Shape-controllable and kinetically miscible Copper–Palladium bimetallic nanozymes with enhanced Fenton-like performance for biocatalysis

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Abstract

Bimetallic nanozymes have been emerging as essential catalysts due to their unique physicochemical properties from the monometallics. However, the access to optimize catalytic performance is often limited by the thermodynamic immiscibility and also heterogeneity. Thus, we present a one-step coreduction strategy to prepare the miscible Cu–Pd bimetallic nanozymes with controllable shape and homogeneously alloyed structure. The homogeneity is systematically explored and luckily, the homogeneous introduction of Cu successfully endows Cu–Pd bimetallic nanozymes with enhanced Fenton-like efficiency. Density functional theory (DFT) theoretical calculation reveals that Cu–Pd bimetallic nanozymes exhibit smaller d-band center compared with Pd nanozymes. Easier adsorption of H₂O₂ molecular contributed by the electronic structure of Cu significantly accelerate the catalytic process together with the strong repulsive interaction between H atom and Pd atom. In vitro cytotoxicity and intracellular ROS generation performance reveal the potential for in vivo biocatalysis. The strategy to construct kinetically miscible Cu–Pd bimetallic nanozymes will guide the development of bimetallic catalysts with excellent Fenton-like efficiency for biocatalytic nanomedicine.

1. Introduction

Bimetallic nanoparticles have attracted broad attentions due to the unique catalytic properties in various fields, especially in chemical sensing, heterogeneous catalysis, and nanomedicine [1–3]. Different from monometallic nanoparticles, bimetallic nanoparticles always present unique multifunctional performances due to the synergistic effects among of corresponded monometallic analogues [4]. For instance, Cu–Ni bimetallic nanoparticles have been obtained via nonequilibrium synthetic strategy and exhibit enhanced C₂H₄ product Faradaic efficiencies (~76%), which is ~20% higher than that of monometallic Cu [5]. Yang and coworkers have precisely explored the activity of Au–Cu bimetallic nanoparticles as a function of their composition and demonstrated that uniform Au₃Cu bimetallic nanoparticles exhibit highest catalytic activity, while pure Cu nanoparticles owned the lowest overall activity [6]. Compared to monometallic materials, the design and development of bimetallic nanoparticles greatly optimize the functionality and broaden the applications.

Due to the fact that various factors like size, morphology, component, and nanoscale arrangement will influence the physicochemical properties, many research interests have been paid to deliberately identify the relationship between enhanced catalytic performances and structures in order to expand the practical applications in the field of catalysis [7,8]. However, there still remains two fundamental challenges: the limited access to obtain homogeneously alloyed bimetallic nanozymes due to the thermodynamic immiscibility and the difficulty to identify and optimize the catalytic performance because of the heterogeneity in bimetallic catalysts [5]. Traditional preparation methods such as seed-mediated growth, galvanic replacement, concurrent thermal decomposition, and impregnation tend to yield bimetallic nanozymes with unfavorable structures, broad size distributions, inhomogeneous alloying, and other heterostructures [2,9]. New methods like surface plasmon resonance

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XPS spectra of samples were measured on the X-Ray Photoelectron D/max-2550, Rigaku) was used to analyze the crystal structure of powder synthesized Pd and Cu. The structural morphology, crystal structure, and element components of as-synthesized Pd and Cu were explored to obtain the high-resolution AC HAADF-STEM images of Pd nanozymes. The aberration-corrected high-angle annular dark-field scanning TEM (AC HAADF-STEM, FEI Titan Cubed G2 300) was performed to obtain the high-resolution AC HAADF-STEM images of Pd and Cu–Pd bimetallic nanozymes at 300 keV. X-ray diffractometer (XRD, D/max-2550, Rigaku) was used to analyze the crystal structure of powder. XPS spectra of samples were measured on the X-Ray Photoelectron Spectroscopy (250Xi, Thermo Fisher Scientific). The electron spin resonance (ESR) spectra were trapped by 5,5-dimethyl-1-pyrroline N-oxide (DMPO) in aqueous solution with CuPd, CuPd2, and Pd (10 μg/mL). The intracellular GSSG level was measured by GSH and GSSG Assay Kit.

