Peroxidase-like activity of water-soluble cupric oxide nanoparticles and its analytical application for detection of hydrogen peroxide and glucose†

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Water-soluble cupric oxide nanoparticles are fabricated via a quick-precipitation method and used as peroxidase mimetics for ultrasensitive detection of hydrogen peroxide and glucose. The water-soluble CuO nanoparticles show much higher catalytic activity than that of commercial CuO nanoparticles due to their higher affinity to hydrogen peroxide. In addition, the as-prepared CuO nanoparticles are stable over a wide range of pH and temperature. This excellent stability in the form of aqueous colloidal suspensions makes the application of the water-soluble CuO nanoparticles easier in aqueous systems. A colorimetric assay for hydrogen peroxide and glucose has been established based on the catalytic oxidation of phenol coupled with 4- amino-atipyrine by the action of hydrogen peroxide. This analytical platform not only confirms the intrinsic peroxidase-like activity of the water-soluble cupric oxide nanoparticles, but also shows its great potential applications in environmental chemistry, biotechnology and medicine.

Introduction

Enzymes are a type of efficient biological catalyst, which are involved in almost all reactions in vivo. Enzyme-catalyzed reactions are of widespread interest due to their high efficiency, high specificity, and mild conditions needed. In addition, enzyme-based analysis has a wide range of applications in clinical diagnosis, biotechnology, chemistry, environmental science, and other fields. However, limited natural sources, difficult and high-cost purification processes, and inherent instability restricts enzyme application to some extent. Therefore, more and more attention has been paid to constructing enzyme mimetics with similar functions to natural enzymes in recent years. 1 Many mimetics have been developed for enzymes such as cytochrome P450, 2 serine protease, 3 dioxygenase, 4,5 phosphodiesterase, 6–9 lipase, 10 acylase, 11 ligase, 12 hydrolase, 13 aldolase, 14 superoxide dismutase, 15 and nitrile hydratases. 16 Peroxidase can efficiently catalyze the oxidation of electron donors by hydrogen peroxide, which is an important intermediate product of many biological reactions. Numerous substrates for horseradish peroxidase (HRP) have been described and commercialized for biochemical applications. The use of HRP with a chromogenic donor has been proven to be very useful for assay systems producing hydrogen peroxide, for example, in the determination of glucose in the presence of glucose oxidase. In addition, peroxidase labelled immunoglobulins have been successfully used as immunohistological probes for the demonstration of tissue antigens, and in enzyme amplified immuno-assay systems for the quantitative determination of soluble and insoluble antigens. As alternatives to natural enzymes, hemein, 17 hemeatin, 18 metal-porphyrin, 19 metal-phthalocyanine, 20 metal hexacyanoferrate, 21–22 Schiff base complex, 23 and carboxyl functionalized mesoporous polymers 24 have been utilized as peroxidase mimetics for enzymatic analysis.

Recently, iron oxide magnetic nanoparticles have been found to possess intrinsic enzyme mimetic activity similar to that of natural peroxidases. 25 Since this report, increasing attention has been paid to the nanoscaled peroxidase mimetics and their potential applications. 26–39 In our recent study we found that cupric oxide nanoparticles are highly effective catalysts to peroxidase substrates, and the binding affinity for the substrate 3,3′,5,5′-tetramethylbenzidine (TMB) is higher than that of HRP and other peroxidase nano-mimics. 40 Furthermore, these cupric oxide nanoparticles are considerably more stable and possess an almost unchanged catalytic activity over a wide range of pH and temperatures. However, the aggregation and settlement of the commercial cupric oxide nanoparticles in aqueous systems will affect their applications to some extent. In this work, water-soluble cupric oxide nanoparticles were fabricated and used as peroxidase nano-mimics. With high peroxidase-like activity, the water-soluble cupric oxide nanoparticles can be facilely applied in enzymatic analysis. As a demonstration, a colorimetric method for hydrogen and glucose detection was developed.

