



Article

Ultrasound-Responsive Calcium Copper Phosphate Nanomaterials Induce Tumor Cell Death via the Synergistic Release of Copper and Calcium

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Abstract

Metastatic breast cancer remains a significant therapeutic challenge due to its high invasiveness and resistance to conventional treatments. In this study, an ultrasound-responsive copper-calcium phosphate ($\text{Ca}_{19}\text{Cu}_2(\text{PO}_4)_{14}$) nanomaterial is developed for synergistic ion-mediated tumor therapy. The $\text{Ca}_{19}\text{Cu}_2(\text{PO}_4)_{14}$ nanomaterials exhibit a uniform morphology and crystalline structure, as well as good colloidal stability. Upon ultrasound irradiation, the release of Cu^{2+} and Ca^{2+} is spatiotemporally controlled via mechanical and cavitation effects. In vitro studies using highly metastatic 4T1 cells demonstrate that a combination of $\text{Ca}_{19}\text{Cu}_2(\text{PO}_4)_{14}$ and ultrasound significantly enhances apoptosis to 37.56%, while inducing 41.37% cell viability at 20 $\mu\text{g/mL}$ of $\text{Ca}_{19}\text{Cu}_2(\text{PO}_4)_{14}$ + US. In contrast, $\text{Ca}_{19}\text{Cu}_2(\text{PO}_4)_{14}$ alone exhibits negligible cytotoxicity. Mechanistic investigations reveal that the combined release of Cu^{2+} and Ca^{2+} induces pronounced mitochondrial stress by suppressing the mitochondrial copper/redox regulator FDX1 and the PDH complex E2 subunit DLAT, thereby impairing mitochondrial metabolic homeostasis and promoting mitochondrial dysfunction. Overall, this study presents an ultrasound-triggered $\text{Ca}_{19}\text{Cu}_2(\text{PO}_4)_{14}$ nanoplatform for the effective ablation of tumor cells.

Keywords: $\text{Ca}_{19}\text{Cu}_2(\text{PO}_4)_{14}$ nanomaterial; ultrasound-responsive; synergistic release of copper and calcium; cancer therapy

1. Introduction

Malignant tumors represent a pressing global public health burden, characterized by high incidence and mortality rates that pose substantial challenges to clinical management. Among female malignancies, breast cancer stands as the most prevalent type, with metastatic subtypes—particularly triple-negative breast cancer (TNBC)—remaining a major clinical dilemma [1–4]. Currently, surgery, chemotherapy, and radiotherapy constitute the standard therapeutic armamentarium for breast cancer; however, these modalities exhibit inherent limitations in the management of highly metastatic breast cancer, as exemplified by the 4T1 murine breast cancer model [5–7]. Surgical resection fails to eradicate micrometastatic lesions, leading to unacceptably high recurrence rates, while systemic chemotherapy and radiotherapy are plagued by off-target toxicity to normal tissues and inadequate efficacy against distant metastases [8–10]. Furthermore, tumor cells can develop therapeutic resistance through multiple mechanisms, including upregulation of multidrug resistance genes (e.g., MDR1) and enhanced DNA damage repair capacity, which result



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in progressive attenuation of therapeutic efficacy and eventual treatment failure [11–13]. Clinically, patients with metastatic TNBC face a dismal prognosis, with a five-year overall survival rate of merely 12–20%, markedly lower than that of non-metastatic counterparts, underscoring the urgent need for novel therapeutic strategies with high tumor specificity, low systemic toxicity, and precise spatiotemporal controllability [14–16].

