Ultrasound-responsive calcium copper phosphate nanomaterials induce tumor cell death via the synergistic release of copper and calcium

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1 Materials

Anhydrous calcium chloride ($CaCl_2$), copper sulfate pentahydrate ($CuSO_4·5H_2O$), disodium hydrogen phosphate (Na_2HPO_4), potassium dihydrogen phosphate (KH_2PO_4), and absolute ethanol were purchased from Sinopharm Chemical Reagent Co., Ltd. (China), all with analytical-grade purity. Dulbecco's Modified Eagle Medium (DMEM, high glucose), fetal bovine serum (FBS), and penicillin–streptomycin solution (100×) were obtained from Wuhan Procell Life Science & Technology Co., Ltd. Annexin V-FITC/PI apoptosis detection kit and Calcein-AM/PI live/dead cell staining kit were purchased from Solarbio Science & Technology Co., Ltd. (Beijing, China). Primary antibodies against Caspase-3, Cleaved Caspase-3, Bax, Bcl-2, and β-actin, as well as horseradish peroxidase (HRP)-conjugated secondary antibodies, were obtained from Cell Signaling Technology (USA). MitoTracker Red CMXRos was purchased from Thermo Fisher Scientific (USA). BCA protein assay kit, RIPA lysis buffer, and protease inhibitor cocktail were supplied by Beyotime Biotechnology (Shanghai, China). Murine breast cancer 4T1 cells were obtained from the Cell Bank of the Chinese Academy of Sciences. Cells were cultured in DMEM high-glucose medium supplemented with 10% FBS and 1% penicillin–streptomycin in a humidified incubator at 37 °C with 5% CO_2. The 4T1 cells were routinely tested for mycoplasma contamination and were authenticated prior to use.

2 Material Characterization

An appropriate amount of $Ca_{19}Cu_2(PO_4)_{14}$ nanopowder was dispersed in deionized water and ultrasonicated for 10 min at a power of 100 W to obtain a dispersion with a concentration of 0.1 mg/mL. A 10 μL aliquot of the dispersion was dropped onto a silicon wafer (for SEM analysis) or a carbon-coated copper grid (for TEM analysis) and dried naturally at room temperature. The morphology and dispersion of the nanomaterials were observed using a scanning electron microscope (SEM, Hitachi SU8010) operated at an accelerating voltage of 10 kV. The microstructure, internal morphology, and particle size distribution were examined using a transmission electron microscope (TEM, Zeiss Libra 200FE) at an accelerating voltage of 200 kV. The crystalline structure of $Ca_{19}Cu_2(PO_4)_{14}$ nanomaterials was analyzed using an X-ray diffractometer (Bruker D8 Advance). Samples were evenly spread on the sample holder and scanned over a 2θ range of 10°–80° with a scanning rate of 5°/min and a step size of 0.02°, using Cu Kα radiation (λ = 1.5406 Å). The obtained diffraction patterns were compared with standard data (JCPDS No. 01-074-1831) to confirm the crystal phase. Surface elemental composition and chemical states of the nanomaterials were characterized using an X-ray photoelectron spectrometer (Thermo Scientific ESCALAB 250Xi) with monochromatic Al Kα radiation as the excitation source at a power of 150 W. The binding energies were calibrated using the C 1s peak at 284.8 eV. The characteristic peaks of Cu, Ca, P, and O were analyzed to determine their chemical valence states. For the characterization of dynamic Light Scattering (DLS) and Zeta Potential, $Ca_{19}Cu_2(PO_4)_{14}$ nanomaterials were dispersed in phosphate-buffered saline (PBS, pH 7.4) and ultrasonicated for 10 min to obtain a dispersion with a concentration of 0.5 mg/mL, consistent with that used in subsequent cellular experiments. The hydrodynamic diameter distribution, polydispersity index (PDI), and zeta potential were measured using a dynamic light scattering analyzer (Malvern Zetasizer Nano ZS90). Each sample was measured in triplicate, and the average values were reported to evaluate the colloidal stability under physiological conditions.

3 Western Blot (WB) Analysis

4T1 cells were seeded in 6-well plates and treated under the same grouping and conditions described above (PBS control, ultrasound alone, $Ca_{19}Cu_2(PO_4)_{14}$ alone, and

$Ca_{19}Cu_2(PO_4)_{14}$+ US; $Ca_{19}Cu_2(PO_4)_{14}$ at 200 μg/mL; ultrasound at 1 W/cm² and 1 MHz for 5 min). After further incubation (typically 24 h), cells were harvested and washed with cold PBS. Total cellular proteins were extracted using RIPA lysis buffer supplemented with protease/phosphatase inhibitors, followed by centrifugation to remove debris. Protein concentrations were determined using a BCA assay. Equal amounts of protein were separated by SDS–PAGE and transferred onto PVDF membranes. Membranes were blocked (e.g., 5% non-fat milk or BSA in TBST) and incubated with primary antibodies against apoptosis- and mitochondria-related proteins, including FDX1, DLAT, cytochrome c, Cleaved-Caspase-3, Bax, and Bcl-2. After washing, membranes were incubated with HRP-conjugated secondary antibodies and visualized using an enhanced chemiluminescence (ECL) substrate. Band intensities were quantified by densitometry (e.g., ImageJ) and normalized to a housekeeping protein (e.g., GAPDH or β-actin).

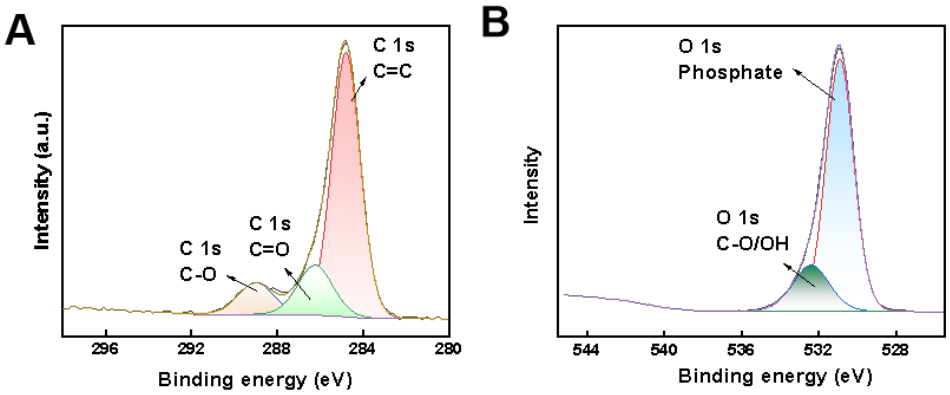

Figure S1. High-resolution XPS spectra for C 1s (A), O 1s (B).



Figure S2. WB analysis of 4T1 cells with PBS, US, $Ca_{19}Cu_2(PO_4)_{14}$, and $Ca_{19}Cu_2(PO_4)_{14}$+US, respectively.