

A $\text{Cu}_2(\text{OH})_3\text{Cl}\text{-CeO}_2$ nanocomposite with peroxidase-like activity, and its application to the determination of hydrogen peroxide, glucose and cholesterol

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Abstract A nanomaterial of the chemical composition $\text{Cu}_2(\text{OH})_3\text{Cl}\text{-CeO}_2$ and with a large surface area is shown to be a viable peroxidase mimetic. It was synthesized by coprecipitation of an aqueous solution containing Ce(III) chloride, Cu(II) chloride and hexamethylenetetramine by adding an ionic liquid. The material was characterized by scanning electron microscopy and X-ray powder diffractometry. The composite possesses peroxidase-like activity and catalyzes the oxidation of the peroxidase substrate 3,3',5,5'-tetramethylbenzidine by H_2O_2 to produce a blue product. Based on this finding, a simple, rapid and selective colorimetric method was worked out for the determination of glucose and cholesterol by using the respective oxidases and by quantifying the H_2O_2 formed. Both glucose and cholesterol can be determined by this method at levels as low as 50 μM .

Keywords Photometry · Peroxidase mimic · Hydrogen peroxide · Copper complex · Enzymatic biosensor

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Introduction

Natural peroxidases serve as catalysts to degrade H_2O_2 in biological systems [1]. H_2O_2 is a spontaneously occurring byproduct of oxygen metabolism in human body [2]. Its concentration is adjusted by peroxidases. Peroxidase is present in high concentration in the kidneys and erythrocytes, and its expression is adjusted at various levels [3]. Peroxidases are crucial for human metabolism, but they catalyze the degradation H_2O_2 under gentle biological conditions only [4]. So, the widespread application of such enzyme is limited by their intrinsic properties such as easily denaturing under ultra pH conditions and high temperatures. Besides, their applications were also restricted by their high cost and rigorous storage requirements [5]. Therefore, the development of artificial mimetic enzymes is indispensable and has been arousing intense interest. Although a number of mimetic enzymes have been applied in this field, the catalytic activities of molecules including cyclodextrin, porphyrin, DNAzyme, molecularly imprinted hydrogels, and functional polymers are much lower compared to those of natural enzymes [6, 7].

Numerous kinds of mimetic enzymes have been synthesized based on metal materials [8, 9]. Among them, Cu-based materials have attracted much attention, because copper ions play an important role in differing biological processes such as dioxygen-activating enzymes process and oxygen oxidation process [10, 11]. Low molecular weight copper complexes were also studied as active centers of copper containing mimetic enzyme [12–15]. Highly dispersed CuO is a well-known component of catalysts in various industrial reactions, and it has also been reported as mimetic enzyme [16]. However, there were few reports about $\text{Cu}_2(\text{OH})_3\text{Cl}$ serving as mimetic enzyme. Here, we synthesized

$\text{Cu}_2(\text{OH})_3\text{Cl}$ and found out that it is a good candidate as a component of mimetic enzyme due to its high activity and low cost [17]. In addition, Ce, a very effective material, is frequently added to the Cu-based catalysts to make them working more efficiently than the commercial noble metal catalysts [18]. Asati et al. [19] reported that cerium oxide nanoparticles showed an intrinsic oxidase-like activity at acidic environment, and it also have been reported that CeO_2 were used as mimetic peroxidase for H_2O_2 and glucose detection [20]. Furthermore, in many processes, the mixed metal oxides have been used as heterogeneous catalysts for oxidation, hydrogenation and condensation reaction [21]. The materials of Cu and Ce hold considerable promise for utilization in catalysts due to their relatively high abundance and the low cost of copper and cerium salt [22]. The catalysts rooted in metal hydroxide precursors are characterized by longer lifetimes and higher activity [23]. Consequently, $\text{Cu}_2(\text{OH})_3\text{Cl}\text{-CeO}_2$ composite material as mimetic enzyme were synthesized in this work.

In addition, ionic liquids have been arising interest in the last decade with a diversified range of applications [24], and they have been investigated for their special solvent properties in a wide range of processes [25]. Herein, instead of using traditional volatile organic solutions, we used ionic liquids, as medium in the synthesis of $\text{Cu}_2(\text{OH})_3\text{Cl}\text{-CeO}_2$ composite material. In this compound reaction, ionic liquid provided a more favorable environment different from other solvents due to its low vapor pressure, high adhesiveness and adjustable dissolving ability [26, 27], which would enhance the stability of catalytic and also improves its activity.

