

Intrinsic Peroxidase-like Activity of Porous CuO Micro-/nanostructures with Clean Surface

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Porous CuO micro-/nanostructures with clean surface, prepared through $\text{Cu}_2(\text{OH})_2\text{CO}_3$ precursor followed by calcination in air, were proven to be an effective peroxidase mimic. They can quickly catalyze oxidation of the peroxidase substrate 3,3',5,5'-tetramethylbenzidine (TMB) in the presence of H_2O_2 , producing a blue color. The obtained porous CuO micro-/nanostructure have potential application in wastewater treatment. The apparent steady-state kinetic parameter was studied with TMB as the substrate. In addition, the potential application of the porous CuO in wastewater treatment was demonstrated with phenol-containing water as an example. Such investigation not only confirms the intrinsic peroxidase-like activity of micro-/nanostructured CuO, but also suggests its potential application in wastewater treatment.

Keywords CuO, 3,3',5,5'-tetramethylbenzidine, peroxidase, enzyme, porous micro-/nanostructures

Introduction

Over the past decades, various physical and chemical methods have been devoted to the preparation of nanoscale solid materials because of their unique size, shape, and structure-dependent properties.^[1,2] Exploiting the new properties and potential applications of the prepared nanoobjects now becomes one of the most attractive aspects in nanoscience. Functional nanomaterials acting as enzyme mimic have recently been known and became a hot research topic because of their intrinsic advantages such as greater resistance to extremes of pH and temperature range.

As a type of efficient biological catalyst, enzymes are involved in almost all reactions *in vivo* and demonstrate high efficiency and high specificity but with mild conditions. Enzymes also have a wide range of applications including clinical diagnosis, chemistry, biotechnology, environmental science.^[3] However, they bear intrinsic drawbacks such as limited natural sources, difficult and high-cost purification processes, low stability, and sensitivity of catalytic activity to external conditions, all of which restrict their wide applications.^[4] Therefore, the searching of efficient enzyme mimics is an important issue.^[3] Among the many enzymes, peroxidase attracts much attention because it can efficiently catalyze the oxidation of electron donors by hydrogen peroxide, which is an important intermediate product of many biological reactions. Since the first report of Fe_3O_4 nanoparticle-based artificial peroxidase,^[5] engi-

neering of nanoscale solids that mimic peroxidase has received great attention.

In recent years, several common nanomaterials have been found to show intrinsic peroxidase-like activity including sheet-like FeS nanostructures,^[6] spherical CeO_2 nanoparticles,^[7] carbon nanomaterials,^[8-10] noble metal nanostructures^[11-13] and V_2O_5 nanowires.^[14] The newly found peroxidase-like functions enable them to be potentially applied in various bio-detections. It is proposed that the peroxidase-like activity of Fe-based nanoparticles relates to the Fenton reactivity of Fe species. This inspired us to explore the peroxidase-like activity of Cu-based micro-/nanostructures, since Cu and Fe species are both common Fenton reagents. Recently, Zheng *et al.*^[15] have shown concave CuS superstructure with peroxidase-like activity. Chen *et al.*^[16] also demonstrated the peroxidase-like activity of CuO nanoparticles. However, few studies were performed in this field with porous Cu-based micro-/nanostructures. For the improvement of catalytic activity, porous micro-/nanostructures are highly preferred, because porous structure will not only increase their accessible surface area but also facilitate the mass transport of reagents in the catalytic material.

Herein, porous CuO micro-/nanostructures were prepared by a precursor route without involving any surfactants. Owing to the subsequent calcination route, the obtained CuO product has clean surface and possesses openly porous structural feature. Thus, they are

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Received September 8, 2013; accepted December 9, 2013; published online January 14, 2014.

expected to show improved performances in catalysis or related applications. Their peroxidase-like activity was then investigated with 3,3',5,5'-tetramethyl-benzidine (TMB) as a model substance. It was demonstrated that the porous CuO micro-/nanostructure with clean surface exhibited intrinsic peroxidase-like activity, as it could catalyze TMB oxidation quickly in the presence of hydrogen peroxide.

Experimental

All chemicals were of analytical reagent grade and used as received without further purification. 3,3',5,5'-Tetramethylbenzidine (TMB) was purchased from Sigma-Aldrich. Copper nitrate, urea, hydrogen peroxide, sodium acetate, and acetic acid were purchased from Sinopharm Chemical Reagent Co. Deionized water was used in the experiment.

