Ultrasmall copper nanoclusters with multi-enzyme activities†

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Reactive oxygen species (ROS) as a key messenger of signal transduction mediate physiological activities, however, oxidative stress produced by excessive ROS can cause the destruction of cell homeostasis, which will result in a series of diseases. Therefore, effective control of ROS level is critical to the homeostasis of the cell. Here, we reported that glutathione (GSH)-stabilized copper nanoclusters (CuNCs) with about 9 Cu atoms can functionally mimic three major antioxidant enzymes, namely catalase (CAT), glutathione peroxidase (GPx) and superoxide dismutase (SOD). The rate of H$_2$O$_2$ decomposition was calculated to be $\sim$0.23 mg L$^{-1}$ s$^{-1}$ when the concentration of CuNCs was 100 $\mu$g mL$^{-1}$. The SOD-like activity by catalyzing the disproportionation of superoxide (O$_2^-$) to H$_2$O$_2$ and O$_2$ reached 25.6 U mg$^{-1}$ when the effective inhibition rate was $\sim$55.4%. Intracellular ROS scavenging studies further identified that CuNCs can obviously protect cells from oxidative stress and the cell viability recovered to above 90%. Hence, we expect that ultrasmall CuNCs will provide good therapeutic potential in the future treatment of ROS-related diseases.

1 Introduction

Aerobic organisms produce reactive oxygen species (ROS) such as superoxide (O$_2^-$), hydrogen peroxide (H$_2$O$_2$) and hydroxyl radicals (‘OH) in order to maintain normal life activities.$^{1-3}$ ROS mediate the transduction mechanisms of various signals, which play an important role in regulating myriad physiological functions in cells.$^4$ Excessive production of ROS leads to oxidative stress, causing the destruction of biomolecules.$^5$ These events cause a variety of pathological and physiological processes involved in neurodegeneration, diabetes, chronic kidney disease, cardiovascular disease and cancer.$^{6-7}$ Therefore, the maintenance of ROS level against oxidative stress is particularly important for sustaining cellular homeostasis.$^8$

Redox equilibrium in cells is achieved by complex endogenous antioxidant defense systems, which include endogenous antioxidant enzymes, e.g., superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx).$^{9,10}$ The regulation of oxidative stress caused by the imbalance of ROS has been recognized as a vital therapeutic target for cancers and neurological diseases. However, the development of natural antioxidant enzymes is still hampered owing to the intrinsic shortcomings such as difficulty in storage, easy inactivation, poor stability and relatively limited sources. To overcome these shortcomings of natural enzymes, researchers are working to develop ideal alternatives to biological enzymes.$^{11,12}$

Scientists have developed various enzymatically active materials including metal complexes, redox polymers and ceria.$^{13-15}$ Since the discovery of Fe$_3$O$_4$ nanoparticles with peroxidase activity in 2007,$^{16}$ the material, widely known as nanozymes, has attracted considerable attention.$^{17}$ Vernekar et al.$^{18}$ demonstrated that V$_2$O$_5$ nanowires displayed remarkable GPx-like antioxidant activity under physiologically conditions. Mechanism study illustrated that the internalization of V$_2$O$_5$ nanowires can equilibrate the ROS and control oxidative stress by catalytically reducing H$_2$O$_2$ in the presence of glutathione (GSH). Zhang and colleagues identified that platinum nanoparticles distributed homogeneously on metal–organic frameworks possessed CAT-like catalytic activity.$^{19}$ This material relied on the O$_2$ that was catalyzed by H$_2$O$_2$ to facilitate production of $^{1}$O$_2$ in hypoxic tumor site, playing a critical role for tumor treatments.

In fact, antioxidant enzymes have different regulatory mechanisms for scavenging ROS. For example, SOD has a very high specificity for dismutase O$_2^-$ into O$_2$ and H$_2$O$_2$ in vivo. Although the presence of H$_2$O$_2$ is still harmful, CAT and peroxidase will immediately break it into harmless water. CAT is responsible for the catalysis of excess H$_2$O$_2$ during oxidative...
stress, while GPx shows the function of fine-tune the concentration of H₂O₂ for cell signalling.²⁰ Inspired by these findings, scientists are no longer satisfy with the development of materials with single enzyme activity and designing material with multi-enzyme activities deserves more attention.