To overcome the limitations of conventional therapies, stimuli-responsive nanotherapeutic systems have garnered considerable attention in cancer research, due to their ability to achieve externally controlled therapeutic activation and improved targeting precision [10,17–19]. Among various external stimuli, ultrasound (US) has emerged as a particularly promising trigger, attributed to its deep tissue penetration, non-invasive nature, superior spatiotemporal controllability, and well-established clinical safety profile [20–22]. Ultrasound-responsive nanomaterials can convert acoustic energy into localized mechanical, thermal, or cavitation effects, thereby enabling on-demand drug release or therapeutic activation at tumor sites while minimizing damage to surrounding normal tissues [20,23]. Recent advances in ultrasound-based therapeutic modalities, such as sonodynamic therapy [24–26] and ultrasound-triggered drug delivery systems [27–29], have demonstrated encouraging antitumor efficacy in preclinical studies. Nevertheless, most existing ultrasound-responsive platforms rely on organic sonosensitizers or chemotherapeutic agents as therapeutic payloads, which are associated with inherent drawbacks including limited in vivo stability, insufficient therapeutic potency, and undesirable systemic toxicity [30,31]. Additionally, the therapeutic efficacy of these systems is frequently compromised by the tumor microenvironment (TME)'s characteristics, such as hypoxia and heterogeneous vascularization, as well as intrinsic or acquired drug resistance mechanisms [32–34]. Therefore, the development of novel ultrasound-responsive nanomaterials that operate through alternative, non-drug-based therapeutic mechanisms is deemed a critical direction for achieving more effective, safe, and controllable tumor therapy.

Inspired by the emerging concept of ion-mediated tumor therapy, recent studies have demonstrated that dysregulation of intracellular metal ion homeostasis can effectively induce tumor cell death through mitochondrial dysfunction and apoptotic signaling. Specifically, copper ions (Cu^{2+}) can catalyze Fenton-like redox reactions to generate reactive oxygen species (ROS), augment intracellular oxidative stress, and trigger mitochondrial damage, while calcium ions (Ca^{2+}), as a key second messenger, regulate mitochondrial permeability transition pore (mPTP) opening and apoptotic pathway activation [35]. Notably, the synergistic disruption of Cu^{2+} and Ca^{2+} homeostasis offers a drug-free therapeutic strategy that circumvents conventional chemoresistance mechanisms, as it targets fundamental cellular metabolic processes rather than specific molecular targets.

Copper-calcium phosphate nanomaterials have been widely explored as biomedical carriers owing to their excellent biocompatibility, biodegradability, and inherent calcium reservoir properties [36–38]. Shen et al. synthesized hydroxyapatite (HAp) nanorods for promoting the differentiation of neural stem cells into GABAergic neurons (accelerating GABAergic neurogenesis) via Ca^{2+} /c-Jun/TXL3 signaling, with potential application in precise stem-cell therapy for neurological diseases [39,40]. Zheng et al. synthesized biodegradable Ca^{2+} nanomodulators for cancer immunotherapy by inducing mitochondrial Ca^{2+} overload-triggered pyroptosis and activating antitumor immune responses [41]. Noori et al. synthesized copper-doped hydroxyapatite nanoparticles for antibacterial and pro-angiogenic bone tissue engineering-related applications [42]. However, uncontrolled ion leakage and insufficient tumor-specific activation remain major challenges for copper-calcium phosphate-based systems. To address these limitations, ultrasound provides an ideal external stimulus to precisely regulate ion release through mechanical and cavitation effects, enabling spatiotemporally confined therapeutic activation. Herein, we develop

an ultrasound-responsive $\text{Ca}_{19}\text{Cu}_2(\text{PO}_4)_{14}$ nanoplatform that integrates the advantages of calcium phosphate-based materials and ion-mediated therapy, enabling on-demand, synergistic release of Cu^{2+} and Ca^{2+} upon ultrasound irradiation. We hypothesize that this nanoplatform can induce pronounced mitochondrial dysfunction and activate the intrinsic apoptotic pathway, thereby achieving efficient ablation of metastatic tumor cells.

In this study, we systematically developed and characterized an ultrasound-responsive copper-calcium phosphate ($\text{Ca}_{19}\text{Cu}_2(\text{PO}_4)_{14}$) nanoplatform for precise, non-drug-based tumor therapy. The synthesized $\text{Ca}_{19}\text{Cu}_2(\text{PO}_4)_{14}$ nanomaterials were comprehensively characterized using scanning electron microscopy (SEM), transmission electron microscopy (TEM), X-ray diffraction (XRD), X-ray photoelectron spectroscopy (XPS), dynamic light scattering (DLS), and zeta potential analysis. The ultrasound-triggered ion release behavior was quantified by inductively coupled plasma mass spectrometry (ICP-MS), confirming spatiotemporally controlled release of Cu^{2+} and Ca^{2+} via mechanical and cavitation effects. In vitro antitumor performance was evaluated using highly metastatic 4T1 breast cancer cells, with apoptosis and cell death rates assessed by flow cytometry and Calcein-AM/PI staining, respectively. Mechanistic investigations were further conducted to elucidate the underlying antitumor mechanisms, focusing on mitochondrial function and apoptotic pathway activation, as evidenced by downregulation of FDX1 and the PDH complex E2 subunit DLAT in Western blots (WBs). Overall, this study establishes a novel non-drug-based, ultrasound-triggered ion intervention strategy and highlights the potential of $\text{Ca}_{19}\text{Cu}_2(\text{PO}_4)_{14}$ nanomaterials as a precise and minimally invasive therapeutic option for metastatic breast cancer.