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Experimental

Chemicals and materials

Cupric acetate, sodium hydroxide, glacial acetic acid, phenol, 4-amino-atipyrine (4-AAP), and 30% H₂O₂ were purchased from Sinopharm Chemical Reagent Co. Ltd (Shanghai, China). Glucose oxidase (GOx), 3,3',5,5'-tetramethylbenzidine (TMB), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 3,3'-diaminobenzidine (DAB), and commercial cupric oxide nanoparticles (30 nm) were purchased from Aladdin Reagent Company (Shanghai, China). Other reagents and chemicals were of at least analytical reagent grade. The water used was purified by a Milli-Q system (Millipore, USA).

Preparation and characterization of water-soluble cupric oxide nanoparticles

The cupric oxide nanoparticles were prepared via a previously reported quick-precipitation method. Briefly, 150 ml of 0.02 M copper acetate aqueous solution was mixed with 0.5 ml glacial acetic acid in a round-bottomed flask equipped with a refluxing device. The solution was heated to boiling with vigorous stirring. Then 10 ml of 0.04 g ml⁻¹ NaOH aqueous solution was rapidly added into the above boiling solution, where a large amount of black precipitate was immediately formed. The precipitate was centrifuged, washed three times with absolute ethanol, and dried in air at room temperature. The morphology and size distribution of the nanoparticles were studied by using a transmission electron microscope (TEM) of Tacnai-12 (Philip, Netherlands). The crystal phase was investigated by X’Pert Pro MPD X-ray diffractometer (Panalytical, Netherlands). Fourier transform infrared (FTIR) spectra were taken in KBr pressed pellets on an AVATR 330 FT-IR spectrometer ( Nicolet Thermo, USA).

Investigation of the peroxidase-like activity of the CuO nanoparticles

To investigate the peroxidase-like activity of the as-prepared CuO nanoparticles, the catalytic oxidation of the peroxidase substrate TMB in the presence of H₂O₂ was tested. A typical experiment was carried out at 37 °C by using 4 μg mL⁻¹ CuO nanoparticles in a reaction volume of 5.0 ml with 665 μM TMB and 1.5 M H₂O₂ as substrates. The blue color that developed as the reaction proceeded was monitored in time scan mode at 652 nm using a Shimadzu UV-2450 spectrophotometer. The solution was heated to boiling with vigorous stirring. Then 10 ml of 0.04 g ml⁻¹ NaOH aqueous solution was rapidly added into the above boiling solution, where a large amount of black precipitate was immediately formed. The precipitate was centrifuged, washed three times with absolute ethanol, and dried in air at room temperature. The morphology and size distribution of the nanoparticles were studied by using a transmission electron microscope (TEM) of Tacnai-12 (Philip, Netherlands). The crystal phase was investigated by X’Pert Pro MPD X-ray diffractometer (Panalytical, Netherlands). Fourier transform infrared (FTIR) spectra were taken in KBr pressed pellets on an AVATR 330 FT-IR spectrometer ( Nicolet Thermo, USA).

H₂O₂ detection

In a typical experiment, (a) 2.0 ml of 6.0 mM phenol, 790 μl of 6.0 mM 4-AAP, 50 μl of the CuO nanoparticles stock solution (0.2 mg ml⁻¹), and 300 μl H₂O₂ of different concentrations were added into 960 μl of 20 mM phosphate buffer (pH = 6.0); (b) the mixed solution was incubated in a 37 °C water bath for 20 min; (c) the resulting reaction solution was measured by using a Shimadzu UV-2450 spectrophotometer.

Glucose detection

Glucose detection was carried out as follows: (a) 65 μl of 1 U ml⁻¹ GOx, 300 μl of glucose aqueous solution of different concentrations, 2.0 ml of 6.0 mM phenol, 790 μl of 6.0 mM 4-AAP, and 50 μl of the CuO nanoparticles stock solution (0.2 mg ml⁻¹) were added into 895 μl of 20 mM phosphate buffer (pH = 5.5); (b) the mixed solution was incubated in a 37 °C water bath for 20 min; (c) the resulting reaction solution was measured by using a Shimadzu UV-2450 spectrophotometer.