2.3 Preparation of Cu–Pd bimetallic nanozymes

Hovenia acerba-like Pd nanozymes: 2.67 mL Na2PdCl4 aqueous solution (122.4 mM) and 200 mg HDA were added into 7 mL ultrapure (UP) water in a 20 mL Wheaton Sample Vial. The mixture was magnetically stirred overnight at ambient temperature for complete dissolution. Then, the solution was transferred into a 100 mL round-bottom flask and another 1 mL glucose aqueous solution (75.7 mM) was added. The system was heated to 110 °C for 2 h under magnetic stirring. After cooling down to room temperature, the product was washed with aqueous ethanol solution (20 vol%) for four times and redispersed in 2 mL UP water for further use.

Cu–Pd bimetallic nanozymes: Following the same strategy as Pd nanozymes, alliums-like CuPd3 nanozymes were prepared by adding 0.42 mL CuCl2 · H2O aqueous solution (100 mM) at the beginning, and concave rhombic dodecahedral-like Cu-Pd nanozymes were prepared by synchronously adding 1.24 mL CuCl2 · H2O aqueous solution (100 mM).

2.4 Fenton-like efficiency and kinetics measurement

In order to evaluate the Fenton-like efficiency and kinetics performance, TMB Single-Component Substrate Solution was employed to detect the generated hydroxide radical (OH) as protocol in our previous study. For testing Cu–Pd bimetallic nanozymes concentration-dependent kinetics, 0.5 mL TMB Single-Component Substrate Solution and 0.5 mL UP water were mixed in a 48-well plate firstly. Then Cu–Pd bimetallic nanozymes were added for a final concentration of 500, 200, 100, and 50 μg/mL quickly. The time-dependent absorbance value at 652 nm was detected in a Microplate Reader (Varioskan LUX, Thermo Scientific). The UV–vis spectra of above mixture were measured when the reaction lasts for 0.5 h. Besides, for measuring H2O2 concentration-dependence kinetics, additional H2O2 was added into the TMB Single-Component Substrate Solution for a final H2O2 concentration of 40, 30, 20, 10, 5, 1 mM. The time-dependent absorbance value at 652 nm was detected immediately after Pd and Cu–Pd bimetallic nanozymes were added (Pd: 500 μg/mL, CuPd3: 300 μg/mL, CuPd2: 150 μg/mL). Meanwhile, the UV–vis spectra of Cu–Pd bimetallic nanozyme in various TMB/H2O2 solution (H2O2 concentration: 40, 30, 20, 10, 5, 1 μM) were measured when the reaction lasts for 0.5 h.

2.5 X-ray absorption spectrum measurement

For X-ray absorption spectrum measurement, Cu–Pd bimetallic nanozyme powders were prepared via lyophilization strategy. Cu–K edge X-ray absorption spectra (XAS) data of Cu–Pd bimetallic nanozyme were collected on beam line Si(111) crystal monochromators at the BL11B beamlines at the Shanghai Synchrotron Radiation Facility (SSRF) (Shanghai, China). X-ray absorption near-edge structure (XANES) was observed between two scans taken for a specific sample and extended X-ray absorption fine structure (EXAFS) data were performed by in transmission mode. The data were analyzed by the software of Athena. Wavelet Transform (WT) was carried out with hamaFortran software.

2.6 DFT theoretical calculation

The structural files of Cu (111) and Pd (111) were downloaded from AMOSSD (American Mineralogist Crystal Structure Database). The substitutional atoms were set, and three metal-organic models were created by GaussView5.0, which had 16 transition metal atoms and two hydrogen peroxide molecules. The optimizations of the systems were carried out by using PBE method and LANL2TZ basis set as employed in Gaussian 16 package. Then the equilibrations in a constant ensemble (NVT) were performed by using GFN2-XTB method in the CP2K package, of which the temperature was 310 K and the pressure was 1 bar. Density of States (DOS) analysis has also been obtained by MULTIWFN 3.8 program, and d-band center was calculated to study the adsorption between the transition metal surface and hydrogen peroxide (H2O2).
2.7. In vitro cytotoxicity evaluation

To evaluate the in vitro cytotoxicity of as-synthesized Pd and Cu–Pd bimetallic nanozyme, 4T1 murine mammary cancer cell line was chosen as model. In detail, cells in exponential phase were seeded in a 96-well plate with a concentration of 5000/well. 12 h later, the medium was replaced with fresh medium (various Pd and Cu–Pd bimetallic nanozyme: 400, 200, 100, 50, 25, 12.5, 6.25, 0 μg/mL). After another 24 h incubation, the cell viability of 4T1 cells was measured by cell count kit-8 (CCK-8).

