Atom-Precise Fluorescent Copper Cluster for Tumor Microenvironment Targeting and Transient Chemodynamic Cancer Therapy

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Abstract

Background: Reactive oxygen species (ROS) have been widely studied for cancer therapy. Nevertheless, instability and aspecific damages to cellular biomolecules limited the application effect. Recently, significant research efforts have been witnessed in the flourishing area of metal nanoclusters (NCs) with atomically precise structures for targeted release of ROS but few achieved success towards targeting tumor microenvironment.

Results: In this work, we reported an atomically precise nanocluster $\text{Cu}_6(\text{C}_4\text{H}_3\text{N}_2\text{S})_6$ (Cu_6NC), which could slowly break and generate ROS once encountered with acidic. The as-prepared Cu_6NC demonstrated high biological safety and efficient chemodynamic anti-tumor properties. Moreover, Cu_6NC enabled transient release of ROS and contained targeting behavior led by the tumor microenvironment. Both in vitro and in vivo experiments confirmed that Cu_6NC demonstrated a low cytotoxicity for normal cells, while presented high cytotoxicity for tumor cells with a concentration-dependent manner.

Conclusions: This work not only reported a promising candidate for chemodynamic cancer therapy, but also paved the route to address clinical issues at the atomic level.

1. Introduction

Reactive oxygen species (ROS) are unstable molecules that contain oxygen, such as singlet oxygen, superoxide, hydroxyl radical and peroxide, etc. For the last decades, both experimental and clinical studies confirm that the alterations of ROS can affect the intracellular redox state.[1–3] Ever since, numerous efforts have been devoted to examine the role of ROS in cellular transformation and tumorigenesis.[4, 5] It has been observed that raising ROS level could effectively promote the apoptosis of cancer cells and modulate the immune response (specifically, inhibition) towards the treatment of cancer as well as autoimmune diseases.[6–8] Moreover, elevation of ROS levels also contribute to tissue regeneration.[9] Many treatments such as the exposure of cancer cells to chemotherapy and radiotherapy could upregulate ROS.[10–12] Nevertheless, caused by the intrinsic instability, it is difficult for ROS to realize sustainable release for persistent anti-cancer effect. Meanwhile, a well-coordinated and balanced redox system is typically present under normal physiological conditions. Thus, promoting ROS may produce cellular stress and damage, since ROS can damage cellular biomolecules (such as proteins, DNA, RNA) and result in mutations or even carcinogenesis. Hence, the controllable presentation of ROS is vital to take advantage of this double-edged sword for cancer therapy.

Over the past decades, remarkable progress has been achieved in the area of nanomaterials such as atomically precise assembled nanoclusters.[13–15] Amongst the nanoclusters, atomically precise metal nanoclusters (NCs) has explored promising potentials with fine-tuned properties.[16–18] The bottom-up programmed assembly of these NCs make it relatively simple to control over the size and to replace the surface organic ligand, providing good opportunities for particular applications.[19–23] By far, various metal based NCs (especially gold) have demonstrated effective anti-cancer properties.[24, 25] For

example, researchers have developed NCs that could make cancer cells more sensitive to radiation and promote the efficiency of radiotherapy.[26–29] Our previous work also explored photothermal behavior from coordinated-metal centers for cancer therapy.[30] By far, our group have designed and synthesized dozens of metal NCs with atomically precise assembly.[31–37] Benefiting from the precise formulas, both the structure and the physicochemical properties of atomically precise metal NCs are controllable.[38, 39] Thus, one could reasonably hypothesize that with rationale design, the additional introduction of the organic ligand could modulate targeting properties while the functional modification may generate sustained releasement of NCs in particular position.

