3.3 Preparation of aZIF-8

4 mL of 2-methylimidazole water solution with a concentration of 80 mmol/L and 4 mL of zinc acetate water solution with a concentration of 20 mmol/L were prepared and subjected to ultrasonic treatment for 10 minutes. Then 4 mL of 2-methylimidazole water solution and 4 mL of zinc acetate water solution were mixed and stirred at room temperature (25 °C) reaction for 2 hours. The turbid liquid after reaction was centrifuged and the precipitate was obtained. After washing the precipitate 3 times with



deionized water, the product was obtained after lyophilization.

#### 2.3.4 Cyt c@aZIF-8 activity assay

In a typical experiment, 900 µL of ABTS aqueous solution of 2.74 mg/mL, 50 µL of hydrogen peroxide solution of volume fraction 0.3% and 50 µL of Cyt c water solution were mixed, followed by determining the absorbance change at 415 nm for 30 s using the ultraviolet spectrophotometer. The same protocol was applied to determine the activity of free Cyt c and immobilized Cyt c at the same protein concentration.

#### 2.3.5 Enzyme concentration assay

BCA method was used to determine the protein concentration. Referring to the bicinchoninic acid (BCA) protein concentration kit, the standard protein was bovine serum albumin, and the absorbance was detected on a *Tecan Sunrise* microplate reader.

#### 2.3.6 Scanning electron microscopy

The samples to be characterized were suspended in deionized water, followed by adding 10-20 µL solution on a silicon wafer, holding for 1-2 minutes, removing the excess liquid and drying at room temperature for 24 h. Then the samples were observed under scanning electron microscope.

#### 2.3.7 Transmission electron microscopy analysis

The samples to be characterized were suspended in deionized water, followed by adding 10-20  $\mu$ L solution on a carbon support film, holding for 1-2 minutes, removing the excess liquid with a filter paper, drying at room temperature for 24 h. Observe under scanning electron microscope. Then the samples were observed under scanning electron microscope.

## 2.3.8 X-ray diffraction



The X-ray diffraction analysis was measured on the Bruker D8 X-ray diffractometer, the scanning speed is 2  $^{\circ}$ /min, and the diffraction angle range is 5  $^{\circ}$  -50  $^{\circ}$ .

#### 2.3.9 Thermogravimetric analysis

Measured on a TGA 2050 thermogravimetric analyzer, the sample was heated in an air atmosphere at a rate of 10 °C/min and a temperature range of 30 °C to 800 °C.

#### 2.3.10 Cyclic voltammetry (CV) analysis

10 µL of ABTS aqueous solution (concentration of 2.74 mg/mL) was added dropwise to the working electrode area of the screen-printing electrode, followed by the drying process at 80 °C in an oven for 8 minutes. After drying, 10 µL of enzyme solution was added dropwise to the working electrode, and subjected to the same 80 °C treatment for 8 minutes as well. At last, a drop of 2 µL Nafion (1%) as a protective layer was applied on the working electrode followed by drying at room temperature. The CV curves were measured on a CHI852D electrochemical workstation with a voltage sweep range of 1.0 V to -1.0 V and a scan rate of 0.5 V/s. Before the start of the CV analysis, 100 µL H<sub>2</sub>O<sub>2</sub> solution was applied on the screen-printed electrode immobilized with enzyme to immerse all the three electrodes.



Figure 2.3 Screen-printing electrode

#### 2.3.11 Chronoamperometry analysis

10 µL of ABTS aqueous solution (concentration of 2.74 mg/mL) was added dropwise to the working electrode area of the screen-printing electrode, followed by the drying process at 80 °C in an oven for 8 minutes. After drying, 10 µL of enzyme solution was added dropwise to the working electrode, and subjected to the same 80 °C treatment for 8 minutes as well. At last, a drop of 2 µL Nafion (1%) as a protective layer was applied on the working electrode followed by drying at room temperature. The chronoamperometry analysis was measured on a CHI852D electrochemical workstation with an operating voltage of -3.0 V. Before the start of the test, 100 µL H<sub>2</sub>O<sub>2</sub> solution was applied on the screen-printed electrode immobilized with enzyme to immerse all the three electrodes. 