2.8. Intracellular hydroxide radical detection

DCFH-DA fluorescent probe was employed to detect the intracellular OH catalyzed by Pd and Cu–Pd bimetallic nanozymes. In general, 4T1 cells in exponential phase were seeded in a 96-well plate with a concentration of 5000/well. 12 h later, the medium was replaced with fresh medium with Pd and Cu–Pd bimetallic nanozymes concentration of 25 μg/mL. After incubation for another 6 h, the Pd and Cu–Pd bimetallic nanozymes were removed thoroughly and the cells were stained with DCFH-DA probe. Finally, confocal laser scanning microscopy (FV3000, Olympus) was used to image the cells with excitation/emission wavelength: 504/529 nm.

2.9. Statistical analysis

Results in this study were presented as mean values ± SD, and the statistical difference was calculated by two-tailed student’s t-test. *p < 0.05, **p < 0.01, ***p < 0.001.

3. Results and discussion

3.1. Synthesis and characterization of Pd and Cu–Pd bimetallic nanozymes

As illustrated in Fig. 1a, the one-step coreduction strategy was introduced to prepare the hovenia acerba-like Pd nanozyme and Cu–Pd
bimetallic nanozymes (alliums-like CuPd₃ nanozyme, and concave rhombic dodecahedral-like Cu₃Pd) for optimized Fenton-like efficiency (catalyzing H₂O₂ into hydroxide radical (OH)). Generally, Na₂PdCl₄ and CuCl₂·2H₂O were employed as metal precursors and glucose was taken as reductant during the one-pot preparation procedures in the presence of hexadecylamine (HDA). By adjusting the molar ratio of Pd and Cu precursor, homogeneously alloyed Cu–Pd bimetallic nanozymes with different morphology and structure were obtained. As shown in Fig. 1b, the transmission electron microscopy (TEM) image of Pd nanozyme presents regular and homogeneous hovenia acerba-like structure with a narrow size distribution. When 1/3 M ratio of Cu/Pd precursor was added, the obtained CuPd₃ nanozyme exhibits agminated and pyknotic alliums-like structure (Fig. 1c). Furthermore, a concave rhombic dodecahedral-like structure (Fig. 1d) appears when the molar ratio of Cu/Pd precursor increases to 3. High-resolution transmission electron microscopy (HRTEM) images of Pd and Cu–Pd bimetallic nanozymes

![Image](https://via.placeholder.com/150)

Fig. 2. (a–f) AC-HAADF-STEM images of Pd (a), CuPd₃ (b), and Cu₃Pd (c). (d–f) Corresponding element mapping (HADDF image, Cu, Pd, merge image) of Pd (d), CuPd₃ (e), and Cu₃Pd (f). (g) Atomistic simulation scheme describes the formation of kinetically trapped homogeneous Cu–Pd bimetallic nanozymes.
clearly demonstrate the fine crystal structure with periodic fringe spaces (Fig. 1e–g). As presented in Fig. 1e, the d-spacing value is measured to be 1.935 Å, which is consistent with the d-spacing value for (200) of pure Pd nanozyme. Meanwhile, the d-spacing of 2.071 Å, and 2.078 Å for CuPd₃ nanozyme (Fig. 1f) reveals the lattice plane of Cu (111) and Pd (200), respectively. The same lattice planes are also detected for Cu₃Pd nanozyme at d-spacing of 2.078 Å and 1.948 Å, indicating the fine crystal structure of Cu–Pd bimetallic nanozymes. As-synthesized Pd and Cu–Pd bimetallic nanozymes show excellent dispersibility and stability in water, phosphate buffer saline (PBS), and DMEM medium (Fig. S1). The size distribution obtained by dynamic light scattering (Fig. S2) is consistent with TEM images and all of them demonstrate negative zeta potential (Fig. S3). Besides, X-ray diffraction (XRD) was performed to explore the crystal structure of as-prepared Cu–Pd bimetallic nanozymes. The XRD