We presented a $\text{Cu}_2(\text{OH})_3\text{Cl}\text{-CeO}_2$ nanocomposite prepared by a facile co-precipitation in the aqueous solution containing ionic liquids as reaction medium. For the more, we used hexamethylenetetramine to provide an alkaline environment in this experiment. Additionally, the morphology and element component of this nanocomposite material were characterized by SEM and XRD. The peroxidase activity of the material, the optimum pH and temperature and catalytic mechanism were studied. Consequently, the calibration curves for H_2O_2 , glucose and cholesterol were obtained. Then the method of colorimetric determination of glucose and cholesterol was established based on the reaction of TMB and H_2O_2 producing blue solution. The detection limit is low enough to determine glucose and cholesterol in human serum. This study will offer a promising strategy in diagnosis and biochemistry fields.

Experimental section

Reagents and materials

Cerium chloride ($\text{CeCl}_3 \cdot 7\text{H}_2\text{O}$), TMB, glucose oxidase (GOx) and cholesterol oxidase were purchased from Aladdin

(Shanghai, China, <http://www.aladdin-reagent.com>). Ionic liquids of 1-Ethyl-3-methylimidazolium chloride were purchased from Chengjie Chemical Reagents Co., Ltd (Shanghai, China, <http://www.shyfx.com>). Copper chloride ($\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$), H_2O_2 , and other chemical reagents were purchased from Kay Tong Chemical Reagents Co., Ltd (Tianjin, China, <http://tjksj.cn.china.cn>). All reagents employed were of analytical grade and used without further purification. Phosphate buffer (pH from 2.0 to 10.0) was used and double distilled deionized water was applied throughout the experiment.

Apparatus

Scanning electron microscope (SEM) was recorded on a JSM-6610 scanning electron microscope (Japan). X-ray powder diffract meter (XRD) data was collected using a Rigaku DLMAX-2550 V diffract meter (40 kv, Cu Ka; 2 range 5–80°; scan speed of 60° min⁻¹). Kinetic measurements and UV–vis absorption spectra were carried out on a UV-2450 Shimadzu Vis-spectrometer (Japan). Photographs were taken using a Canon G11 digital camera. A Guohua SHA-C constant-temperature shaker (Shanghai, China) and a Jingli Ld4-2 low-speed centrifuge (Beijing, China) were used.

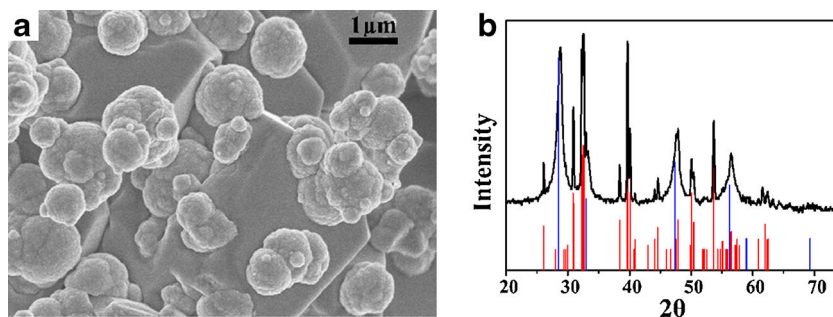
Synthesis of $\text{Cu}_2(\text{OH})_3\text{Cl}\text{-CeO}_2$ composite material

The material was prepared by a facile co-precipitation method in the aqueous solution adding $\text{CeCl}_3 \cdot 7\text{H}_2\text{O}$ and $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, containing ionic liquids as green medium. The molecular ratio of copper salt to cerium salt was at a fixed constant ion concentration (2:1). 0.4672 g of $\text{CeCl}_3 \cdot 7\text{H}_2\text{O}$ and 0.4276 g of $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ were dissolved in 200 mL distilled deionized water and then brought to oil bath heating at 95 °C while magnetic stirring in a round bottom flask fitted with a reflux condenser, and 2.3945 g of ionic liquids were rapidly added to the solution. The solution was heated to 95 °C under nitrogen. Two hours later, 50 mL 90 mM hexamethylenetetramine was added to the solution drop by drop and drop over a period of 30 min. The solution was additionally reacted for 5 h with constant fluxing under nitrogen and magnetic stirring. The samples were separated by filtration and further washed with water after the reaction completed, and then naturally dried at room temperature. Then the material was stored at room temperature and redispersed in water when used.