Figure 2.4 Chronoamperometry analysis was on the CHI852D electrochemical workstation



## **3 Results and discussion**

## **3.1 Structural characterizations**

The morphology of the prepared samples was characterized by dynamic light scattering (DLS), scanning electron microscopy (SEM), transmission electron microscopy (TEM), X-ray diffraction (XRD), and thermogravimetric analysis (TGA).

#### **3.1.1 Gold nanoparticles**

Gold nanoparticles (AuNPs) were characterized by DLS and TEM. As shown in Figure 3.1 and Figure 3.2, the average size of AuNPs is around 20-25 nm and the size distribution is narrow.



Figure 3.1 DLS of AuNPs in water solution





Figure 3.2 TEM of AuNPs

## 3.1.2 Structure of Cyt c@aZIF-8

The morphology of the as-prepared Cyt *c*@aZIF-8 was observed under SEM and TEM. As shown in Figure 3.4, compared with blank aZIF-8 (without embedding proteins) (Figure 3.3), the as-prepared Cyt *c*@aZIF-8 showed similar morphology as very regular nanospheres with size less than 100 nm. Some unsmooth islands were observed on the surface (Figure 3.3), which might be due the presence of protein molecules. This was further proven by TEM observation. As shown in Figure 3.6, protein molecules with light electron density can be clearly observed, demonstrating the presence of protein the composites. In contrast, blank aZIF-8 has no such regions with light electron density (Figure 3.5).





Figure 3.3 SEM of blank a ZIF-8  $\,$ 



Figure 3.4 SEM of Cyt c@aZIF-8



Figure 3.5 TEM of blank aZIF-8



Figure 3.6 TEM of Cyt c@aZIF-8

As can be analyzed from the SEM and TEM images, the blank aZIF-8 and Cyt *c*@aZIF-8 were presented as small spheres, with diameters about 50-80 nm. The size of the blank aZIF-8 was uniform. The Cyt c @ aZIF-8 exhibited slightly irregular in shape, which indicated that the presence of

Kanning Hulle



Cyt c had some effects on the formation of aZIF-8 during the co-precipitation process.

To futher prove the presence of Cyt c in the aMOFs, the blank aZIF-8 and Cyt c@aZIF-8 were respectively subjected to thermogravimetric analysis. The results are given in Figure 3.7.



Figure 3.7 TGA curves of Cyt c@aZIF-8 and blank aZIF-8

As shown in Figure 3.7, starting from 100 ° C, the water in the blank aZIF-8 and Cyt c@aZIF-8 began to evaporate. After higher than 200 ° C, Cyt c@aZIF-8 was more rapidly degraded than the blank aZIF-8 due to the presence of protein which has a lower degradation temperature than ZIF-8. This result indicated that Cyt c was successfully embedded in aZIF-8. By calculating the weight loss in TGA curves, the embedded amount of Cyt c (i.e., the protein content in the immobilized enzyme product) was about 5%.

#### 3.1.3 Structure of Cyt c-AuNP@aZIF-8



The morphology of Cyt *c*-AuNP@aZIF-8 was observed under SEM and TEM, showing in Figure 3.8 - Figure 3.9.



Figure 3.8 SEM of Cyt c-AuNP@aZIF-8



Figure 3.9 TEM of Cyt c-AuNP@aZIF-8

From the SEM image, it can be observed that the addition of AuNPs



didn't affect the formation of Cyt *c*@aZIF-8. Cyt *c*-AuNP@aZIF-8 was still in spherical shape with a diameter of 50-100 nm, similar as Cyt *c*@aZIF-8. The TEM image further confirmed that some particles had cavities in the middle, and there are small nanoparticles with size of about 20 nm appeared. These nanoparticles were confirmed to be the incorporated AuNPs, although we still cannot determine whether these AuNPs were embedded inside aZIF-8 or just adsorbed on the surface of the composite.

## 3.1.4 Crystal structural of Cyt c (-AuNP)@aMOFs

To further determine the structural properties of Cyt *c* (-AuNP)@aMOFs, Cyt *c*@aMOFs and Cyt *c*-AuNP@aMOFs were analyzed by XRD. The XRD patterns were shown in Figure 3.10.