![X-ray absorption evaluation for Cu–Pd bimetallic nanozymes. (a, b) XANES and magnified pre-edge XANES spectra taken at Cu K-edge of CuPd₃ (a) and Cu₃Pd (b). (c, d) Fourier transform of Cu-edge EXAFS of CuPd₃ (c) and Cu₃Pd (d) in R-space. (e, f) Wavelet Transform image at the Cu-edge of CuPd₃ (e) and Cu₃Pd (f). Cu-foil and CuPc were taken as reference.](image-url)
pattern in Fig. S4a shows the characteristic peaks of Pd at about 40.1° for (111), 46.7° for (200), and 68.1° for (220), which is consistent with the standard PDF card (PDF#46–1043). For the samples were loaded by a transparent glass slide, the strong peak at about 21.6° is responsible for the (111) of silicon dioxide (PDF#27–0605). For the samples were loaded by a transparent glass slide, the strong peak at about 21.6° is responsible for the (111) of silicon dioxide (PDF#27–0605). The XRD pattern of CuPd₃ (Fig. S4b) and Cu₃Pd (Fig. S4c) nanozymes present obvious diffraction peak shifting to high angle positions, indicating the increasing doping amount of Cu in Pd lattice. Meanwhile, the characteristic peaks of (111), (200), and (220) of Cu at about 43.3°, 50.4°, and 74.1° appear (PDF#04–0836), demonstrating that cubic crystal system of Cu become the main phase. It is well known that Cu and Pd have the same crystal system (cubic), similar space group (Fm-3m and Fd-3m), and approximately equal atomic diameter (1.28 Å and 1.37 Å), which making it easy to form the homogenous Cu–Pd bimetallic nanozymes [5].

According to the Cu–Pd binary phase diagrams (Fig. S5), there are two superlattices (L1₂ and B₂) for the low-temperature ordered phases [14,15]. And the L1₂ structure is the ordered phase Cu₃Pd with a composition range of homogeneity.

In order to further study the atomic spatial information of Cu and Pd (single-atom or intermetallic compound), we tested the Pd and Cu–Pd nanozymes using the high-angle-annular-dark-field scanning transmission electron microscopy (HAADF-STEM) (Fig. 2a–c). Compared with the HAADF-STEM images of pure Pd nanozyme (Fig. 2a), there is no evident nanoparticle and metallic cluster in both CuPd₃ (Fig. 2b) and Cu₃Pd (Fig. 2c) nanozymes, indicating the homogenous alloying of Cu and Pd, which is consistent with the results of element mapping. Meanwhile, none small bright/dark dots appear in the aberration-corrected HAADF-STEM (AC-HAADF-STEM) images with sub-Angstrom resolution of CuPd₃ (Fig. 2b) and Cu₃Pd (Fig. 2c) nanozymes, demonstrating the spacing state of Cu and Pd atom in lattice is not single-atom, but intermetallic compound. The simultaneously obtained energy-dispersive X-ray spectroscopy (EDS) spectra (Figs. S6a–c) confirms the presence of Cu and Pd in CuPd₃ and Cu₃Pd nanozymes. To intuitively verify the successful synthesis of Pd and Cu–Pd bimetallic nanozymes, the EDS mapping on HRTEM was carried out (Fig. 2d–f). The results clearly exhibit the pure Pd nanozyme and the homogenous distribution of Cu.

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**Fig. 4.** Fenton-like reaction activity and kinetics performance of Cu–Pd bimetallic nanozymes. (a–c) Time-dependent UV–vis absorbance of CuPd₃ (a), CuPd₃ (b), and Pd (c) in TMB/H₂O₂ solution with various concentrations (500, 200, 100, 50 μg/mL). (d) Time-dependent UV–vis absorbance of Cu–Pd bimetallic nanozymes in TMB/H₂O₂ solution with concentration of 200 μg/mL (e) ESR spectra of hydroxide radical trapped by 5,5-dimethyl-1-pyrroline N-oxide (DMPO) in aqueous solution with CuPd₃, CuPd₃, and Pd (10 μg/mL). (f) UV–vis absorbance value at 652 nm of TMB/H₂O₂ solutions after incubation with Cu–Pd bimetallic nanozymes (200, 150, 100, 50 μg/mL) for 1.5 h. (g) Photographs of TMB/H₂O₂ solutions after incubation with Cu–Pd bimetallic nanozymes (500, 200, 100, 50 μg/mL) for 1.5 h. (h) UV–vis absorbance value at 652 nm of TMB/H₂O₂ solutions (H₂O₂ concentration: 40, 30, 20, 10, 5, 1 mM) after incubation with Cu–Pd bimetallic nanozymes for 0.5 h. (i) Time-dependent UV–vis absorbance of CuPd₃ in various TMB/H₂O₂ solution (H₂O₂ concentration: 40, 30, 20, 10, 5, 1 μM).
and Pd in both CuPd₃ and Cu₃Pd nanozymes. Furthermore, the atomic ratio of Cu/Pd in Cu–Pd bimetallic nanozymes is qualitatively verified by the EDS mapping, which are 1:3.4 for CuPd₃ and 3.4:1 for Cu₃Pd nanozyme (Fig. S6d&e).