Studies demonstrated that the CeO₂ and MnO₂ nanoparticles showed inherent multi-enzyme activities.²¹,²² Fan et al.²³ synthesized highly stable platinum nanoparticles and identified that this material showed CAT and peroxidase activities for various substrates. Furthermore, Huang et al.²⁴ constructed a novel nanozyme cooperative platform consisting of V₂O₅ nanowire and MnO₂ nanoparticles. V₂O₅ nanowire acted as a GPx mimic, while MnO₂ nanoparticle was used to mimic SOD and CAT. In a cellular model of Parkinson disease, Mn₃O₄ nanozymes protected cells from oxidative damage by its redox regulation, thus preventing neurological diseases caused by ROS imbalance.²⁵

Copper, an essential micronutrient in human body, plays a biological role in many enzymes.²⁶ Cu-based nanoparticles, especially cuprous and cupric oxides (Cu₂O and CuO), have been studied extensively. Recently, Liu et al.²⁷ prepared ultrasmall Cu-based nanoparticles for scavenging ROS and alleviating inflammation-related diseases. Hao et al.²⁸ synthesized chiral molecules-mediated porous Cu₉O nanoclusters with CAT, SOD and GPx activities for ameliorating Parkinson's disease. In this context, we attempted to design and construct ultrasmall copper nanoclusters (CuNCs) by a one-step method (Scheme 1). Of particular importance was that these CuNCs could functionally mimic three major cellular antioxidant enzymes (namely CAT, GPx and SOD). To the best of our knowledge, developing ultrasmall CuNCs with excellent multi-enzyme catalytic activities has not been reported before.

2 Experiment

2.1 Materials
All the chemicals were commercially available and used without further purification. Glutathione in the reduced form (GSH) was obtained from ACROS ORGANICS. Copper nitrate trihydrate, sodium citrate, 3,3',5,5'-tetramethylbenzidine (TMB), sodium hydroxide and ethanol were purchased from Sinopharm Chemical Reagent Co. Ltd. Terephthalic acid (TPA) was from URCHEM (Shanghai, China). SOD assay kit (WST-1 method) and total antioxidant capacity detection kit (T-AOC) were purchased from Nanjing Jiancheng Bioengineering Institute. Human embryonic kidney (HEK 293), Dulbecco’s modified Eagle's medium (DMEM), Fetal Bovine Serum (FBS) and Penicillin-Streptomycin were bought from GIBCO. SOD from bovine erythrocytes and 2',7’-dichlorofluorescein diacetate (DCFH-DA) were obtained from Sigma-Aldrich. Cell Counting Kit-8 (CCK-8) was purchased in Beyotime Biotechnology Co. Ltd. 5,5’-Dithiobis-(2-nitrobenzoic acid) (DTNB) was obtained from Aladdin Reagent Co. Ltd. Milli-Q ultrapure water was used in all needed experiments.

2.2 Synthesis of ultrasmall CuNCs
In a typical procedure,²⁷ GSH (1.22 g) and Cu(NO₃)₂ (188 mg) were dissolved in 50 mL of deionized water. The solution was stirred at 25 °C until a white hydrogel was formed. Then, the hydrogel solution was heated to 90 °C for 10 min under magnetic stirring. Thereafter, the white hydrogel solution turned into a light yellow by dropping 1 mL of NaOH (1 M) solution. After cooling to 25 °C, the products were collected by precipitating with ethanol and centrifugation at 8000 rpm. By discarding the supernatant liquid, the solid at the bottom was dissolved in water and then precipitated with ethanol. The entire purification process was repeated three times. The resultant CuNCs were freeze-dried under vacuum and stored in 4 °C refrigerators for further use.

2.3 Cytotoxicity experiment
Cell culture. The HEK 293 cells were cultured in DMEM with 10% FBS and 1% Penicillin-Streptomycin at 37 °C and 5% CO₂.