Figure 3.10 XRD patterns of blank a ZIF-8 and Cyt c (-AuNP)@a ZIF-8

From the X-ray diffraction patterns, it can be seen that the crystal



diffraction peaks did not appear in the synthesized Cyt *c*@aZIF-8, Cyt *c*-AuNP@aZIF-8 and aZIF-8, indicating that the synthesized products were amorphous. In addition, XRD patterns of the products with Cyt *c* and AuNPs were consistent with the diffraction peak pattern of blank aZIF-8, which indicated that the embedding of enzyme and AuNP didn't the crystal structure of the composites.

### 3.2 Performance of Cyt c(-AuNP)@aZIF-8

The loading of enzyme in the composites and the enzyme activity are important for studying the performance of the immobilized enzymes. In this study, the protein concentration in the supernatant was measured by using the BCA protein concentration test kit. After subtracting the protein content in the supernatant from the total amount of the added enzyme, we can calculate the loading amount of Cyt c in the composites. The activity of enzyme was measured by using ABTS and H<sub>2</sub>O<sub>2</sub> as the reaction substrates. The change of absorbance at 415 nm was detected by a UV spectrophotometer.

The loading amount Cyt *c* in the composites was determined as about 5% in this study for the samples of Cyt *c*@aZIF-8 and Cyt *c*-AuNP@aZIF-8, indicating that the presence of AuNPs didn't affect the loading of protein.

From the results of enzyme activity assay, the apparent enzyme activity of Cyt *c*@aZIF-8 was significantly higher than that of natural enzyme, by an incensement of 500%-600%. The addition of AuNPs has no impact on the activity of immobilized enzyme, which was still maintaining at 500%-600% of natural enzyme.

### 3.3 Optimization of synthesis conditions

To investigate the optimum condition for the preparation of Cyt c(-AuNP)@aZIF-8 by the co-precipitation method, the reaction time, the



amount of AuNPs and the amount of enzyme were optimized respectively.

#### 3.3.1 Reaction time

The reaction time may have influence on the morphology, structure and aggregation degree of aMOFs. Therefore, the reaction time of the coprecipitation process was optimized in this experiment. At the fixed concentrations of reactants (2-methylimidazole: 80 mmol/L, 4mL; zinc acetate: 20mmol/L, 4mL; Cyt c: 10 mg/mL, 0.333 mL), the reaction time from 1 h to 5 h was investigated. The enzyme activities of composites obtained at different reaction times were measured, and the results were shown in Figure 3.11.



Figure 3.11 The activity at different reaction times

The result indicated that the reaction time had some effect on the activity of enzyme. After the reaction for 1 h, enzyme activity was relatively



low; for 2-4 h, enzyme activity was relatively high, about 500%-600% of native Cyt *c*. Further increase of reaction time can result in decrease of activity. Therefore, an optimized reaction time is 2 h, which can achieve 562.0% activity of natural enzyme.

#### 3.3.2 Enzyme concentration

To obtain a higher enzyme activity of the Cyt c@aZIF-8, the feeding concentration of enzyme in the co-precipitation method was optimized. The reaction time was set to 2 h, the volume of Cyt c was 0.333 mL, and the concentration of Cyt c was varied as 10, 5, 4, 3 and 1 mg/mL. The enzyme activities of the as-prepared composites at different concentrations of Cyt c were measured respectively. The results were shown in Figure 3.12.



Figure 3.12 The activity at different enzyme concentrations

From the above results, we can see that when the concentration of Cyt c was 5 mg/mL, the enzyme activity was higher, which was 635% of the



natural enzyme. Therefore, the optimized reaction time was 2 h and the concentration of Cyt c was 5 mg/mL.

#### 3.3.3 Amount of AuNPs

To investigate the effect of the addition amount of AuNPs on the activity of immobilized Cyt c, the addition of 100, 200, 300, 500 µL AuNPs to the coprecipitation process was carried out. As shown in Figure 3.13, the effect of different amounts of AuNPs on the activity of immobilized enzyme was studied.