Herein, we reported an atomically precise nanocluster $\text{Cu}_6(\text{C}_4\text{H}_3\text{N}_2\text{S})_6$, which were determined by the single crystal X-ray diffraction (SC-XRD). 2-mercaptopyrimidine was used to assemble the precise structures and led to a size of ~ 1 nm, which was much smaller than the kidney filtration threshold. Consistently, none of cardiotoxicity, kidney toxicity or liver toxicity were observed the main organs and peripheral blood, suggesting the good compatibility of our Cu_6NC . Specially, the structure $\text{Cu}_6(\text{C}_4\text{H}_3\text{N}_2\text{S})_6$ was quite stable unless mixed in acidic environment. The slight addition of acid ($\text{pH} \sim 6$) would slowly break the structure and generate the burst of radicals, realizing the sustained releasement of ROS (Scheme 1). The *in vitro* and *in vivo* experiments confirmed that Cu_6NC demonstrated a low cytotoxicity for normal cells, while presented high cytotoxicity for tumor cells with a concentration-dependent manner (Scheme 1). Such observations not only confirmed the biological safety and chemodynamic anti-tumor properties, but also indicated the targeting behavior led by the tumor microenvironment.

2. Results And Discussions

2.1 Synthesis and characterization of $\text{Cu}_6(\text{C}_4\text{H}_3\text{N}_2\text{S})_6$ nanoclusters. Typically, the $\text{Cu}_6(\text{C}_4\text{H}_3\text{N}_2\text{S})_6$ nanocluster (Cu_6NC) was synthesized *via* one-pot route by blending 2-mercaptopyrimidine with $\text{Cu}(\text{NO}_3)_2 \cdot 3\text{H}_2\text{O}$ (ratio of 1/1 by mole) in dimethyl formamide (detailed in experimental section). As revealed by SC-XRD, the hexagonal prismatic structure of $\text{Cu}_6(\text{C}_4\text{H}_3\text{N}_2\text{S})_6$ was composed of a hexagonal prismatic Cu_6 kernel which was stabilized and sealed by the introduced six 2-mercaptopyrimidine (Fig. 1a and Additional file 1: Table S1). As shown in Fig. 1b, the experimental Powder-XRD (PXRD) patterns were good agreement with the simulated ones from the SC-XRD, indicating the phase purity of the as-synthesized crystalline products of Cu_6NC . X-Ray photoelectron spectroscopy (XPS) (Additional file 1: Figure S1a) confirmed that Cu_6NC contained C, N, S and Cu elements. The peaks at 932.6 and 952.5 eV in the high-resolution Cu 2p spectrum (Additional file 1: Figure S1b) could be attributed to the $2p_{2/3}$ and $2p_{1/2}$ of Cu^+ , respectively,[40, 41] which was generated by the reductive mercapto groups. In the Fourier transform infrared (FTIR) spectra (Additional file 1: Figure S2), the adsorption peaks of S-H stretch were absent in the spectra of Cu_6NC and the $\text{C}=\text{C}/\text{C}=\text{N}$ peaks were apparently red-shifted, suggesting the formation of the coordination between mercaptopyrimidine and Cu ions.[42] Such coordinated ligands led to considerable thermal stabilities. Under nitrogen atmosphere, the

thermogravimetric analysis (TGA) revealed that Cu₆NC could be maintained from room temperature to nearly 400 °C and the total weight loss of Cu₆NC achieved ~ 50 % at 800 °C (Additional file 1: Figure S3).

Notably, Cu₆NC demonstrated crucial luminescent properties, indicating potential applications for biomedical labeling and targeting. The normalized emission spectra of the Cu₆NC showed long-wavelength emission bands of 700 nm (Additional file 1: Figure S4), which was appealing for the biological test. According to dynamic light scattering (DLS) analysis, the lateral size of Cu₆NC components in aqueous was ~ 23 nm (Additional file 1: Figure S5), which was within the range enabling efficient uptake by tumors. Moreover, obvious light absorption of Cu₆NC was observed at the wavelength of 370 nm during the solid state UV-vis test (Additional file 1: Figure S6). Since the structure was quite stable in water, we further tracked the UV-vis curves of Cu₆NC in the HCl solution (pH ~ 6), observing a slowly structure changes (Fig. 1c). Meanwhile, the PXRD revealed that the crystalline structures of Cu₆NC were totally broken (Additional file 1: Figure S7). More importantly, electron paramagnetic response (EPR) of Cu₆NC displayed significantly changed signals with the addition of HCl solution (pH ~ 6) (Fig. 1d), proving that a large number of ROS was produced during the structural fragmentation of Cu₆NC. The morphology analysis by scanning electron microscopy (SEM) and the associated energy dispersive spectroscopy (EDS) also revealed that the crystalline Cu₆NC with clear shape (Fig. 1e and Additional file 1: Figure S8) was totally pulverized (Fig. 1f and Additional file 1: Figure S9) after the treatment of acidic solution (pH ~ 6).