2. Methods and Experimental Section

2.1. Synthesis and Characterization of $\text{Ca}_{19}\text{Cu}_2(\text{PO}_4)_{14}$ Nanomaterials

$\text{Ca}_{19}\text{Cu}_2(\text{PO}_4)_{14}$ nanomaterials were synthesized via a modified co-precipitation method (Figure 1A) with precise control of stoichiometric ratios and reaction conditions [38,42,43]. All chemical reagents ($\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, $(\text{NH}_4)_2\text{HPO}_4$, $\text{Cu}(\text{NO}_3)_2 \cdot 3\text{H}_2\text{O}$, aqueous ammonia, and absolute ethanol, etc.) were of analytical grade (Sigma-Aldrich, New York, NY, USA) and used without further purification. Briefly, 3.54 g $\text{Cu}(\text{NO}_3)_2 \cdot 3\text{H}_2\text{O}$ (15 mmol) was dissolved in 50 mL absolute ethanol under magnetic stirring (500 rpm) to form a homogeneous calcium precursor solution. The pH of the solution was adjusted to 10.0 ± 0.1 using 25% (v/v) aqueous ammonia, which was added dropwise to avoid local pH fluctuations. Subsequently, 50 mL aqueous solution containing $(\text{NH}_4)_2\text{HPO}_4$ (9 mmol) was added dropwise to the calcium precursor solution at a rate of 1 mL/min under continuous magnetic stirring (800 rpm) to ensure uniform mixing. After complete addition of the phosphate source, 0.49 g $\text{Cu}(\text{NO}_3)_2 \cdot 3\text{H}_2\text{O}$ (2 mmol) was added to the mixed system, followed by vigorous stirring at room temperature ($25 \pm 2^\circ\text{C}$) for 2 h to facilitate co-precipitation. The resulting mixture was transferred to a sealed glass vessel and allowed to age statically for 24 h at $25 \pm 2^\circ\text{C}$ to form a homogeneous gel. The gel product was collected by centrifugation at 8000 rpm for 10 min, and washed repeatedly with absolute ethanol (3 times, 50 mL each) until the supernatant reached a neutral pH (6.8–7.2). The collected precipitate was dried in a vacuum oven at 100°C for 12 h to remove residual solvents, ground into fine powder using an agate mortar (Jiangxi, China), and calcined in a muffle furnace (Nabertherm, Berlin, Germany) at 900°C for 2 h with a heating rate of $5^\circ\text{C}/\text{min}$ to induce crystal phase formation. After natural cooling to room temperature, the final $\text{Ca}_{19}\text{Cu}_2(\text{PO}_4)_{14}$ nanomaterial powder was collected and stored in a desiccator for subsequent experiments.