Assay for catalytic activity study

To investigate the peroxidase-like activity of the material, the catalytic oxidation of the peroxidase substrate TMB in the presence of H_2O_2 was tested. Kinetic measurements were carried out by photometry at an analytical wavelength of 652 nm [28]. Experiments were carried out using 40 $\mu\text{g} \cdot \text{mL}^{-1}$

Fig. 1 **a** SEM image and **b** XRD image of $\text{Cu}_2(\text{OH})_3\text{Cl}-\text{CeO}_2$ composite material. The red line and blue line represent the standard XRD pattern of $\text{Cu}_2(\text{OH})_3\text{Cl}$ and CeO_2 , respectively



material in a reaction volume of 400 μL phosphate buffer (25 mM PBS, pH 4.0, 25 $^\circ\text{C}$) with 800 μM TMB as substrate, and H_2O_2 concentration was varied. The Michaelis–Menten constant was calculated using the Lineweaver–Burk plot.

To examine the influence of reaction buffer pH and reaction temperature on the material activity, 0.1 M phosphate buffer from pH 2.0 to 7.0 and temperature from 20 to 50 $^\circ\text{C}$ were investigated, under condition identical to these used above. The final reaction solution was used to perform the adsorption spectroscopy measurement.

Assay for glucose and cholesterol detection

Glucose detection was taken place in air-saturated solution as previously reported [29, 30]. Firstly, 10 μL of 40 $\text{mg}\cdot\text{mL}^{-1}$ GOx and 100 μL glucose of different concentrations in 0.5 mM phosphate buffer (pH 7.0) were reacted at 37 $^\circ\text{C}$ for 1 h. In addition, 50 μL of 8 mM TMB, 160 μL of 0.1 M phosphate buffer (pH 3.0), and 80 μL of the material colloidal suspension were added into above 110 μL glucose reaction solution. Finally, the solution was used for adsorption spectroscopy measurement. Cholesterol detection was similar to glucose detection, but the difference was that the concentration of cholesterol oxidase was 13 $\text{mg}\cdot\text{mL}^{-1}$.

Results and discussion

Characterization of the product

The material was prepared using co-precipitation method by adding the ionic liquid (1-Ethyl-3-methylimidazolium chloride) to the aqueous solution of CeCl_3 and CuCl_2 . The terminal product was a very fine green powder and can be well dispersed in water. The morphology, shape and size of the samples were characterized by SEM. SEM images (Fig. 1a) indicated that the spheres with diameter about 0.8 μm were adhered on the large crystals with diameter of 5–10 μm . Subsequently, the phase and structures of the as-prepared powders were characterized by XRD. As shown in Fig. 1b, all the diffraction peaks of the powders match well with the $\text{Cu}_2(\text{OH})_3\text{Cl}$ Phase (JCPDS 18–0886) as shown in the red line

and cerium oxide Phase (JCPDS 43–1002) as shown in the blue line, indicating the formation of a pure $\text{Cu}_2(\text{OH})_3\text{Cl}-\text{CeO}_2$ composite material.

Peroxidase-like activity of $\text{Cu}_2(\text{OH})_3\text{Cl}-\text{CeO}_2$ composite material

To explore the peroxidase-like activity of the material, its catalytic oxidation of TMB was detected in the presence of H_2O_2 . As shown in the insert in Fig. 2, the color changes of TMB in different systems were observed. In the $\text{H}_2\text{O}_2+\text{TMB}$ system without catalysts, the solution was colorless and showed a negligible color change. So did TMB+catalyst system. But the solution presented an obvious blue in the $\text{H}_2\text{O}_2+\text{TMB}+\text{catalysts}$ system. The phenomenon of the solutions' color changing from colorless to blue indicated that the material indeed exhibits a catalytic behavior toward TMB oxidation by H_2O_2 . Similarly, as shown in Fig. 2, the material possesses excellent peroxidase-like activity with the spectra of TMB in different systems. The $\text{H}_2\text{O}_2+\text{TMB}$ system without catalyst presents almost no absorption corresponding to the black line in the graph. Also the solution shows a small negligible absorption in TMB+catalysts system corresponding to the red line in the graph. The main reason must be the role of catalysts. In addition, the $\text{H}_2\text{O}_2+\text{TMB}+\text{catalysts}$ system has a great absorbance at 652 nm corresponding to the blue line in