2.2 Cytotoxicity studies of Cu₆ NC. The biological safety of Cu₆NC was evaluated by cell viability. Both normal (cardiomyocytes, H9C2) and cancer cell lines were used as models to assess cellular responses by CCK-8 assay kit. Cu₆NC demonstrated high cytotoxicity to tumor cells (Additional file 1: Figure S10 and Fig. 2a). After treatment with 20 μM Cu₆NC for 48 h, the cell viability of A375, MCF-7 and Kyse30 cancer cell lines were 26.3%, 19.6%, and 20.9%, respectively (Fig. 2a). On the contrary, low cytotoxicity was observed to normal cardiomyocytes (Fig. 2b). After 48 h of incubation, the inhibition rate of 20 μM Cu₆NC was only 49.3% and the cell viability was 78.5% at the 15 μM (administration level), which was much higher than tumor cells at the same level. Notably, we also measured the cellular uptake capacity of Cu₆NC into both A375 cells and normal cardiomyocytes (Fig. 2c and 2d) during flow cytometric test. Along with the increase of incubation durations, the proportion of positive cells was slightly raised in cardiomyocytes while obviously promoted in cancer cells, suggesting the much more effective adsorption of Cu₆NC by tumor cells. These phenomena confirmed that Cu₆NC was tumor-specific.

2.3 Investigations of anti-tumor properties. Due to the selective cytotoxic property, Cu₆NC was thoroughly evaluated for chemodynamic therapy (CDT) in vitro. The four groups of cells treated with different concentrations of Cu₆NC (0, 2, 8 and 16 μM) were analyzed. Subsequently, the apoptosis and cycle were evaluated by flow cytometry 24 h. As shown in Fig. 3a and Additional file 1: Figure S11, Cu₆NC promoted the apoptosis of A375 cells, thereby causing cell proliferation inhibition. Particularly, Cu₆NC could mediate cell arrest in G2 phase (Fig. 3b and Additional file 1: Figure S12). Live/dead stain imaging

showed more red fluorescence appeared with the increased concentration of Cu₆NC (Fig. 2e), suggesting that the viability of cells in the Cu₆NC decreased in a dose-dependent manner. Meanwhile, live/dead detection associated with flow cytometry (Additional file 1: Figure S13 and S14) also demonstrated that after incubation with 0, 2, 8 and 16 μM Cu₆NC, the cell death rates were 3.63%, 7.14%, 23.0% and 42.2%, respectively. Since the formidable metastasis ability is one of the reasons that tumor cells are difficult to completely clean, we also performed transwell migration assay in vitro and observed that Cu₆NC could significantly inhibit tumor cells migration (Fig. 3c and Additional file 1: Figure S15). Furthermore, the cell colony formation assay was conducted to assess the chemodynamic efficacy of Cu₆NC over a longer period of time. The corresponding results revealed similar dose-dependent (Fig. 3d and Additional file 1: Figure S16). In the control group (0 μM), the colonies were densely packed and none effect on cell proliferation was observed. Specifically, the cell survival fraction obviously decreased with the addition of Cu₆NC. In the 16 μM group, even almost no colonies appeared, indicating that Cu₆NC were excellent for CDT.