Cytotoxicity. In vitro CCK-8 assay was used to detect cytotoxicity. HEK 293 cells were placed into 96-well plate with 5 × 10⁸ cells per well and cultured for 24 h. Cell culture medium was extracted and fresh culture medium containing various concentrations of CuNCs was added. After incubation for 24 h and 48 h respectively, the medium was then treated with fresh medium (100 μL) and CCK-8 solution (10 μL), incubated at 37 °C
After inserting HEK 293 cells into 96-well plate for 24 h, CuNCs (0, 10, 20, 50, 100 ng mL$^{-1}$) were added and incubated for 30 min. Then, the cells were treated with H$_2$O$_2$ (100 μM) and cultured at 37 °C for 24 h. CCK-8 assay was used to detect cell viability. The group without H$_2$O$_2$ was set as a negative control.

Intracellular ROS scavenging. Intracellular ROS levels were detected using DCFH-DA, an oxidation-sensitive fluorescent dye. After incubation with H$_2$O$_2$ for 24 h as described above, the cells were gently washed three times in serum-free medium to remove free CuNCs. Then, DCFH-DA (10 μM) was added to the cells and incubated in the dark at 37 °C for 30 min. By Washing three times in serum-free medium removed unloaded DCFH-DA probes, and imaging. Notably, the operating table and tools used in this experiment should be sterilized in advance.

2.4 Characterizations

Fourier transform infrared spectroscopy (FT-IR) spectra were recorded in the wavelength range of 4000–400 cm$^{-1}$ with Vertex 80v (Bruker, Germany). The UV-Vis optical spectra were recorded with UV-2000 (Shimadzu, Japan). High-resolution TEM images were obtained from JEM-2100F STEM operating at 200 kV (JEOL, Japan). Photoluminescence (PL) experiments were performed with a FLS1000 fluorescence spectrometer (Edinburgh Instruments, England). X-ray photoelectron spectroscopy (XPS) study was performed on an ESCALAB 250Xi spectrometer (ThermoFisher Scientific, USA). Mass spectrometry studies were performed on an ESI-Q-TOF MS spectrometer (Bruker, Germany). The isotopic simulation pattern was calculated by the IsoPro 3.1 MS/MS software. Dynamic light scattering were performed on a NanoZS ZEN3600 (Malvern England). Dissolved oxygen was recorded by JPBJ-609 Dissolved Oxygen Meter (Shanghai INESA Scientific Instrument Co. Ltd.). Microplate reader was conducted on a Cytation 5 (Biotek, USA). Electron paramagnetic resonance (EPR) spectra were measured by a Bruker A300 (X-band) spectrometer (Bruker, Germany). Atomic force microscope (AFM) was recorded on Fastscan (Bruker, Germany). Inductively coupled plasma spectrometry (ICP) was performed on a Prodigy 7 (Liman-Lebers, USA).

2.5 Catalase-like activity of CuNCs

Of note, the dissolved oxygen meter was activated for 1 h before measurement, then the full and zero oxygen calibrations were performed by using ultrapure water and 5% sodium sulfite solution, respectively. After calibration, the test interval was set as 5 s. At 25 °C, 1.7 mL of PBS buffer and 2 mL of 10 mM H$_2$O$_2$ were added to an amber laboratory bottle. After that, electrode probe was immersed below the liquid level and the test was started. The testing of the variation of O$_2$ was accomplished within 5 min after addition of 0.3 mL of CuNCs. This operation was repeated three times and took the average values.

To verify the mechanism of CuNCs-induced decomposition of H$_2$O$_2$, TPA was adopted as a fluorescence probe, which reacts with ‘OH from H$_2$O$_2$, forming highly fluorescent 2-hydroxyterephthalic acid with an emission peak at 425 nm upon exposure to the excitation wavelength of 315 nm. The solutions contained CuNCs (0–100 μg mL$^{-1}$), TPA (5 mM) and H$_2$O$_2$ (10 mM). The mixtures were incubated for 4 h.