Besides the elemental components measurement, the X-ray photoelectron spectroscopy (XPS) was performed to further investigate the surface chemical composition of Pd and Cu–Pd bimetallic nanozymes. As shown in Figs. S7a–c, none peak is detected for Cu 2p spectrum, but the narrow-scan Pd 3d spectra exhibits characteristic high (343.2 eV) and low (337.8 eV) energy regions that assigning to the 3d₃/2 and 3d₅/2 regions. For CuPd₃ nanozyme, the Cu 2p spectra appears and is divided into two regions (high energy region of 952.3 eV and low energy region of 932.5 eV), which are attributed to 2p₁/₂ and 2p₃/₂ (Figs. S8a–c). Meanwhile, the divided regions of Pd 3d remain (Fig. S8c) similar with that of

![Diagram](image)

Fig. 5. Density Functional Theory (DFT) theoretical calculation for the Fenton-like efficiency of Cu–Pd bimetallic nanozymes. (a–f) The critical intermediate structures and corresponding total energy diagram of Fenton-like reaction path catalyzed by Pd (a, b), CuPd₃ (c, d) and Cu₃Pd (e, f) nanozymes. (g–i) The pDOS of d-band center analysis of Pd (g), CuPd₃ (h) and Cu₃Pd (i) nanozymes.
Pd nanzyme. With the increase of Cu amount, Cu$_2$Pd nanzyme exhibits more obvious Cu 2p peak in XPS survey spectra (Fig. 5a&b). Importantly, the peaks of narrow-scan Cu 2p spectra at Cu 2p$_{3/2}$ shows the Cu(0) (931.9 eV)/Cu(I) (933.5 eV) species (Fig. 5b), indicating that the partial Cu oxidation on the surface. The narrow-scan Pd 3d spectra of Cu$_3$Pd nanzyme is similar with that of CuPd$_3$ nanzyme (Fig. 5c), revealing the similar atomic state. Besides, the zero-valent metal peaks for the Cu 2p spectra in both CuPd$_3$ and Cu$_2$Pd nanzymes and Pd 3d spectra in all samples further confirming the homogenous alloying of Cu–Pd bimetallic nanozymes [16]. Based on above results, we speculate that both Cu and Pd atom are kinetically trapped in bimetallic lattice, as shown in Fig. 2g. Because the thermodynamic equilibrium state eliminates the phase segregation of Cu or Pd, homogeneity of Cu–Pd bimetallic nanozymes is obtained during the coreduction process, which has been verified by the AC-HAADF-STEM images.

### 3.2. XAS measurements for coordination structure

For further authenticating the kinetically trapped structure in Fig. 2g, X-ray absorption spectroscopy (XAS) was performed to investigate the precise coordination structure of the Cu–Pd bimetallic nanozymes. The Cu K-edge X-ray absorption near-edge structure (XANES) of both CuPd$_3$ (Fig. 3a) and Cu$_2$Pd (Fig. 3b) bimetallic nanozymes exhibit that the pre-edge peaks are located close to Cu-foil and far away from CuPc, indicating that the average valence of Cu in both CuPd$_3$ and Cu$_2$Pd nanozymes is Cu(0), which is consistent with the results of XPS spectra. Furthermore, the k$^2$-weighted Fourier-transformed extended X-ray absorption fine structure spectra (FT-EXAFS) of both CuPd$_3$ (Fig. 3c) and Cu$_2$Pd (Fig. 3d) bimetallic nanozymes show that the major peak locates at about 2.25 Å, which means that the existence of Cu atom in lattice is not single-atom state. This is also verified by none significant peaks appear at about 1.56 Å as that in CuPc. Furthermore, the wavelet transform (WT) of the EXAFS plot (Fig. 3e&f) is employed to examine the atomic configuration via the information of k- and R-spaces. As shown in Fig. 3e, both the centers of CuPd$_3$ nanzyme and Cu-foil are located at k-space (about 7.95 Å$^{-1}$) and R-space (about 2.25 Å), which are attributed by the Cu–Cu scattering signal [17,18]. The WT image of CuPc presents a center at k-space (about 6.25 Å$^{-1}$) and R-space (about 1.02 Å), revealing the typical signal of Cu–N coordinated center. In contrast to the WT image of Cu-foil and CuPc, it can be concluded that the Cu atoms in CuPd$_3$ nanzyme are Cu–Cu pair, a form of miscible bimetallic phase. Similarly, the WT-EXAFS of Cu$_2$Pd nanzyme (Fig. 3f) shows an average bond distance of 2.26 Å, indicating the typical Cu–Cu pair. All of the results confirm the as-speculated homogeneously alloyed atomic arrangement structure of Cu–Pd bimetallic nanozymes (Fig. 2g).