Figure 3.12 The activity at different amounts AuNPs

From Figure 3.12, it was found that the addition of AuNPs had no effect on the activity of immobilized enzyme, and the enzyme activity remained at 500%-600% of the activity of natural enzyme.

## 3.4 Detection of hydrogen peroxide by Cyt c (-



## AuNP)@aMOFs

To investigate the electrochemical detection of  $H_2O_2$ , the immobilized enzyme Cyt *c*@aZIF-8 and Cyt *c*-AuNP@aZIF-8 were coated on the threeelectrode screen-printed electrode and subjected to electrochemical analysis. The material of working electrode and counter electrode is carbon, and the material of reference electrode material is silver / silver chloride. The electron mediator and the enzyme sample were fixed in the working electrode region before detection). The electrochemical detection performance was evaluated by cyclic voltammetry and chronoamperometry.

### **3.4.1 Cyclic voltammetry**

First, cyclic voltammetry (CV) was used to test the performance of Cyt c and Cyt c@aZIF-8. The CV was measured to determine the cathode peak current.



Figure 3.13 CV curve of natural Cyt c



Figure 3.14 CV curve of Cyt c@aZIF-8

Figure 3.13 and Figure 3.14 showed the CV curves of natural Cyt c and Cyt c@aZIF-8, respectively. The black line represented the experiment with no electron mediator ABTS added, but with enzyme and H<sub>2</sub>O<sub>2</sub> solution (0.3%) added. The blue line represented the experiment only the electron mediator ABTS and H<sub>2</sub>O<sub>2</sub> solution (0.3%) added. And the red line represented the experiment with the addition of ABTS, enzyme and H<sub>2</sub>O<sub>2</sub> solution.

From the CV curves, it can be found that the current response of the native Cyt *c* and the immobilized enzyme was neglectable and no significant redox peak was observed without the addition of the electron mediator ABTS. It is shown that the direct electron transfer efficiency between the enzyme and the electrode is very low in the absence of electron mediator, which is due to the fact that the active site of enzyme is encapsulated inside the protein and is difficult to contact with the electrode for electron transport. After the addition of the electron mediator ABTS, the current



response values of the native Cyt c and Cyt c@aZIF-8 were both increased, and the apparent redox peak appeared. Compared with natural enzymes, the peak current value of the Cyt c@aZIF-8 was relatively low and the position of the reduction peak was slightly shifted. This phenomenon might be caused by the fact that the immobilization material which is aZIF-8 can hinder the electron transfer. The cathode peak of the natural Cyt c is located at -0.15 V and the cathode peak of Cyt c@aZIF-8 is at -0.30 V.

The cyclic voltammetry curves of Cyt *c*-AuNP@aZIF-8 with AuNPs were further analyzed to explore the effect of AuNP addition on the electrochemical process.



Figure 3.15 CV curves of Cyt c-AuNP@aZIF-8 with addition of AuNPs

It can be seen from Figure 3.15 that, for the Cyt c-AuNP@aZIF-8,



without the assistance of electron mediator ABTS, the current response was still weak and no significant redox peak appeared. It is still difficult to achieve the direct electron transfer between the enzyme and the electrode. The addition of ABTS resulted in a significant increase in the current response for Cyt *c*-AuNP@aZIF-8. The addition of 500  $\mu$ L AuNPs, compared with Cyt *c*@aZIF-8, resulted in an enhanced current response, which can be contributed to the promotion of electron transfer with the assistance of conductive AuNPs.

#### 3.4.2 Sensitivity

For sensitivity test, 10 µL of ABTS aqueous solution (concentration of 2.74 mg/mL) was added dropwise to the working electrode area of the screen-printing electrode, followed by the drying process at 80 °C in an oven for 8 minutes. After drying, 10 µL of enzyme solution was added dropwise to the working electrode, and subjected to the same 80 °C treatment for 8 minutes as well. At last, a drop of 2 µL Nafion (1%) as a protective layer was applied on the working electrode followed by drying at room temperature. The chronoamperometry analysis was measured on a CHI852D electrochemical workstation with an operating voltage of -3.0 V. The current response was measured for 60 s, and the response current at 60 s was recorded. The calibration curves for the detection of  $H_2O_2$  using different enzyme samples were shown in Figure 3.16.