2.6 GPx-like activity of CuNCs

GPx-like catalytic activity was determined by a modified Hafe-man method.$^{28}$ Sulfhydryl compounds can react with DTNB to break the disulfide bonds of DTNB to produce 2-nitro-5-thiobenzoic acid (NTB$^-$), which can be ionized in neutral and alkaline environments to generate NTB$^2-$ This response can be quantified by the optical absorbance at 412 nm. In a typical assay, GSH (1 mM), H$_2$O$_2$ (1 mM) and CuNCs (20 μg mL$^{-1}$) were added and reacted for 6 min at 37 °C. After the reaction, 1 mL of sodium citrate buffer of DTNB (1 mM) was added and recorded by UV-Vis spectrophotometer. The control experiment was performed by replacing CuNCs with PBS buffer under otherwise identical experiment factors.

2.7 SOD-like activity of CuNCs

The addition of SOD and nanozymes with SOD activity will cause a decrease of absorption at 450 nm. The SOD inhibition rate of nanozymes was obtained by this method based on the UV absorption values at 450 nm before and after catalysis. SOD-like activity was also verified by EPR. In a typical assay, PBS (10 mM), xanthine (5 mM), and xanthine oxidase (0.5 U mL$^{-1}$) were incubated at 37 °C. CuNCs (0, 10, 30, 50, 100 μg mL$^{-1}$) and DMPO were added 10 min later and then recorded.

2.8 Peroxidase-like activity of CuNCs

The peroxidase activity was characterized by chromogenic substrate TMB and H$_2$O$_2$. CuNCs catalyzed H$_2$O$_2$ to oxidize TMB to form a blue mixture with maximum absorbance at 652 nm. Typically, TMB (5 mM), H$_2$O$_2$ (5 mM) and CuNCs (0–200 μg mL$^{-1}$) were mixed evenly and detected on the microplate reader.

2.9 ‘OH scavenging activity of CuNCs

The combination of ‘OH and DMPO usually results in a 1 : 2 : 2 : 1 peak area by the EPR spectroscopy. Typically, H$_2$O$_2$ (10 mM) and FeSO$_4$ (20 μM) were added into the PBS (10 mM, pH 7.2) and incubated for 1.5 min. Then, 50 μL of DMPO and CuNCs (0, 10, 30, 50, 100 μg mL$^{-1}$) were mixed and recorded immediately.

2.10 ABTS radical scavenging activity of CuNCs

ABTS radical scavenging capacity was measured by the T-AOC kit. ABTS can be oxidized into ABTS$^{+}$ with maximum absorption at 405 nm. The presence of antioxidants will inhibit the production of ABTS$^{+}$. The total antioxidant capacity of CuNCs can be calculated by the absorbance of ABTS$^{+}$ at 405 nm. The ABTS radical scavenging activity was calculated as follows:

$$\text{Scavenging ratio(%) = } \left( \frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}} \right) \times 100\%$$

where $A_{\text{blank}}$ represents the absorbance without CuNCs, and $A_{\text{sample}}$ represents the absorbance after adding different concentrations of CuNCs.
3 Results and discussion