The porous CuO micro-/nanostructures were synthesized by a precursor route reported previously by our group.^[17] Typically, 2.3 g of $\text{Cu}(\text{NO}_3)_2 \cdot 3\text{H}_2\text{O}$ and 1.7 g of $\text{CO}(\text{NH}_2)_2$ were orderly dissolved into 25 mL of distilled water. The obtained mixture was then vigorously stirred for 30 min and then sealed into 30 mL Teflon-lined stainless steel autoclave, which was heated to 180 °C and maintained at that temperature for 5 h. The as-formed precipitate, $\text{Cu}_2(\text{OH})_2\text{CO}_3$ precursor, was separated by centrifugation, washed with distilled water and ethanol for several times, and dried in vacuum. Control experiment shows that the $\text{Cu}_2(\text{OH})_2\text{CO}_3$ precursor can be obtained with reaction time of 5–12 h. Porous CuO micro-/nanostructures were obtained by further calcining the precursor in air at 450 °C for 4 h.

Peroxidase-like activity investigation: For a typical catalytic reaction, 5.5 mL of acetate buffer (pH=4.0) was incubated at 25 °C. Firstly, 3 mg catalyst (CuO) was dispersed in the above solution. Then, TMB (40 μL , 10 mg/mL in dimethylsulfoxide) and H_2O_2 (7 μL , 10 wt%) were added as substances with their concentrations of 0.3 and 3.6 mmol/L, respectively. With the reaction proceeding, the solution turns to blue color. After 30 min, the ultraviolet-visible (UV-vis) spectra measurements and photographs were taken. The absorbance of the TMB-derived oxidation product was examined at 652 nm. Controlled experiments were conducted with different concentrations of TMB, commercial CuO powder, different incubation time. Michaelis constants were obtained by varying the concentrations of TMB.

The phase of the as-synthesized product was characterized using X-ray powder diffraction (XRD, Shimadzu XRD-6000) with Cu K α radiation ($\lambda=1.5406 \text{ \AA}$). The morphology and size of the product were examined by scanning electron microscopy (SEM, H-4800) and transmission electron microscopy (TEM, JEOL-2100). UV-vis spectra were obtained on an UV-3000 spectrophotometer. The electron spin resonance (ESR) measurement was performed on an EMX-10/12 electron-spin resonance spectrometer. The Brunauer-Emmett-Teller

(BET) surface area of the CuO sample was tested using an ASAP 2010 sorption analyzer.

Results and Discussion

A simple precursor route was used for the preparation of the porous CuO micro/nanostructures.^[17] First, $\text{Cu}_2(\text{OH})_2\text{CO}_3$ precursor composed of platelet-like building blocks was prepared.^[17] The following calcination of the precursor produces CuO architectures with openly porous feature and preservation of their pre-morphologies. The obtained CuO product was firstly characterized by using XRD, which is shown in Figure 1. For comparison, the standard pattern of CuO is also shown. The XRD pattern agrees well with the standard one with JCPDS No. 65–2309, suggesting pure phase CuO product was obtained (Figure 1). The sharp peak indicates its high crystalline. No impurities were detected.

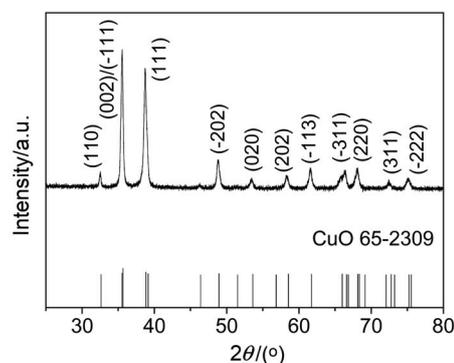


Figure 1 XRD pattern of the CuO product. The standard pattern of CuO is also shown.

Figure 2 shows the SEM and TEM images of the obtained CuO sample, which reveals the porous structural feature with pores size of 20–30 nm. BET investigation of the CuO product provides a surface area of about 18 m^2/g , with main pore size of about 20 nm and pore volume of 0.13 cm^3/g .^[17] This is consistent with the TEM observation. The surface area is quite larger than that of commercial CuO powder (*ca.* 0.1 m^2/g). The openly porous structure provides a bigger surface area and facilitates the mass transport in it. Benefited from the calcination procedure in air, the porous CuO product has good crystalline and clean surface without any bonding or adsorbing of surfactant molecules. This is in contrast to the samples obtained with direct solution route, on the surface of which some organic or surfactant molecules often exist. In addition, the calcination in air with suitable temperature fully oxidizes the product and so makes the sample comprise of pure CuO phase without the presence of Cu_2O . These factors make the obtained CuO product an excellent model to study the intrinsic peroxidase-like activity of Cu-based nano-materials.