Cu₆NC induced considerable oxidative stress in vitro, leading to specific tumor killing effect. Since ROS was detected during EPR test, we explored the underlying mechanisms of Cu₆NC for CDT and detected the intracellular ROS by the probe DCFH-DA, which displayed green fluorescence upon oxidation by ROS. For A375 cells exposed to Cu₆NC 12 h, the observed green fluorescence intensity was enhanced with the increasing of concentration (Fig. 3e and Additional file 1: Figure S17), suggesting production of ROS *via* Cu⁺-mediated Fenton reaction. Moreover, we found that the ratio of GSH/GSSG in A375 cells treated with Cu₆NC for 6 h was significantly reduced in a concentration-dependent manner (Additional file 1: Figure S18), which also indicated that the higher intracellular GSH levels in tumor cells could also be one of the possible mechanisms.

2.4 In vivo cancer therapy. Inspired by the good in vitro CDT efficacy, tumor formation assay was performed in vivo by using human melanoma A375 tumor-bearing mice model. Mice with tumors of about 100 mm³ were randomly divided into three groups: (1) Control group, (2) Cu₆NC group (10 mg/kg), and (3) Cu₆NC group (20 mg/kg). According to the corresponding dose of each group, Cu₆NC were administered intraperitoneally every two days. The tumor volume was measured every other day (Fig. 4a and 4b). At the time of final harvest, the tumor volume in the Control group increased rapidly, while in the dose of 20 mg/kg group the tumor volume was markedly reduced by about 4.5 times (compared with the Control group). Besides, the measured tumor weight also decreased significantly with the increase of the treatment dose (Fig. 4d). To verify the inhibitory effect of Cu₆NC, the proliferation and apoptosis in tumor tissues from each group was further detected. According to the immunohistochemical staining, the Ki67 protein level of the Cu₆NC treatment group was significantly lower than that of the Control group (Fig. 4c and 4e). Consistently, the TUNEL staining also demonstrated a promotion with the increase of the administration concentration (Fig. 4c and 4f), further proving the ability of Cu₆NC to induce apoptosis.

To evaluate the in vivo biocompatibility of Cu₆NC, the main organs and peripheral blood of the mice were investigated after the mice were sacrificed. Specifically, H&E staining results of the mice in every groups

showed that no major pathological abnormalities were induced in main organs of mice (Fig. 4g). Concomitantly, none of cardiotoxicity, kidney toxicity or liver toxicity from Cu₆NC was observed in the blood samples (Additional file 1: Figure S19), indicating the low in vivo toxicity of Cu₆NC.

3. Conclusions

ROS are unstable oxygen containing species, which make it difficult to be persistently subsistent and effective in clinical treatment. On the other hand, lacking of natural targeted properties, the surplus ROS are harmful to normal cells, generating damages or carcinogenesis. In this study, we designed and prepared Cu₆NC with atomically precise structures of Cu₆(C₄H₃N₂S)₆, which could generate large amount of ROS in acidic environment. Such characters address the above conundrum: (1) Cu₆NC could slowly degrade and sustainably release ROS; (2) the fragmentation of Cu₆NC are induced under acidic environment, providing targeted properties. Thus, Cu₆NC could be fragmented under the acidic tumor microenvironment with sustainable burst of ROS. Both in vitro and in vivo experiments confirm that Cu₆NC not only present high cytotoxicity to tumor cells, but also ensure the biological safety. Our intelligent strategy not only develops a promising candidate for the targeted chemodynamic therapy of cancer cells, but also paves the way for addressing clinical issues at the atomic level.

4. Experimental Section

Synthesis of Cu₆(C₄H₃N₂S)₆. 2-Mercaptopyrimidine (≥ 99%) were purchased from Alfa Aesar. DMF and Copper nitrate trihydrate (Cu(NO₃)₃, ≥ 99%) were bought from Sinopharm Chemical Reagent Co., Ltd., China. All reagents and solvents used were of commercially available reagent grade and used without further purification. 2-Mercaptopyrimidine (112mg, 1mmol) and Cu(NO₃)₂·3H₂O (240mg, 1mmol) were dissolved in 20ml DMF solution respectively, stir thoroughly to dissolve completely. Subsequently, add the DMF solution of 2-Mercaptopyrimidine dropwise to the solvent of Cu(NO₃)₂·3H₂O. Cu₆L₆ powder crystals (C₂₄H₁₈Cu₆N₁₂S₆) were obtained by filtration after vigorous stirring at room temperature for two hours. (A single crystal suitable for SCXRD measurements can be obtained by reducing the dosage in the same proportion).