3.1 Characterizations of CuNCs

UV-Vis and photoluminescence spectra of as-prepared CuNCs were shown in Fig. 1a and b. There was no absorption peak at about 610 nm corresponding to the surface plasmon resonance of common copper nanoparticles (Fig. S1†), suggesting the small size of obtained CuNCs. Besides, the presence of absorption signals below 300 nm indicated the formation of CuNCs. The emission and excitation spectra were shown in Fig. 1b. In aqueous solution, CuNCs exhibited a symmetrical emission at ~595 nm. The corresponding excitation peak was ~365 nm. In order to obtain the optimal synthetic recipes, a series of molar ratios of GSH/Cu$^{2+}$ were selected to synthesize CuNCs and the corresponding fluorescence emission spectra were recorded. In Fig. S2a,† we can find that when the ratio of GSH/Cu$^{2+}$ was lower than 4, the fluorescence intensity was proportional to the increase of GSH/Cu$^{2+}$ ratios. Subsequently, when the ratios of GSH/Cu$^{2+}$ continued to increase, the fluorescence emission intensity was decreased. We speculated that once the amount of GSH was in excess, most of free GSH molecules remained in solution, producing a cloudy mixture and causing the fluorescence quenching of CuNCs. Moreover, the thiol group plays a critical role in reducing size and improving size distribution, while amino and carboxyl groups in GSH can protect the CuNCs from aggregation and increase their colloidal stability.° Compared with the $\delta$(S–H) absorption band of GSH at 2524 cm$^{-1}$, this peak disappeared for the as-obtained CuNCs (Fig. 1c), indicating that GSH combined with Cu atoms in the form of Cu–S bonds. Fig. 1d showed the $^1$H-NMR spectra of GSH and CuNCs in the range of 1.5–5.0 ppm, respectively. The peak at 4.69 ppm can be attributed to the residual D$_2$O solvent.° Based on the chemical structure of GSH (inset of Fig. 1d), the signals at 2.12 and 2.51 ppm were assigned to the protons on 3 and 4. Besides, the peak from proton on 7 can split into two peaks at 3.25 and 2.91 ppm, which may be caused by the adjacent chiral 6. Because the protons on 6 and 7 were very close to the copper core, the corresponding proton signals significantly downfield shifted.°

For a more intuitive observation of CuNCs, high-resolution TEM (HR-TEM) image (Fig. 2a) showed that the average size was 1.5 ± 0.5 nm. Under the intense e-beam from TEM instruments, the fusion of metal core may occur due to the e-beam-induced heating and the breaking of metal–S bonds, resulting in an increased polydispersity of CuNCs’ diameters.° AFM was also employed to show a panoramic view of CuNCs (Fig. 2b and S5†). The size distribution of CuNCs coincided well with the analysis of HR-TEM. Owing to the rapid measurement and good repeatability, zeta potential was further used to study the dispersibility of CuNCs in aqueous solution (Fig. 2c and S6†). The dynamic diameter of CuNCs was determined to be
larger than that obtained from HR-TEM (Fig. 2a) because of the presence of hydrated shells.

Mainly owing to the ultrasmall size of CuNCs, mass spectroscopy (MS) was then utilized to analyse their chemical compositions and the exact number of atoms. The mass spectrum of ultrasmall CuNCs was measured by the middle molecular weight mode in the range from 0 to 3000 (Fig. 2g). The largest peak ($m/z = 1183.91$) can be attributed to the Cu$_9$L$_2$ (L = C$_{10}$H$_{16}$O$_6$N$_3$S). In view of the diameter of a single Cu atom centering at about 0.255 nm, the size of CuNCs with compositions of Cu$_9$L$_2$ was very consistent with the size analysis with the HR-TEM image. It was worth mentioning that large energy ionization process may produce the formation of ion fragments. Hence, the peaks with lower mass values including Cu$_3$L$_2$ ($m/z = 800.91$) and Cu$_1$L$_1$ ($m/z = 367.99$) were assigned to the fragment ion signals. Moreover, this assignment was further supported by the precise match of simulated and experimental isotopic distributions indicated that the attribution of peak was correct (Fig. 2d–f and S7†). Besides, Cu loading capacity of CuNCs was up to 10.9% determined by ICP measurement.

To verify the oxidation states of copper in CuNCs, X-ray photoelectron spectroscopy (XPS) was carried out. The XPS survey spectrum shown in Fig. 3a indicated the presence of Cu, C, N and S elements in the product. The absence of satellite peak around 942.0 eV implied the lack of Cu(II) (Fig. 3b). Meanwhile, two peaks at 931.98 and 951.88 eV can be easily observed, corresponding to the binding energies of the 2p$_{3/2}$ and 2p$_{1/2}$ of the electrons of Cu(0), respectively. Another point worth noting was that it was difficult to distinguish Cu(0) and Cu(I) because the binding energy of Cu(0) is very close to Cu(I), Accordingly, we cannot rule out the possible existence of Cu(I) in CuNCs.