To investigate the peroxidase-like activity of the synthesized porous CuO micro-/nanostructures, the

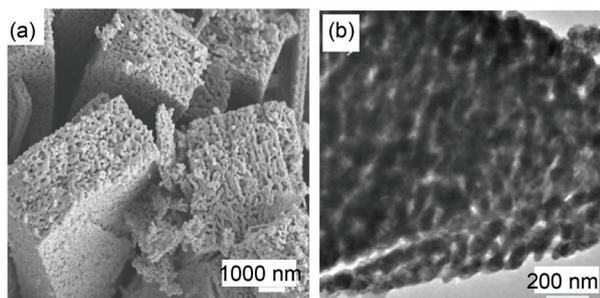
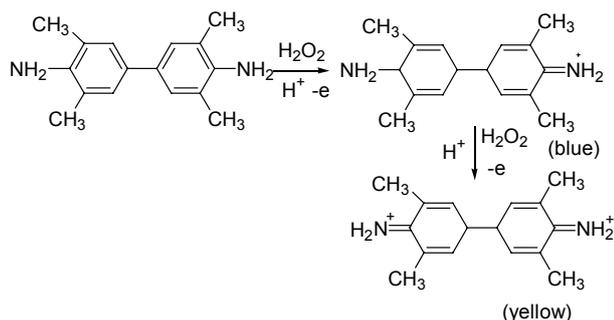


Figure 2 (a) SEM and (b) TEM images of the porous CuO micro-/nanostructures.

catalytic oxidation of the peroxidase substrate TMB in the presence of H_2O_2 was tested. The reaction scheme is shown as follows. With the catalysis of peroxidase, TMB can be oxidized by H_2O_2 forming a blue intermediate, which is a charge-transfer complex and usually observed quickly. If with stronger acidic condition, the final product, a yellow diimine, will be obtained.^[18] The formation of the blue intermediate provides an obvious colorimetric method and is often used as a model reaction to investigate the peroxidase-like activity of various samples.



The experiments were firstly conducted with TMB and CuO product in the presence and absence of H_2O_2 , respectively. As shown in the digital figure in the inset of Figure 3A, the porous CuO product does not show obvious catalytic activity for the substrate in the absence of H_2O_2 . Then, we studied the reaction between TMB and H_2O_2 with or without CuO. Figure 3A shows the UV-vis spectra of the reaction system in acetate buffer. It is clear that the solution system with CuO product has stronger absorption peak at 652 nm, in contrast to the lower absorption without CuO, suggesting that the porous CuO micro-/nanostructures have peroxidase-like activity. The oxidation intermediate product of TMB shows a typical absorbance at 652 nm and so the initial oxidation rates can be evaluated by monitoring the absorbance increase at this wavelength.^[18]

Similar to other enzyme mimics, the catalytic activity of the porous CuO micro-/nanostructures is dependent on pH. Acetate buffers with pH range of 2.6–5.8 were selected for studying the reaction. Figure 3B shows the pH dependent peroxidase-like activity. The catalytic oxidation of TMB with H_2O_2 in the presence of

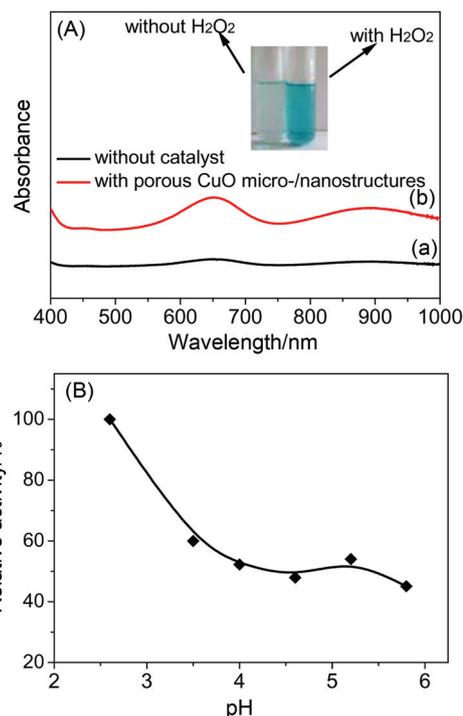


Figure 3 (A) UV-vis spectra of TMB- H_2O_2 solution (a) without porous CuO micro-/nanostructures and (b) with them. The insets show the digital pictures of TMB-CuO systems with or without H_2O_2 . (B) Dependence of the peroxidase-like activity on pH.