Results and discussions

Characterization of the water-soluble cupric oxide nanoparticles

The cupric oxide nanoparticles were prepared via a quick-precipitation method. Fig. 1 shows a typical XRD pattern for the as-prepared nanoparticles which is identical to the single-phase CuO with a monoclinic structure with lattice constants of a = 0.46837 nm, b = 0.34226 nm, c = 0.51288 nm, and β = 99.54° from the standard card JCPDS 72-0629. No peaks of impurity were found in the XRD pattern, indicating the high phase purity of the CuO nanoparticles. The broadening of the peaks indicates that the crystal size is small. The average size of the CuO nanoparticles was estimated from the diffraction peak (200/111) by using the Scherer formula as about 6 nm.

The size and morphology of the product was analyzed by transmission electron microscopy (TEM). The TEM image (Fig. 2) reveals that the product consists of spherical particles with a regular morphology and a significantly narrow size distribution. The average particle diameter is found to be approximately 6.3 ± 0.7 nm, which is in good agreement with that estimated by the Scherer formula from an XRD result.

Fourier transform infrared (FTIR) spectroscopy is a powerful tool providing supplementary information on the nature of copper oxides. The FTIR spectrum of the as-prepared CuO nanoparticles was studied by using a Shimadzu FTIR spectrometer (Nicolet Thermo, USA).
nanoparticles is presented in Fig. 3. The high frequency modes at about 585, 535, and 480 cm$^{-1}$ can be assigned to the vibrations of Cu(II)–O.$^{44}$ The frequency mode due to Cu$_2$O at about 610 cm$^{-1}$ is not seen, which further proves that the as-prepared nanoparticles comprise of a purely CuO phase, without any trace of Cu$_2$O being present.

The UV-vis absorption spectrum was used in order to resolve the excitonic or interband transitions of the CuO nanoparticles. As shown in Fig. 4a, the absorption peak of the CuO nanoparticles dispersed in distilled water is at about 280 nm. The optical band gap is estimated using the following equation for a semiconductor:

$$A = \frac{K(h\nu - \varepsilon)^{m/2}}{h\nu}$$

where, $A$ is the absorbance, $K$ is a constant, $h$ is Planck’s constant, $\nu$ is the frequency, and $m$ equals 1 for a direct transition. The energy intercept of a plot of $(Ah\nu)^2$ versus $E_{\text{phot}}$ yields $E_g$ for a direct transition (Fig. 4b).$^{45}$ The band gap of the as-prepared CuO nanoparticles is estimated to be 3.11 eV from the UV-vis absorption spectrum, which is larger than the reported value for bulk CuO ($E_g = 1.85$ eV).$^{46}$ The increased band gap is attributed to the quantum size effects for semiconductors when the particles become comparable to the de Broglie wavelength of a charge carrier.$^{47}$

Considering the fact that most applications require the use of this material in the form of aqueous colloidal suspensions, it is worth studying the stability of the particles in water. The as-prepared CuO nanoparticles, even without any surface modification, can disperse well in distilled water to form a transparent solution. Both the appearance and the UV-vis absorption spectrum of the solution do not change even after 6 months (Fig. S1 and S2, ESI†). In contrast, the commercial CuO nanoparticles with diameter of 30 nm, which are dispersed in distilled water by sonicating, precipitate from the aqueous suspension in 10 min. Therefore, the water-soluble CuO nanoparticles are more easily applied in aqueous systems.