3.2 CAT-like activity of CuNCs

The ability of CuNCs to functionally mimic cellular antioxidant enzymes was investigated as follows. Based on the mechanism that the CAT catalyses the decomposition of H$_2$O$_2$ into O$_2$ and H$_2$O, CAT-like activity of CuNCs was determined by monitoring the contents of dissolved O$_2$ in solution. As shown in Fig. 4a, when the concentration of CuNCs increased from 0 to 400 μg
mL\(^{-1}\) at a fixed concentration of H\(_2\)O\(_2\), there was a significant increase in the dissolved O\(_2\), indicating the CAT-like catalytic function of CuNCs. For example, the rate of H\(_2\)O\(_2\) decomposition was calculated to be 0.23 mg L\(^{-1}\) s\(^{-1}\) when the concentration of CuNCs was 100 \(\mu\)g mL\(^{-1}\) (Fig. S8a\(^\dagger\)). More importantly, we found that catalytic efficiency almost maintained constant when the reaction temperature varied in the range of 25–40 °C, showing excellent catalytic stability of CuNCs.

Fig. 3 (a) XPS survey spectrum of CuNCs. (b) XPS spectra of Cu 2p.

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Fig. 4 (a) Plot of dissolved O\(_2\) versus time of H\(_2\)O\(_2\) (10 mM) in the presence of CuNCs (0–400 \(\mu\)g mL\(^{-1}\)). (b) H\(_2\)O\(_2\) scavenging of CuNCs. Fluorescence spectra of CuNCs (from top to bottom: 0, 10, 20, 50, 80, 100 \(\mu\)g mL\(^{-1}\), H\(_2\)O\(_2\) (10 mM) and TPA (0.5 mM). (c) GPx activity of different concentrations of CuNCs (2–200 \(\mu\)g mL\(^{-1}\)). (d) Scavenging efficiency of O\(_2^-\) with different concentrations of CuNCs. (e) Comparison of SOD-like catalytic activities of CuNCs and GSH. (f) Peroxidase activity of varied concentrations of CuNCs (0–200 \(\mu\)g mL\(^{-1}\)) in PBS (pH 5.5, 10 mM). (g) *OH scavenging of CuNCs. Fe\(^{2+}\) (20 \(\mu\)M) and H\(_2\)O\(_2\) (10 mM) were mixed for 1.5 min and then CuNCs (0–50 \(\mu\)g mL\(^{-1}\)) and DMPO (50 \(\mu\)L) were added for EPR spectra detection. (h) O\(_2^-\) scavenging of CuNCs. EPR spectra was recorded from samples containing xanthine (5 \(\mu\)M) and xanthine oxidase (0.5 U mL\(^{-1}\)), DMPO (50 \(\mu\)L) and CuNCs (0–100 \(\mu\)g mL\(^{-1}\)). (i) ABTS radical scavenging of CuNCs.

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(Fig. S8b and S9a†). In order to rule out the contribution of GSH on CAT-like activity of CuNCs, we further replaced CuNCs with GSH [0–300 μg mL⁻¹] and performed the catalytic tests under the same conditions (Fig. S9b†). GSH could hardly catalyse H₂O₂ to produce O₂ compared with the CuNCs (Fig. S10†), which suggested that the CAT-like activity of CuNCs mainly originated from CuNCs rather than the surrounding ligand molecules of GSH.

We used TPA as a fluorescent probe, which can generate strong fluorescent substances in the presence of ·OH, to verify the mechanism of CuNCs catalysing H₂O₂. If the content of ·OH in the system decreases, the corresponding fluorescence intensity will be weakened. As shown in Fig. 4b, the increased concentrations of CuNCs caused a gradient drop in the fluorescence intensity. Such results revealed that CuNCs did not promote the generation of ·OH during the CuNCs-induced decomposition of H₂O₂, directly identifying the chemical pathways of CAT-like activity.