Characterization. Powder X-ray diffraction (PXRD) patterns of the samples were acquired on a Rigaku D/Max-2500PC X-ray diffractometer with Cu radiation (λ = 1.54178 Å). Fourier transform infrared (FT-IR) spectroscopy was conducted using a Bruker ALPHA II FT-IR spectrometer. X-ray photoelectron spectroscopy (XPS) analysis was obtained on an ESCALAB 250 instrument operated at 150 W and 200 eV with mono chromated Al Kα radiation. Thermogravimetric analyses (TGA) were performed on an SDT 2960 thermal analyzer from room temperature (RT) to 800°C at a heating rate of 10°C/min under a nitrogen atmosphere. Dynamic light scattering (DLS) data were obtained by Horiba nano Partica SZ-100V2. UV-vis absorption spectra were recorded using a Hitachi UH4150 UV-visible spectrophotometer in the range of 200–700 nm. Emission and excitation spectra at RT were recorded with an Edinburgh FLS 1000 fluorescence spectrometer, and luminescence microscopy images were recorded on an Olympus

BX53 microscope. Electron paramagnetic resonance (EPR) spectra were recorded by a Bruker A 300 EPR spectrometer.

Single-Crystal X-ray Diffraction (SCXRD) analysis. SCXRD was performed on a Rigaku XtaLAB Pro diffractometer Cu-K α radiation ($\lambda = 1.54184 \text{ \AA}$) at 200 K. Data collection and reduction were conducted with CrysAlisPro software. The structures were solved with intrinsic phasing methods (SHELXT-2015) and refined by full-matrix least squares on F² using OLEX2, which utilizes the SHELXL-2015 module. The imposed restraints in least-squares refinement of each structure were commented in the corresponding CIF files. All non-hydrogen atoms were refined anisotropically, and the hydrogen atoms were included on idealized positions. The crystal structures are visualized by DIAMOND 3.2. The detailed information of the crystal data, data collection and refinement results are summarized in Table S1.

Cell Culture. Cell lines including human melanoma cells (A375), human esophageal cancer cells (Kyse30), human breast cancer cells (MCF-7) and Rat cardiomyocytes cells (H9C2) were purchased from the cell bank of the Chinese Academy of Sciences (Shanghai, China). A375 and H9C2 cells were cultured in DMEM medium containing 10% FBS and 1% penicillin-streptomycin. MCF-7 and Kyse30 cells incubated in 1640 medium containing 10% FBS and 1% penicillin-streptomycin.

Cytotoxicity Analysis. A375, MCF-7 and Kyse30 cells were seeded in 96-well plates (8×10^3 cells per well), respectively, and cultured for overnight. Then these cells were incubated with different concentrations of Cu₆NC (0, 2, 4, 8, 12, 15, 20 and 25 μM), the incubation time was 24 hours and 48 hours, respectively. Finally, add 10 μL of CCK-8 solution to each well and incubate for another 1 h. The absorbance of cells in each well at 450 nm was measured by a microplate reader. The cytotoxicity test of Cu₆NC on H9C2 cells was also carried out according to the above description.

Cellular Uptake of Cu₆NC

A375 and H9C2 cells were seeded in culture dishes and incubated for 24 h. Subsequently, cells were incubated with 8 μM Cu₆NC and harvested after 3 h and 6 h. The percentage of positive cells was tested by the flow cytometry (ACEA NovoCyte3130, USA) with an excitation wavelength of 405 nm and an emission wavelength of 780 nm. Data were processed by FlowJo software.

Apoptosis and Cell Cycle Analysis. A375 (2.5×10^5 cells per well) cells were seeded in 6-well plates with 0, 2, 8 and 16 μM Cu₆NC for 24 hours. The cells were collected using trypsin-EDTA and stained with annexin V-FITC/PI (BD Biosciences, USA) for 15 min, and then apoptotic cells were identified by flow cytometry. The cell cycle was measured by PI staining using a cell cycle analysis kit (KeyGen Biotech, China). After staining following the manufacturer's instructions, the cells were analyzed by flow cytometry (FACS Aria III, USA). Data were processed by ModFit LT software.