### 3.3. Fenton-like activity evaluation of Pd and Cu–Pd bimetallic nanozymes

The Fenton-like reaction activity of as-synthesized Pd and Cu–Pd bimetallic nanozymes were systematically explored by 3,3’,5,5’-tetramethylbenzidine (TMB) colorimetric assays. The TMB could be efficiently catalyzed into oxidized TMB (oxTMB) by the Fenton-like reaction products (hydroxide radical: −OH) and generated oxTMB exhibits a characteristic absorption peak at about 652 nm, which is proportional to the amount of −OH [19]. Firstly, the Fenton-like reaction kinetics of Pd and Cu–Pd bimetallic nanozymes was investigated with various concentrations (Fig. 4a-c). As shown in Fig. 4a, although Pd nanzyme exhibits weak catalytic activity below 200 μg/mL, time-dependent oxTMB accumulation under 500 μg/mL shows significant enhancement, indicating the possible Fenton-like reaction catalyzed by Pd nanzyme. Compared with Pd nanzyme, CuPd$_3$ nanzyme reveals efficient catalytic activity (Fig. 4b). All the substrates (TMB/H$_2$O$_2$) were consumed by 200 μg/mL CuPd$_3$ nanzyme within about 20,000 s. And when the concentration was increased to 500 μg/mL, the reaction time would shorten to 11,000 s. Surprisingly, Cu$_2$Pd nanzyme presents highest Fenton-like efficiency for the reaction time is only about 8000 s with low concentration of 100 μg/mL (Fig. 4c). Meanwhile, the kinetic curve of Pd and Cu–Pd bimetallic nanozymes with same concentration (200 μg/mL) further clearly confirms the results that the introduction of Cu will efficiently optimize the Fenton-like reaction performance of Cu–Pd bimetallic nanozymes (Fig. 4d). Meanwhile, Electron spin resonance (ESR) was applied to detect the hydroxide radical trapped by 5,5-dimethyl-1-pyrroline N-oxide (DMPO) in aqueous solution. Compared with alone H$_2$O$_2$ group, no significant OH yield is detected for Pd + H$_2$O$_2$ group with concentration of 10 μg/mL (Fig. 4e). However, the strongly characteristic ESR spectra of spin adduct DMPO/OH are observed in both CuPd$_3$ + H$_2$O$_2$ and Cu$_2$Pd + H$_2$O$_2$ groups, indicating the successful generation of OH via Fenton-like reaction. Meanwhile, CuPd + H$_2$O$_2$ group exhibits the strongest peak intensity compared with Pd and CuPd$_3$, which is consistent with the results of kinetics assay. UV–vis absorbance value at 652 nm of TMB/H$_2$O$_2$ solutions after incubation with Cu–Pd bimetallic nanozymes (200, 150, 100, 50 μg/mL) for 1.5 h (Fig. 4f) demonstrates the concentration-dependent catalysis process and enhancement of Fenton-like efficiency via Cu-introduction, which is further confirmed by the UV–vis spectra in Fig. S10. And the photographs of TMB/H$_2$O$_2$ solutions after incubation with Cu–Pd bimetallic nanozymes (200, 150, 100, 50 μg/mL) for 1.5 h (Fig. 4g) intuitively illustrate the difference of reaction activity. The substrate concentration is one of the main variables to influence the activity of nanozymes [20,21]. Thus, the Fenton-like efficiency of Cu–Pd bimetallic nanozymes was evaluated in various H$_2$O$_2$ concentration system (Fig. 4h). With the increase of H$_2$O$_2$ concentration, all Cu–Pd bimetallic nanozymes present excellent catalytic response. However, compared with Pd, CuPd$_3$ and Cu$_2$Pd demonstrate faster reaction under higher H$_2$O$_2$ concentration system (Fig. S11). The catalytic kinetics were also measured to compare the catalytic activity of Pd and Cu–Pd bimetallic nanozymes in different substrate environment (Fig. 4l, Fig. S12). The results show that higher substrate concentration will bring faster reaction efficiency, indicating that substrate do not influence the activity of nanozymes. Meanwhile, the kinetics results under 40 μM H$_2$O$_2$ concentration increasingly confirms the enhanced Fenton-like property of Cu–Pd bimetallic nanozymes (Fig. S13). All above results reveal that as-synthesized Cu–Pd bimetallic nanozymes possess excellent Fenton-like reaction performance, and the reaction efficiency is effectively enhanced via increasing level of Cu.