3.3 GPx-like activity of CuNCs

Generally, GPx takes advantage of cellular GSH as a reducing agent for catalysing the reduction of H₂O₂ to water along with the production of GSSG. The free thiol group decreases with the increase of GSSG, in which the reduction of thiol group can be linearly related to NTB³⁻ with typical optical absorbance at 412 nm. As shown in Fig. 4c, the GPx activities of CuNCs were investigated by independently varying the concentrations of CuNCs from 0 to 200 μg mL⁻¹. As the majority of GSH was oxidized into GSSG by H₂O₂ in reaction system, the content of the remaining GSH that can react with DTNB will be decreased. In addition, we investigated the variation trend of DTNB absorbance with time in the presence of CuNCs (0–60 μg mL⁻¹), and found that with the increase of concentrations of CuNCs, UV absorption at 412 nm decreased significantly, indicating the good GPx-like activity of CuNCs (Fig. S11†). Furthermore, in the presence of CuNCs, the kinetic constants of CuNCs nanozymes were determined by changing the concentrations of H₂O₂ and GSH, showing typical Michaelis-Menten kinetics (Fig. S12†). For example, the Kₘ value of CuNCs with H₂O₂ as substrate was ~0.10 mM, and the Vₘₐₓ value was ~6.78 μM min⁻¹. The Kₘ and Vₘₐₓ values of CuNCs with GSH as the substrate were ~0.91 mM and ~4.76 μM min⁻¹, respectively.

3.4 SOD-like activity of CuNCs

CuNCs exhibited SOD-like activity by catalysing the disproportionation of superoxide (O₂⁻) to H₂O₂ and O₂. As displayed in Fig. 4d, the inhibition rate of SOD increased rapidly with the increased concentration of CuNCs (60–180 μg mL⁻¹), indicating the SOD-like activity of CuNCs under physiologically relevant conditions. Notably, two groups with inhibition rates of 44.8% and 55.4% were selected and the final SOD activity was calculated to be 25.6 U mg⁻¹. Considering the possible contribution of GSH in the SOD-like activity of CuNCs, we replaced the CuNCs with GSH to further determine the corresponding SOD activity. As shown in the Fig. 4e, we observed that irrespective of concentrations, the SOD inhibition rate of CuNCs was about 3 times higher than that of GSH. For example, at 180 μg mL⁻¹, the SOD inhibition rate of CuNCs was about 55.4%, while this value obviously dropped to about 17.7% for GSH. Furthermore, the effect of catalytic time on SOD activity was also investigated. The UV absorption values of the system solution gradually decreased responding to the increased concentration of CuNCs (Fig. 4d). In the first 20 min, the UV absorption values of the solutions for all selected concentrations of CuNCs showed a positive correlation growth trend. As the catalytic reaction time continued to elongate, the increase in UV absorption will slow down and remained constant after 35 min. The possible reason was that due to the certain content of superoxide anion free radicals produced by the reaction system, accompanying with the gradual completion of catalytic reaction, the UV absorption value gradually decreased and eventually approached a fixed value.

3.5 Peroxidase-like activity of CuNCs

To prove the peroxidase activity of CuNCs, the catalysis of TMB was tested in the presence of H₂O₂. As shown in Fig. 4f, different concentrations of CuNCs can catalyse H₂O₂ to oxidise TMB, while no oxidation reaction was detected without CuNCs or H₂O₂, verifying that both components were necessary. We employed steady-state kinetics to further explore the peroxidase-like activity of CuNCs and observed that the oxidation reaction catalysed by CuNCs followed the typical Michaelis-Menten kinetics for TMB and H₂O₂ (Fig. S13†). The Kₘ value of CuNCs with H₂O₂ as the substrate was significantly lower than that of Horseradish peroxidase (HRP, Kₘ = 3.70)³⁸ revealing that the catalysis of TMB by CuNCs only required a lower concentration of H₂O₂. Turnover number (TON), also termed as Kₘₐₓ, was defined as the maximum number of chemical conversions of the substrate molecules per second.³⁹ It can be calculated from the following equation:

\[
\text{TON or } K_{\text{cat}} = \frac{V_{\text{max}}}{[E]}
\]

where Vₘₐₓ is the maximum reaction rate; [E] is the concentration of enzymes or CuNCs.⁴⁰,⁴¹

The Vₘₐₓ were obtained from Lineweaver-Burk plot and calculated to be \(8.8 \times 10^{-8} \text{ M s}^{-1}\) and \(5.15 \times 10^{-8} \text{ M s}^{-1}\) for H₂O₂ and TMB substrates. From Table S1,† we can find that Vₘₐₓ of CuNCs was even higher than HRP.⁴² Overall, CuNCs performed good catalytic activities compared with other previously reported nanozymes (Table S2 and S3†).