Cell Dead/Live Assay. A375 cells were treated with Cu₆NC (0, 2, 8 and 16 μM) for 24 h. Before being observed with a fluorescence microscope, the cells were washed twice with PBS and incubated with 2 μM calcein AM and 4.5 μM PI for 20 minutes. For flow cytometry detection, after being digested and

harvested, the cells are washed twice by PBS and then tested by a FACS Aria III flow cytometer. Data were processed and analyzed by FlowJo software.

Transwell migration assay. Migration assays of A375 in vitro were performed using a 24-well plate with a transwell polycarbonate permeable support (pore size, 8 μm ; Corning Incorporated, Corning, USA). A375 cells were incubated and treated as described in the Apoptosis Assay. The harvested cells were resuspended in FBS-free medium and seeded in the upper chamber with 1×10^5 cells per well. The lower chamber was filled with 20% FBS medium (600 μL /well). The cells were then cultured for 48 hours. Finally, the cells in the lower layer of the upper chambers were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. Numbers of migrated cells were photographed with a fluorescence microscopy and processed by ImageJ software.

Colony Formation Ability Identification. A375 cells were incubated and treated as described in the Apoptosis Assay. The cells were digested and seeded in 6-well plates (1000 cells per well). Then the cells are cultured in complete medium, and the medium was changed every 4 days. At 12 days, the cells were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. The cell colonies were observed and photographed with a microscope, and the colonies containing more than 50 cells were counted using ImageJ software.

Intracellular Reactive Oxygen Specie (ROS) Generation. A375 cells (5×10^4 cells/well) were incubated in a 24-well plate with different concentrations of Cu_6NC (0, 2, 8 and 16 μM) for 12 h. Then, the level of cellular ROS was measured by 2',7-dichlorodihydrofluorescein diacetate (DCFH-DA) probe (Beyotime Biotechnology, Jiangsu, China). Firstly, the cells were washed twice with PBS, incubated with DCFH-DA (10 μM) for 25 minutes, and then washed with PBS three times. Finally, the ROS signal of the cells was observed by a fluorescence microscopy.

Assessment of Glutathione (GSH). Levels of GSH in A375 cells after treatment with Cu_6NC were evaluated with GSH and GSSG assay kits (Beyotime Biotechnology, Jiangsu, China). In short, A375 cells were seeded in 6-well dishes (2×10^5 cells per well) and treated with different concentrations of Cu_6NC (0, 2, 8 and 16 μM) for 6 h. Then, the cells were collected with centrifugation, and the supernatant was discarded. Samples were added protein remover M (10 mg/30 μL), subjected to 3 cycles of freezing – thawing, and then centrifugated at 1000 g for 10 min at 4°C. The supernatant was reserved for GSH measurement with GSH and GSSG assay kits according to the manufacturer's protocol.

In vivo Chemodynamic Therapy. All animal procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and were approved by the Welfare and Ethics Review Committee of Zhengzhou University Laboratory Animal Center (Approval number: ZZU-LAC20200911[14]). In order to generate a xenograft mouse model, on day 0, we planted A375 cells (5×10^6 tumor cells in 200 μL of PBS) in 6-week-old female BALB/c nude mice (Beijing Vital River Laboratory Animal Technology Co. Ltd, China) subcutaneously in the right flank. When the tumor volume reached about 100mm^3 on the 4th day, we randomly divided the mice into three groups: normal control group (NC), Cu_6 NPs group (10 mg/kg), and

Cu₆NC group (20 mg/kg), at the same time by intraperitoneal injection corresponding dose of Cu₆ NPs to treat mice. Mice were exposed to these treatments every other day for a total of nine administrations. The length (L) and width (W) of the tumor were measured with a vernier caliper every two days, and the tumor volume (V) was calculated using the formula: $V = L \times W^2 / 2$. On the 20th day, the tumor tissues, peripheral blood, and major organs (heart, liver, spleen, lungs and kidneys) of mice were harvested.