**Peroxidase-like activity of the water-soluble CuO nanoparticles**

The synthesized water-soluble cupric oxide nanoparticles have peroxidase-like catalytic activity. They can catalyze the oxidation of peroxidase substrates, such as 3,3',5,5'-tetramethylbenzidine (TMB), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diaminonitromethylamine salt (ABTS), and 3,3'-diaminobenzidine (DAB) in the presence of hydrogen peroxide to produce a color change. In addition, the water-soluble CuO nanoparticles show significantly higher peroxidase-like activity than the commercial ones. Upon addition of cupric oxide nanoparticles to the peroxidase substrate TMB in the presence of H$_2$O$_2$, a blue color product showing a maximum absorbance at 652 nm was formed. As shown in Fig. 5, much more blue product is formed in the water-soluble CuO nanoparticle system than that of commercial CuO.
nanoparticle system under the same experimental conditions. As estimated from the absorbance of the catalytic products, the catalytic activity of the water-soluble CuO nanoparticles was about 100 times that of the commercial product. The higher peroxidase-like activity of the water-soluble CuO nanoparticles can be ascribed to the size effect.

To rule out the possibility that the observed peroxidase-like catalytic activity is caused by copper ions leaching from CuO nanoparticles in the solution, a leaching solution was used instead of CuO nanoparticles in the reaction. As shown in Fig. 6, absorbance at 652 nm increases continuously during the reaction process in the presence of CuO nanoparticles. However, there is no obvious change of absorbance that can be observed when the leaching solution was used under the same reaction conditions. These experimental results reveal that the observed peroxidase-like activity is due to intact nanoparticles.

The apparent steady-state kinetic parameters for the peroxidase-like enzymatic color reaction were determined by changing the concentrations of TMB and H2O2 in the system, respectively. Typical Michaelis–Menten curves can be obtained for the water-soluble CuO nanoparticles with both TMB and H2O2 (Fig. 7). Obtained from the Lineweaver–Burk plot, the Michaelis constant (Km) value for the water-soluble CuO nanoparticles with TMB is 0.016 mM, similar to that of the commercial CuO nanoparticles. This result shows that the water-soluble CuO nanoparticles also have high affinity to TMB compared to HRP and other peroxidase nano-mimetics. On the other hand, the apparent Km value of water-soluble CuO nanoparticles with H2O2 as the substrate is 41.0 mM, which is significantly lower than that of commercial CuO nanoparticles. This difference leads to the higher catalytic activity of the water-soluble CuO nanoparticles compared to the commercial CuO nanoparticles.

The main problem in the application of enzymes in vitro is the great impact of environmental factors such as temperature and pH on the structure and catalytic activity of enzyme. For example, after treatment at pH lower than 5 or temperatures greater than 40 °C for only 2 h, the catalytic activity of HRP dramatically declined. However, except for the decomposition in strong acidic condition (pH < 2), the CuO nanoparticles are stable over a wide range of pH from 3 to 12, and temperature from 4 to 90 °C (Fig. 8). The robustness of CuO NPs makes them suitable for a wide range of applications in biomedicine and environmental chemistry fields.

Analytical application in determination of hydrogen peroxide and glucose

The analytical determination of hydrogen peroxide is of considerable importance for medical diagnosis since hydrogen peroxide is formed as an intermediate product in the case of a large number of important detection processes. One of the most frequently used spectrophotometric detection systems is that reported by Trinder, in which phenol is oxidatively coupled with 4-amino-atipyrine (4-AAP) in the presence of hydrogen peroxide to give a red product. However, even if immobilized on a carrier, peroxidase still has some disadvantages regarding stability, handling, and storage. In addition, peroxidase will be inactivated in an irreversible process in the presence of both hydrogen peroxide and phenol. Thus, the development of an effective artificial mimic with high peroxidase-like catalytic activity has been desired to overcome these disadvantages. Based on the 4-AAP-phenol colorimetric
system and the peroxidase-like activity of CuO nanoparticles, a novel method for the determination of hydrogen peroxide was established. The chromogenic reaction proceeds as follows:

\[
2\text{H}_2\text{O}_2 + \text{PhOH} \rightarrow \text{PhO}^{	ext{OH}}\text{O} + 4\text{H}_2\text{O} \tag{2}
\]

As shown in Fig. 9, the as-prepared CuO nanoparticles indeed exhibit a catalytic behavior toward phenol oxidation coupled with 4-AAP in the presence of hydrogen peroxide. The absorption spectra indicates that the presence of the water-soluble CuO nanoparticles gives a 1940% response when compared with the one in the absence of the catalyst.