### 3.4. DFT theoretical calculation for enhanced Fenton-like performance

The above optimized Fenton-like efficiency of Cu–Pd bimetallic nanozymes compared with Pd nanzyme inspires us to disclose the catalytic mechanism. Therefore, we performed the molecular dynamic simulation in a canonical ensemble (NVT) by using GEM2-XTB method in the CP2K package. Density of States (DOS) analysis has also been obtained by MULTIWfn 3.8 program, and d-band center was calculated to study the adsorption between the transition metal surface and H$_2$O$_2$. Fig. 5a-f shows the critical intermediate structures for the generation of -OH from H$_2$O$_2$ catalyzed by Cu–Pd bimetallic nanozymes. The initial H$_2$O$_2$ molecular will absorb on the Pd and Cu–Pd bimetallic nanozymes and then dissociate into -OH and OH$^-$ homogeneously. Thereafter, one -OH will react with H$_2$O$_2$ molecular to produce OOH$^-$ and H$_2$O. Finally, the Cu–Pd bimetallic nanozymes will return to the original state via desorption of H$_2$O$_2$ molecular [22]. Along this reaction routine, the total energy diagrams of Cu–Pd bimetallic nanozymes was calculated and

| eV     | Pd | CuPd$_3$ | Cu$_2$Pd |
|--------|----|----------|----------|
| tDOS   | -13.69 | -9.806 | -8.828 |
| pDOS   | -14.633 | -10.041 | -9.366 |
| HOMO level | -13.143 | -8.170 | -6.064 |
| d-band center | -1.490 | -1.871 | -3.302 |
depicted in Fig. 5b,d,f. It is obviously that Cu$_3$Pd system presents the largest total energy decrease (0.648 eV) compared with that of CuPd$_3$ (0.448 eV) and Pd (0.443 eV), indicating the highest Fenton-like efficiency, which is consistent with previous experimental results. To further disclose catalytic mechanism, the partial density of states (pDOS) is explored to analyze the d-band of Cu–Pd bimetallic nanozymes. As shown in Fig. 5g–I, and Table 1, Pd nanozyme has a larger absolute pDOS value (14.633 eV) than that of CuPd$_3$ (10.041 eV) and Cu$_3$Pd (9.366 eV) bimetallic nanozymes, indicating the weaker interaction between Pd nanozyme with H$_2$O$_2$. Meanwhile, the order of d-band center is Cu$_3$Pd ($-3.302$ eV) < CuPd$_3$ ($-1.871$ eV) < Pd ($-1.490$ eV), demonstrating the easier absorption of H$_2$O$_2$ on the transition metal surface of Cu$_3$Pd then both CuPd$_3$ and Pd nanozymes. Our previous study has demonstrated that Pd atom has strong repulsive interaction to H atom in H$_2$O$_2$ due to...
the electronic structure [23]. Herein, we speculate the easier absorption effect for CuPd3 and Cu3Pd is contributed by the optimized electronic structure of Cu compared with Pd nanozyme. Therefore, contributing by the optimized electronic structure by Cu to absorb H₂O₂ molecular and largest total energy decrease to produce -OH, CuPd nanozyme displays highest Fenton-like efficiency than both CuPd₃ and Pd nanozymes.