porous CuO micro-/nanostructures was much faster in acidic solution with lower pH values. The CuO product shows the isoelectric point of about 9.5.^[19] In the NaAc-HAc buffer solution with pH of 2.6–5.8 (lower than the isoelectric point of CuO), a layer of $-\text{OH}_2^+$ would absorb on the CuO surface. This would facilitate the absorption of TMB molecules to CuO surface and thus promote the catalytic oxidation of TMB with H_2O_2 . The buffer with pH of 4.0 was then selected for the following experiments considering the possible instability of CuO in stronger acidic solution, although no dissolution of CuO product was observed in these buffer solutions during the test process. To investigate the dissolving of Cu^{2+} ions from the CuO product in the reaction buffer solution, the CuO product was incubated in the buffer solution with pH of 4.0 for 30 min, then the CuO solid sample was removed. The catalytic activity of the remaining solution was tested with the same conditions. No obvious catalytic activity was observed. This reveals that the observed peroxidase-like activity originates from the CuO solid rather than the leached Cu^{2+} ions.^[15,16]

The improved catalytic activity of the porous CuO micro-/nanostructures is revealed by the comparison experiment. Figure 4 presents the time course curves of the reaction system catalyzed by porous CuO micro-/nanostructures and commercial CuO powder within 30 min. The commercial CuO powder with BET specific surface area of about $0.1 \text{ m}^2/\text{g}$ does not show obvi-

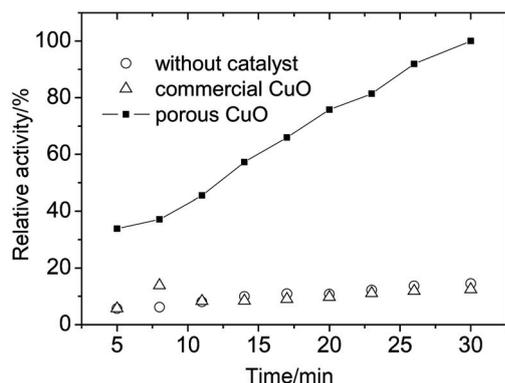


Figure 4 A time-dependent catalytic activity of porous CuO micro-/nanostructures and commercial CuO powder.

ous peroxidase-like activity. Thus, the higher peroxidase-like activity of porous CuO micro-/nanostucture can be ascribed to its openly porous microstructure, which enables it to have larger specific surface area, providing more active sites for the activity reaction.^[15] This further demonstrates that the peroxidase-like activity is based on the intact CuO product. A Fenten-like mechanism may act in the catalytic oxidation process, where the porous CuO product catalyzes the transformation of H_2O_2 to hydroxyl radicals. The latter then oxidizes the TMB molecules. To clarify this issue, an ESR technique was used. First, we investigated the aqueous H_2O_2 solution by ESR technique, which showed no signal. Then, porous CuO product was added in the H_2O_2 solution. As shown in Figure 5, a relatively obvious $\text{HO}\cdot$ signal was detected in H_2O_2 solution (as shown by the noted frame). This indicates that $\text{HO}\cdot$ radicals are formed in the system, as also observed in the system of $\text{H}_2\text{O}_2 + \text{Fe}_3\text{O}_4$,^[20,21] and suggests that the CuO products catalyze the formation of $\text{HO}\cdot$. Thus, a Fenten-like mechanism may act in the TMB catalytic oxidation process. In addition, the CuO micro-/nanostuctures may also facilitate the electron transfer process between TMB and H_2O_2 , promoting the catalytic reaction. While, indeed, a definite catalytic mechanism needs further research.