3.6 Free radical scavenging of CuNCs

We further selected three representative free radicals (e.g., ·OH, O₂⁻ and ABTS) to verify the antioxidant performance of CuNCs. It is difficult to detect free radicals since the life span was extremely short, so DMPO was used to capture free radicals to form stable adducts (DMPO/·OH), which results in a 1 : 2 : 2 : 1 peak area on the EPR. From Fig. 4g, we can find that the signal was the strongest in absence of CuNCs, indicating that H₂O₂ produces a large number of ·OH through Fenton effect under the action of Fe²⁺. When CuNCs (10 μg mL⁻¹) were added into
In the system, the intensity of DMPO/•OH was significantly reduced, indicating that CuNCs could effectively scavenging •OH. When the final concentration of CuNCs reaches 50 μg mL⁻¹, the signal strength is only 10% of the original, indicating that CuNCs have been able to remove most •OH. Similarly, DMPO was also used to capture O₂⁻ to form stable adducts (DMPO/O₂⁻), which results in a 1 : 1 : 1 : 1 peak area on the EPR. As shown in Fig. 4h, the scavenging efficiency of O₂⁻ was proportional to the concentrations of CuNCs. CuNCs with the concentration of 100 μg mL⁻¹ could drastically eliminate the O₂⁻. In addition, we also compared the SOD natural enzyme scavenging O₂⁻ under the same conditions (Fig. S14†), demonstrating that 50 μg mL⁻¹ of CuNCs was close to the SOD at 5 U mL⁻¹. Finally, we verified the scavenging ability of CuNCs on ABTS free radicals and established that CuNCs (0.5 mg mL⁻¹) can reach 78% of scavenging effect (Fig. 4i).

3.7 Intracellular ROS scavenging of CuNCs

We selected HEK 293 cell line to identify the protective ability of CuNCs against ROS injury in vitro. First, HEK 293 cells with different concentrations of CuNCs were incubated for 24 and 48 h, respectively (Fig. 5b and S15†). The cell viability studies showed that CuNCs had no cytotoxicity to HEK 293 cells. Moreover, we used H₂O₂ (100 μM) to simulate the damage caused by ROS to cells. After incubating the cells with H₂O₂, the ROS signals (green fluorescence) increased significantly. Interestingly, the decline of fluorescence indicated that the ROS could be gradually eliminated after adding CuNCs. Almost all the ROS signals disappeared and returned to the level of negative control when the CuNCs concentration reached 100 ng mL⁻¹ (Fig. 5a and S16†). After that, we used the CCK-8 assay to quantify the cell viability. Nearly 50% of cells were damaged after incubating with H₂O₂, and the damaged cells can be successfully repaired after introducing CuNCs. The cell viability recovered to above 91% (Fig. 5c), confirming that CuNCs can protect cells from oxidative stress.

4 Conclusion

In summary, this study described a one-pot method to prepare ultrasmall GSH-capped CuNCs with obvious aggregation-induced emission. We, for the first time, demonstrated that ultrasmall CuNCs had remarkable multienzyme catalytic activities, which can mimic three major structurally and functionally
different antioxidant enzymes. Intracellular ROS scavenging of CuNCs indicated that CuNCs could protect neuronal cells from oxidative damage. Taken together, CuNCs with ROS scavenging capability show great potential in the future treatment of ROS-related diseases.

**Author contributions**

B. C. and T. S. conceived and designed the study, supervised the project and wrote the manuscript. Y. P. performed all the experiments. Y. R. and H. Z. helped the design of the study and analyzed the data. Y. A. contributed to structural analysis by TEM. All authors discussed the results and commented on the manuscript.

**Conflicts of interest**

The authors declare no competing financial interests.

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