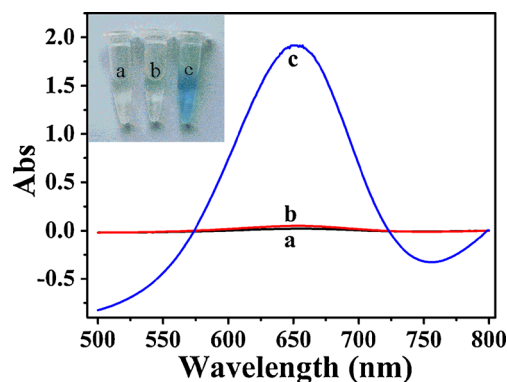


Fig. 2 Absorbance spectra in different reaction systems. The solutions incubated in 25 mM phosphate buffer (pH 3.0) for 10 min at the room temperature. Insert: change in color **a**: $\text{H}_2\text{O}_2+\text{TMB}$ **b**: TMB+catalysts ($<1 \text{ mg}\cdot\text{mL}^{-1}$) **c**: $\text{H}_2\text{O}_2+\text{TMB}+\text{catalysts}$ ($<1 \text{ mg}\cdot\text{mL}^{-1}$)

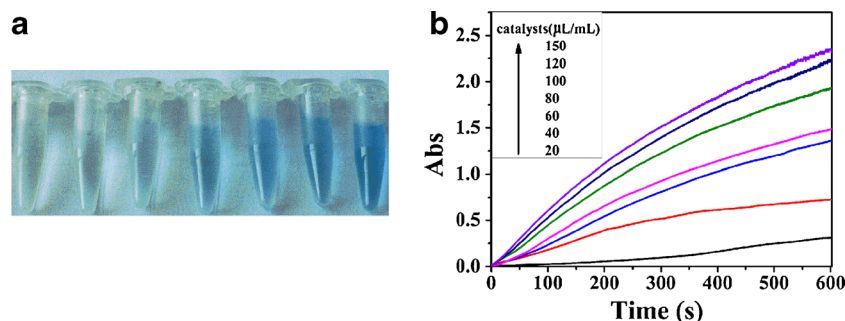


Fig. 3 **a** Time-dependent color changes of 800 μM TMB (10 mM H_2O_2) in the catalytic assay with different concentration of material after placing at the room temperature for 10 min. Concentration from left to right: 20, 40, 60, 80, 100, 120, 150 $\mu\text{L}\cdot\text{mL}^{-1}$, respectively. **b** Time-dependent

absorbance changes at 652 nm of 800 μM TMB reaction solutions in the absence or presence of different doses of material in 25 mM phosphate buffer (pH 3.0) at the room temperature

the graph which indicates the production of oxidized TMB. The conclusion is that the material exhibits excellent peroxidase-like activity when it catalyzed H_2O_2 oxidizing the chromomeric substrate TMB producing a blue color reaction, which has maximum absorbance at 652 nm.

Reaction conditions

It is important to study the catalytic activity of material influenced by optimum pH, optimum temperature, and catalyst concentration. Primarily, the effect of pH and temperature was investigated (Fig. S1, Electronic Supplementary Material, ESM). When the catalyzing reaction of this material is taken place in pH 3.0 and 40 $^\circ\text{C}$, the absorbance at 652 nm reached its peak. Thus phosphate buffer saline (pH 3.0) at 40 $^\circ\text{C}$ was chosen as the reaction environment in this experiment.

Additionally, the intrinsic catalytic activity of this material was examined for further study under the optimum pH and temperature. Figure 3a shows the different color changes coupling with the increasing material concentration in different reaction system. As the material's concentration increased, the reaction solution displayed color changing from light to dark blue, which provided a colorimetric method for H_2O_2 detection. Figure 3b shows the changes of the time course-

dependent absorbance by adding different concentration of material. When the catalyst's concentration increased from 20 to 150 $\mu\text{L}\cdot\text{mL}^{-1}$, the reaction rate increased coupling with it, and thus 150 $\mu\text{L}\cdot\text{mL}^{-1}$ was used as the concentration of this material in the following experiment.