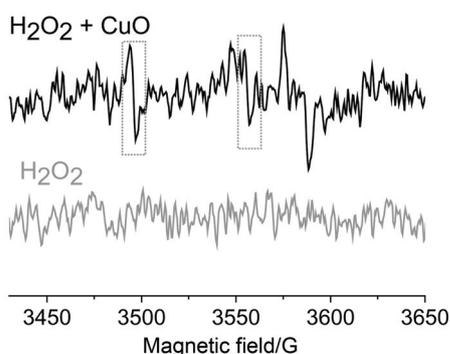


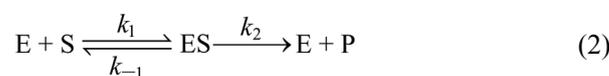
Figure 5 ESR spectra of $\cdot\text{OH}$ radicals in the systems of H_2O_2 or $\text{H}_2\text{O}_2 + \text{CuO}$. Conditions: $3.6 \text{ mmol}\cdot\text{L}^{-1}$ of H_2O_2 or with 3 mg of porous CuO product.

The apparent steady-state kinetic parameters for the reaction with porous CuO micro-/nanostuctures as catalyst were then determined. Absorbance data were back-calculated to concentration through the Beer-Lambert Law and a molar absorption coefficient of $39\,000 \text{ L}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}$ for TMB-derived oxidation product was used for the calculation.^[22] According to the Michaelis-Menten equation, the apparent kinetic parameters could be calculated.

$$V_o = V_{\max}[\text{S}]/([\text{S}] + K_m) \quad (1)$$

where V_o is the reaction rate, V_{\max} is the maximum reaction rate, $[\text{S}]$ is the substrate concentration, and K_m is the Michaelis constant which is equivalent to the substrate concentration at which the rate of conversion is half of V_{\max} and denotes the affinity of enzyme to the catalyst. Figure 6a shows the TMB concentration dependent absorbencies. The corresponding $1/V-1/[\text{S}]$ curve is shown in Figure 6b. Through the slope, the K_m value of porous CuO was determined to be 0.14 mmol/L , which is lower than those of horseradish peroxidase (HRP) and Fe_3O_4 nanoparticles.^[23,24]

The K_m value obtained with porous CuO is a little higher than the reported CuO nanoparticles (Table 1).^[16] Usually, an enzymatic reaction is modeled as a two-step process:^[25] the binding of substrate (S) to enzyme (E) and formation of an enzyme-substrate (ES) complex, followed by an irreversible breakdown of the ES complex to free enzyme and product (P).



$$K_m = \frac{k_{-1} + k_1}{k_2} \quad (3)$$

Therefore, K_m is determined by k_1 , k_{-1} and k_2 as shown in Equation (3). Decreasing k_1 or increasing k_{-1} and/or k_2 will result in an increase in K_m . In our case, porous CuO has a bigger surface area, which causes a bigger k_1 and so a smaller K_m . On the other hand, the bigger surface area also results in an enhancement in catalytic activity, inducing a bigger k_2 . If the influence of k_2 is larger than that of k_1 , it will give rise to a bigger K_m .

The K_{cat} value of the porous CuO was also calculated and compared with that of HRP, CuO nanoparticles, and Fe_3O_4 nanoparticles (Table 1). Commercial CuO powder was not compared because no obvious catalytic activity was observed in our experiment. The porous CuO shows a relatively lower K_m value, while it has a comparative V_{\max} value with Fe_3O_4 nanoparticles.^[5] The reported K_{cat} values have a wide range even with the same material. The porous CuO shows relatively lower K_{cat} value when compared with HRP or Fe_3O_4 nanoparticles, which would relate to the lower concentration of H_2O_2