The absorbance at 505 nm of the reaction mixture consisting of 1.2 mM 4-AAP, 1.0 mM H₂O₂, and varying concentrations of phenol in the presence of 4.0 μg mL⁻¹ CuO nanoparticles was determined after incubation at 37 °C for 20 min. The dependence of the attained color intensity as a function of phenol concentration was examined between 0.25 and 6.0 mM. As shown in Fig. 10, the slope for low phenol concentrations is much steeper than in the higher concentration range. In the steeper region, the
product formation could be limited by the quantity of available phenol in the system and therefore directly depends on the phenol concentration within 3.0 mM. At higher concentrations, the absorbance of the system reaches the plateau of maxima.

A similar study of the influence of 4-AAP concentration was also performed. The reaction mixture consisted of 3.0 mM phenol, 1.0 mM H$_2$O$_2$, and 4.0 $\mu$g mL$^{-1}$ CuO nanoparticles. As shown in Fig. 11, the absorbance increases with increasing the concentration of 4-AAP and reaches a maximum value at 1.2 mM.

The 4-AAP/phenol/CuO NPs colorimetric system is also affected by solution pH over a wide range. As shown in Fig. 12, variation in color attenuation of the colorimetric system is very small between pH 4.0 and 7.0. This makes the present system interesting for investigation of combined multi-enzyme assays with oxidases that require a narrower pH range for their optimum activity.

The calibration graph for the determination of H$_2$O$_2$ is constructed under the optimum reaction conditions (1.2 mM 4-AAP, 3.0 mM phenol, and pH 6.0). Good linearity is found in the range 0.01–1.0 mM. The linear regression equation is $A = 0.119 + 0.269 \, C$ (mM) and the correlation coefficient $r$ is 0.9991. The relative standard deviation is 1.2% for 1.0 mM H$_2$O$_2$ ($n = 6$).

When the catalytic reaction (eqn (2)) is coupled with the glucose catalytic reaction by GOx, colorimetric determination of glucose can be readily realized. Differently from the ABTS or TMB system, the phenol/4-AAP system can be successfully performed at pH 5.5, which is the optimum pH for the activity of glucose oxidase. Therefore, the glucose detection can be performed in one step as mentioned in the experimental section. Fig. 13 shows the calibration graph for glucose obtained under the recommended conditions. The linear range is 0.1–8 mM and linear regression equation is $A = 0.176 + 0.024 \, C$ (mM), with a correlation coefficient of 0.999. The relative standard deviation is 1.9% for 5.0 mM glucose ($n = 10$).

For testing if the detection of glucose is specific, control experiments were taken using fructose, lactose, maltose and sucrose. No detectable signals were obtained for the control samples with concentrations of 5 mM. Thus the colorimetric method developed here shows high selectivity towards glucose detection.

**Conclusions**

In summary, water-soluble cupric oxide nanoparticles were fabricated and investigated as peroxidase mimetics. The catalytic activity of the water-soluble CuO nanoparticles is about 100 times that of the commercial ones, which can be ascribed to the high affinity of water-soluble cupric oxide to hydrogen peroxide. Except for decomposition under strongly acidic conditions (pH < 2), the as-prepared CuO nanoparticles are stable over a wide range of pH and temperature. Moreover, the excellent stability in the form of aqueous colloidal suspensions makes the water-soluble CuO nanoparticles more easily applied in aqueous systems. The catalytic oxidation of phenol coupled with 4-amino-atipyrine by the action of hydrogen peroxide was realized as a colorimetric assay for hydrogen peroxide and glucose. This analytical platform not only confirms the intrinsic peroxidase-like activity of the water-soluble cupric oxide nanoparticles,
but also shows great potential applications in environmental chemistry, biotechnology and medicine.

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Notes and references