3.5. In vitro evaluation of Fenton-like efficiency

As one of the tumor-specific treatments, chemodynamic therapy (CDT) mediated by both Fenton or Fenton-like reaction in tumor microenvironment (TME) has exhibited various advantages such as high selectivity, deep tissue penetration, and excellent sensitivity [24–26]. It is well known that the intracellular H₂O₂ level in tumor cells (about 50–100 μM) is higher than that of normal cells [27,28]. Therefore, the optimized Fenton-like efficiency of Cu-Pd bimetallic nanozymes inspire us to investigate in vivo antitumor effect. 4T1 murine mammary cancer cell line was chosen as model to coinoculation with various concentrations of Pd and Cu-Pd bimetallic nanozymes (400, 200, 100, 50, 25, 12.5, 6.25, 0 μg/mL) for 24 h. The relative cell viabilities were evaluated by Cell Count Kit-8 (CCK-8) and the results were shown in Fig. 6a-f. CuPd₃ (Fig. 6a), CuPd (Fig. 6a), Cu₃Pd (Fig. 6b), and Pd (Fig. 6c) nanozymes demonstrate the concentration-dependent cytotoxicity to 4T1 cells with corresponding IC50 value of 59.55 μg/mL, 68.18 μg/mL, and 95.82 μg/mL, respectively. As comparison, CuPd and CuPd₃ nanozymes have 1.6 and 1.4 folds cytotoxicity lower than Pd nanozymes, which is agreement with the kinetic results (Fig. 4). When incubation 4T1 cells with CuPd₃, CuPd, and Pd nanozymes with concentration of 100 μg/mL for 24 h, there are about 88.55%, 78.43%, and 44.15% cells were killed compared with Control group (Fig. 6d). And the cytotoxic difference among of them is remarkable due to the different Fenton-like efficiency. Once the concentration raises to above 200 μg/mL, practically all cells are unable to survival in both CuPd and Cu₃Pd group (Fig. 6e), indicating the optimized Fenton-like performance in tumor microenvironment. It is notable that 29.75% of cells are still alive even incubating with 400 μg/mL Pd nanozymes, demonstrating the low biocatalytic performance and also good biocompatibility (Fig. 6f). Meanwhile, the biocompatibility of as-synthesized Pd and Cu-Pd bimetallic nanozymes were evaluated on mouse fibroblast cell line (L929), and the results (Fig. S14) show that few toxicities are observed even under 400 μg/mL nanozyme treatment. Furthermore, to verify the Fenton-like reaction catalyzed by Cu-Pd bimetallic nanozymes in 4T1 cells, 2-7 dichlorofluorescin diacetate (DCFH-DA) which is a cell-permeant reagent fluorogenic dye that tests peroxyl, hydroxyl, and other ROS activity was employed to intuitively evaluate the generated -OH [29]. After incubation with Cu-Pd bimetallic nanozymes (25 μg/mL) for 6 h, a few green signals are detected in Pd group compared with Control group (Fig. 6g, Fig. S15), indicating the successful generation of -OH. Both CuPd₃ and CuPd₃ groups exhibit obviously enhanced green signal, demonstrating higher -OH level contributed by the enhanced Fenton-like efficiency, which is consistent with cell viability results. It is well-known that higher -OH generation will cause the intracellular oxidative stress, which then upregulate the GSSG level [29]. It is clear that Cu-Pd bimetallic nanozymes treated 4T1 cells produce higher GSSG level compared with Pd nanozyme treated group (Fig. S10), which confirms the higher level of -OH. All above results reveal the optimized Fenton-like efficiency of Cu-Pd bimetallic nanozymes compared with Pd nanozymes and great potential as agents for cancer chemodynamic treatment.

4. Conclusions

In summary, we presented a one-step coreduction strategy to prepare the miscible Cu-Pd bimetallic nanozymes with controllable shape and homogeneously alloyed structure to avoid the immiscibility and heterogeneity. The homogeneity is systematically explored through HRTEM, AC-HAADF-STEM with sub-Angstrom resolution, XPS, and XAS. Wavelet Transform images at the Cu-edge clearly demonstrate the homogenously alloyed structure of Cu-Pd bimetallic nanozymes via the x-space and R-space. TMB colorimetric assays exhibit homogeneous introduction of Cu successfully endows Cu-Pd bimetallic nanozymes with enhanced Fenton-like efficiency, especially for Cu₃Pd nanozymes. Further density functional theory (DFT) theoretical calculation reveals that Cu-Pd bimetallic nanozymes exhibit smaller d-band center compared with Pd nanozymes. Easier adsorption of H₂O₂ molecular contributed by the electronic structure of Cu significantly accelerate the catalytic process together with the strong repulsive interaction between H atom and Pd atom. In vitro cytotoxicity and intracellular ROS generation performance are evaluated to reveal the potential for in vivo biocatalysis. The strategy to construct kinetically miscible Cu-Pd bimetallic nanozymes will guide the development of bimetallic catalysts with excellent Fenton-like efficiency for biocatalytic nanomedicine.

Credit author statement

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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