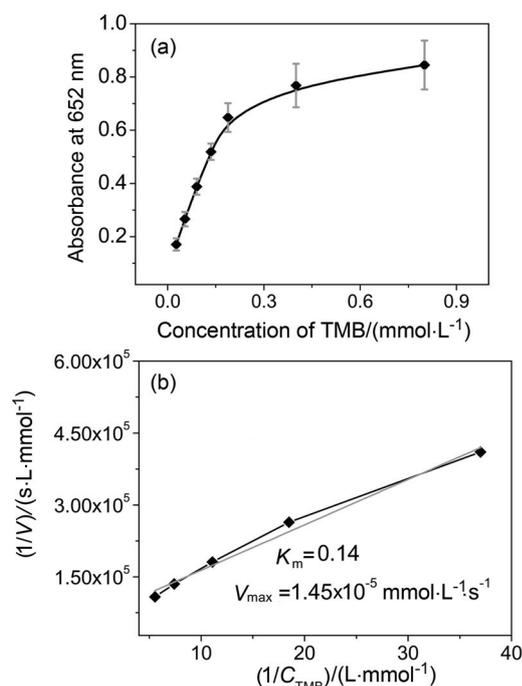


Figure 6 Kinetic analysis for porous CuO product. (a) The absorbance obtained in 5.5 mL acetate buffer (pH=4.0) at 25 °C in the presence of 15 mmol/L H₂O₂, 3 mg porous CuO and TMB with different concentrations. (b) Double-reciprocal plots of activity at a fixed concentration of H₂O₂ (15 mmol/L) and varying concentration of TMB.

used in our experiment. It is believed that the catalytic activity of porous CuO can be further improved by a suitable chemical modification, since the activity is also surface group and charge dependent.^[26]

Table 1 Comparison of the kinetic parameters

	$K_m/(\text{mmol}\cdot\text{L}^{-1})$	$V_{\text{max}}/(\text{mmol}\cdot\text{L}^{-1}\cdot\text{s}^{-1})$	$K_{\text{cat}}^a/\text{s}^{-1}$
HRP ^[5]	0.434	1.0×10^{-4}	4×10^3
CuO nanoparticles ^[16]	0.016	4.0×10^{-4}	8×10^{-3}
Fe ₃ O ₄ nanoparticles ^[5]	0.098	3.44×10^{-5}	3.02×10^4
Fe ₃ O ₄ nanoparticles ^[27]	0.17	1.7×10^{-4}	7.68×10^{-4}
Fe ₃ O ₄ nanoparticles ^[26]	0.22–0.71	$0.42–2.4\times 10^{-4}$	$2.0–11.4\times 10^{-5}$
Porous CuO ^b	0.14	1.45×10^{-5}	2.12×10^{-6}

^a Catalytic constant derived from $k_{\text{cat}}=V_{\text{max}}/[\text{cat}]$. ^b Conditions: 25 °C, 5.5 mL acetate buffer (pH=4.0), 15 mmol/L H₂O₂.

The observed catalytic activity of CuO would be used for the treatment of waste water. We then tested the applicability of the porous CuO for catalytic treatment of phenol-containing water (Figure 7). After the porous CuO, H₂O₂ were added into the phenol-contain-

ing water, the obtained mixture was stirred for 60 min. The phenol removal was studied by UV-vis spectrum monitored at about 275 nm. After the treat process, the characteristic peak at 275 nm decreases, accompanied with the increasing absorption between 300–500 nm possibly corresponding to the oxidation products.^[28] This demonstrates the potential application of the porous CuO with intrinsic peroxidase activity.

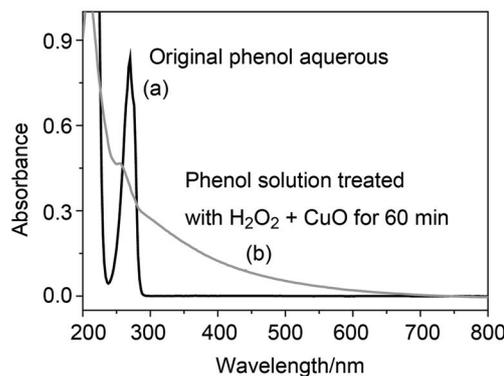


Figure 7 UV-vis spectra monitoring phenol treatment. (a) The original phenol aqueous solution (50 mg/L), (b) the phenol solution treated with H₂O₂ (0.5 mol/L)+CuO (0.3 g/L) for 60 min.

Conclusions

In summary, through a precursor route, porous CuO micro-/nanostructures with clean surface were prepared, which show intrinsic peroxidase-like activity demonstrated with TMB as the substrate. The porous CuO micro-/nanostructures have higher peroxidase-like catalytic activity than commercial CuO powder. In addition, the potential application of the porous CuO in waste water treatment was demonstrated with phenol as a model pollutant. Our present study provides a new peroxidase catalyst, which has potential application in colorimetric detection of H₂O₂, glucose, cholesterol and in waste water treatment.

Acknowledgement

The authors are grateful for financial support from the National Natural Science Foundation of China (Nos. 51203069, 51102117, 51072071), and the China Post-doctoral Science Foundation (Nos. 2011M500085, 2012T50439).

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