All harvested tissues were fixed with 4% formalin at least for 48 hours. The fixed tissue is used for experiments such as hematoxylin and eosin (H&E) and immunohistochemistry staining. Apoptosis of tumor tissue sections was detected by staining with terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL). The proliferation of tumor tissue was detected by immunohistochemical Ki67 staining. H&E were used to stain sections of major organs in each group. All these stained sections could be observed with a fluorescence microscopy. After the peripheral blood was collected, a serum sample was obtained by centrifugation. Then, an automatic biochemical analyzer (Chemray 240 or 840, Rayto, China) was used to detect the indexes of liver function, kidney function and myocardial enzymes.

Statistical Analysis. All results are presented as the mean $\pm$ standard deviation (SD). Two treatment groups were compared by Student's t test. Multiple group comparisons were performed by two-way analysis of variance with Tukey's post hoc test. All statistical analyses were carried out using GraphPad Prism 5. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$ were deemed as significant differences. "NS" indicates no significant differences.

Declarations

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Authors' contributions

P.P., B.L. and S.Z. conceived various aspects of the project. Z.Y. and A.Y. performed all experiments on synthesis and characterize of metal nanoclusters, in vitro and in vivo experiments, data collection and analysis. W.M, K.M and Y.L performed experiments on chemical and morphological characterizations of nanoclusters and worked on data analysis. P.P. and Z.Y. wrote the manuscript with input from other authors. #Z.Y. and A.Y. contributed equally to this work.

Availability of data and materials

The datasets supporting the conclusions of this article are included within the article and its additional file.

Ethics approval and consent to participate

All animal procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and were approved by the Welfare and Ethics Review Committee of Zhengzhou University Laboratory Animal Center (Approval number: ZZU-LAC20200911[14])

Consent for publication

All authors consent to publish.

Competing interests

The authors declare no conflict of interest.

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Figures

Figure 1

In vitro characterization of Cu₆NC. a) The molecular structure of Cu₆NC. b) The experimental PXRD patterns and the simulated ones from the SC-XRD of Cu₆NC, which were in good agreement. c) The tracking monitoring of UV-vis for Cu₆NC in HCl solution (pH~6). d) EPR of Cu₆NC with and without the addition of HCl solution (pH~6). The emerging peaks indicated the generation of ROS once HCl was

added. e) SEM images of the crystalline Cu6NC. Clearly shape could be observed. f) SEM images of Cu6NC after the treatment of HCl solution (pH~6). The crystals were totally pulverized.

Figure 2

In vitro toxicity and cellular uptake of Cu6NC. a) CCK-8 results of the viability of different cancer cells cultured with Cu6NC for 48 h. b) CCK-8 results of the viability of H9C2 cells cultured with Cu6NC for 24 h and 48 h. c) and d) Flow cytometric results reflect the proportion of positive cells corresponding to the

cellular uptake capacity of Cu6NC after the incubation of different durations for A375 cells and H9C2 cells. e) Live/dead imaging of A735 cells after receiving different treatments. Scale bar: 50 μ m.

Figure 3
Chemokinetic anti-tumor properties of Cu6NC. Cell flow cytometry analysis for apoptosis a) and cell cycle b) distribution in A735 cells after different concentrations (0, 2, 8 and16 μ M) of Cu6NC treatment. c) Representative images of transwell migration assay. Scale bar: 50 μ m. d) Typical images representing the

colony formation ability of A735 cells with different treatments. e) ROS production in A375 cells after Cu6NC treatment. Scale bar: 100 μ m.

Figure 4

In vivo cancer chemotherapy results of Cu6NC. a) Photographs of mice after 16 days of different formulations. b) Tumor growth curves of mice in different treatment groups. c) Ki67 staining and TUNEL staining of tumor sections from different treatment groups. Scale bar: 100 μ m. d) Comparison of tumor

weight in mice after therapy. The quantitative analysis of Ki67 e) and TUNEL f) performed by Image J software. g) Pathological analysis of various organs in mice injected with different formulations. Scale bar: 50 μ m.

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