Figure 3.16 Calibration curve of  $H_2O_2$  detection

(Black for natural Cyt c, red for Cyt c @ aZIF-8, blue for Cyt c-AuNP @ aZIF-8)

It can be seen from the calibration curves that using the natural enzyme Cyt c, Cyt *c*@aZIF-8 and Cyt *c*-AuNP500@aZIF-8 as the enzyme samples have good linearity in the 5-30 mmol/L H<sub>2</sub>O<sub>2</sub> solution concentration range. The detection sensitivity of the Cyt *c*@aZIF-8 enzyme electrode (as reflected by the slope of the standard curve) was increased by 72% compared with the enzyme electrode prepared by natural Cyt *c*. After further co-embedding the AuNPs, the sensitivity of Cyt *c*-AuNP@aZIF-8 enzyme electrode was further enhanced by 23% compared to the Cyt c@aZIF-8 enzyme electrode.

Compared with the natural enzyme Cyt c, the increase of sensitivity for Cyt c@ZIF-8 might be due to the enrichment of H<sub>2</sub>O<sub>2</sub> in the surrounding environment of the immobilization carriers. In addition, because of the amorphous metal-organic frameworks, the composites had more mesopores, which displayed less influence on the conformational change of enzyme



during the catalysis, and therefor increased enzyme activity. For Cyt *c*-AuNP@aZIF-8, the presence of AuNPs further increased the current response, showing that AuNPs can enhance the electron transfer, making the detection even more sensitive.



## 4. Conclusion

In this study, the composite composed of Cyt c and aZIF-8 was prepared by an aqueous co-precipitation method. AuNPs were co-embedded in the composite to form Cyt c-AuNP@aZIF-8. The composites of Cyt c@aZIF-8 and Cyt c-AuNP@aZIF-8 were applied to prepare the screen-printed enzyme electrode for the fast detection of H<sub>2</sub>O<sub>2</sub>. It is expected to both increase enzyme activity and enhance the electron transfer efficiency between the enzyme and the electrode, and therefore improve the detection sensitivity. The conclusion of this study is as follows:

- (1) The immobilized enzyme Cyt c@aZIF-8 was successfully prepared by the aqueous co-precipitation method. It was proved that the composite was in an amorphous form. The activity of Cyt c@aZIF-8 was significantly increased to 500% -600% of the natural enzyme. The optimal reaction time for the co-precipitation process and concentration of enzyme were respectively 2 h and 5 mg/mL. At this condition, the particle size of Cyt c@aZIF-8 was around 50-80 nm, and the enzyme activity was 635% of natural enzyme.
- (2) AuNPs were added to prepare the Cyt *c*-AuNP@aZIF-8. It was proved that the addition of 500 µL 0.005 w% AuNPs aqueous solution did not affect the morphology and amorphous structure of Cyt *c*@aZIF-8 and the particle size of Cyt *c*-AuNP@aZIF-8 was around 50-100 nm. The enzyme activity was still at a high level, which was 500%-600% of the natural enzyme.
- (3) The electrochemical detection of H<sub>2</sub>O<sub>2</sub> based on the screen-printed enzyme electrode using Cyt c, Cyt c@aZIF-8 and Cyt c-AuNP@aZIF-8 as the enzyme samples was investigated. Within the detection range of 5-30 mmol/L H<sub>2</sub>O<sub>2</sub>, the calibration curves for all enzyme samples showed



good linearity. The detection sensitivity of the Cyt c@aZIF-8 enzyme electrode was increased by 72% compared with the enzyme electrode prepared by natural Cyt c. After further co-embedding AuNPs, the sensitivity of Cyt c-AuNP@aZIF-8 enzyme electrode was further enhanced by 23% compared to the Cyt c@aZIF-8 enzyme electrode. From this study we can conclude that with enzyme and AuNPs co-embedding in aMOFs, the enzyme activity and electron transfer can be promoted and therefore the detection sensitivity can be significantly increased, indicating that the composites of enzyme-AuNP-aMOFs could be very promising for constructing highly sensitive enzyme-based electrochemical biosensors.



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