The peroxidase-like activity of the material was further studied using steady-state kinetic with H_2O_2 and TMB at the last. The plots of the reciprocal initial velocity versus the reciprocal substrate concentration were obtained by varying concentration of the other substrate (Fig. S2, ESM). The approximate parallel lines demonstrate that the slopes are similar and conform to the Ping-Pong mechanism [30], which is similar to that of horseradish peroxidase. The equations and R^2 of TMB and H_2O_2 were obtained using the Lineweaver-Burk plot in this system (Table S1, ESM).

Detection of H_2O_2 , glucose and cholesterol

Figure 4a shows the absorbance spectra at 652 nm of oxidized TMB in the presence of different concentrations of H_2O_2 under the optimum conditions. H_2O_2 can be simply detected by using a UV-vis spectrometer or even the naked eye. We designed a colorimetric method for the detection of H_2O_2 , glucose and cholesterol using this material catalyzing blue color

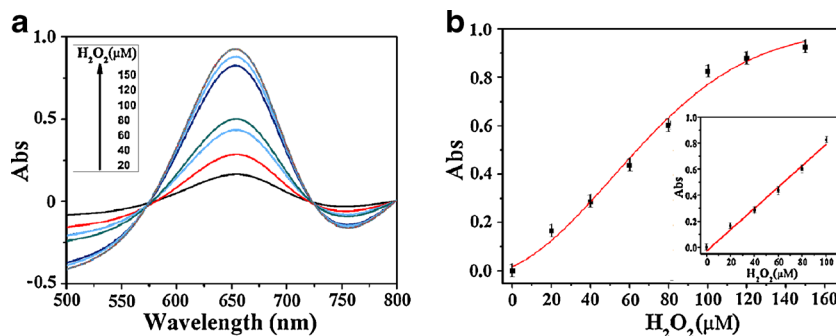


Fig. 4 **a** Absorbance spectra of TMB reaction solutions catalyzed by material in the presence of different concentration of H_2O_2 in 25 mM phosphate buffer (pH 3.0) at the room temperature. **b** A dose-response

curve for H_2O_2 detection. Inset: linear calibration plot for H_2O_2 . The error bars shown are the standard errors derived from three measurements

reaction based on its intrinsic peroxidase property. Figure 4b shows the H₂O₂ concentration-response curves gained from optimum absorbance in Fig. 4a. The linear range is from 0.02 to 0.05 mM and H₂O₂ can be detected as low as 0.01 mM.

Hydrogen peroxide can be the product of glucose oxidase (GOx) catalyzed and cholesterol oxidase-catalyzed reaction. Therefore, colorimetric detection of glucose and cholesterol can be realized using this material instead of natural peroxidase enzymes. Figure S3a and S3c shows the absorbance spectra curve of different concentrations of glucose and cholesterol. Glucose can be detected as low as 0.05 mM and the linear range was from 0.1 to 2 mM in the calibration curve (Fig. S3b, ESM) and cholesterol can be detected as low as 0.05 mM and the linear range was from 0.1 to 2 mM in the calibration curve (Fig. S3d, ESM). These results demonstrate that the sensing system had a high selectivity for glucose and cholesterol detection (Table S2 and S3, ESM). Therefore, the proposed method can be applied to detect glucose and cholesterol in real samples.

Conclusion

In summary, a fast and selective colorimetric methods were established to detect H₂O₂, glucose, cholesterol using the Cu₂(OH)₃Cl-CeO₂ composite material that were successfully synthesized by a facile co-precipitation in the aqueous solution containing ionic liquids. In addition, the composite material having intrinsic peroxidase-like activity can fleetly catalyze oxidation of the peroxidase substrate TMB in the presence of H₂O₂ to produce a blue color reaction. On this basis, simple and selective colorimetric methods for the glucose and cholesterol detection were developed, and the detection limit of the glucose and cholesterol was 0.05 mM. This result will be used in clinical diagnosis and biochemistry.

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