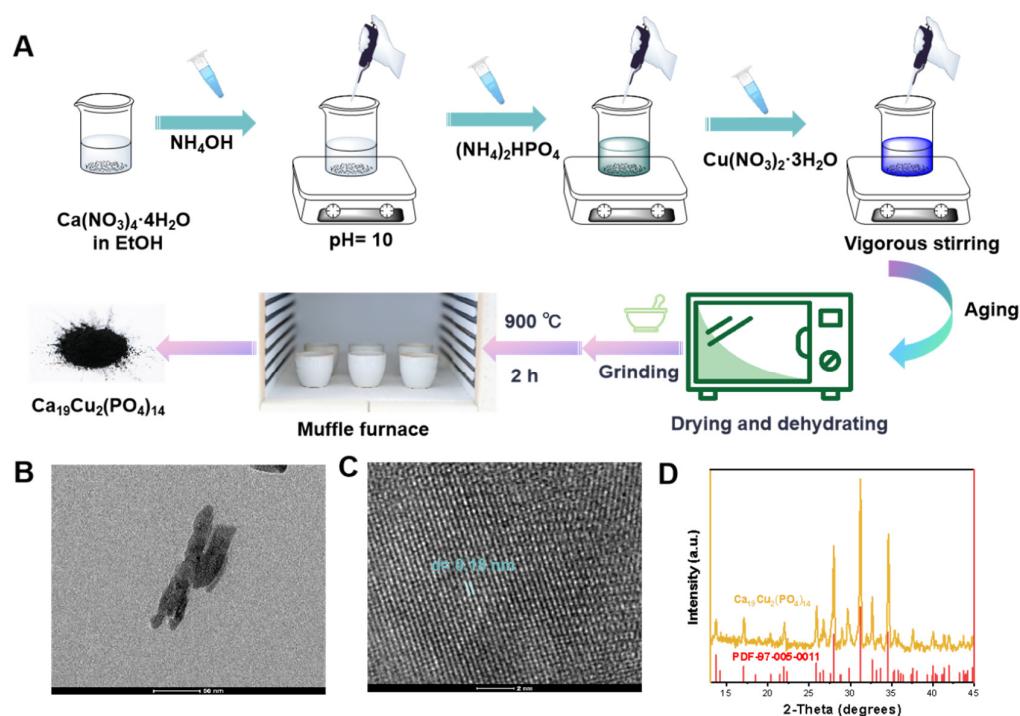


Figure 1. Schematic illustration of the synthesis (A), TEM image (B), HRTEM image (C) and XRD (D) of $\text{Ca}_{19}\text{Cu}_2(\text{PO}_4)_{14}$ nanomaterials.

For the characterization of dynamic Light Scattering (DLS) and Zeta Potential, $\text{Ca}_{19}\text{Cu}_2(\text{PO}_4)_{14}$ 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, Malvern, UK). Each sample was measured in triplicate, and the average values were reported to evaluate the colloidal stability under physiological conditions.

2.2. In Vitro Ultrasound Responsiveness Ion Release Evaluation

The ultrasound-triggered ion release behavior of $\text{Ca}_{19}\text{Cu}_2(\text{PO}_4)_{14}$ nanomaterials was evaluated in phosphate-buffered saline (PBS, pH 7.4, 10 mM), which mimics the physiological environment. In brief, $\text{Ca}_{19}\text{Cu}_2(\text{PO}_4)_{14}$ nanomaterials were dispersed in PBS via ultrasonic homogenization (100 W, 5 min) to obtain a final concentration of 20 $\mu\text{g/mL}$. The dispersion was aliquoted into 1.5 mL centrifuge tubes (1 mL per tube) and divided into two experimental groups: (1) $\text{Ca}_{19}\text{Cu}_2(\text{PO}_4)_{14}$ alone (non-ultrasound group); (2) $\text{Ca}_{19}\text{Cu}_2(\text{PO}_4)_{14}$ + ultrasound (US group). For the US group, samples were irradiated using a therapeutic ultrasound device (Sonitron 2000, Berlin, Germany) with predefined parameters (frequency: 1 MHz, power density: 1 W/cm^2 , duty cycle: 50%) for 5 min at predetermined time points (2, 4, 8, and 12 h) during incubation at 37 °C with gentle shaking (100 rpm). After each treatment, all samples were centrifuged at 12,000 rpm for 15 min to remove undissolved nanomaterials, and the supernatants were collected for ion concentration analysis. The concentrations of released Cu^{2+} and Ca^{2+} ions were quantified using inductively coupled plasma optical emission spectroscopy (ICP-OES, Agilent 7250, New York, NY, USA) with calibration curves established using standard solutions of Cu^{2+} (1–100 $\mu\text{g/L}$) and Ca^{2+} (1–1000 $\mu\text{g/L}$). Each experimental condition was performed in triplicate, and the ion release efficiency was calculated as the percentage of released ions relative to the total ion content in the initial nanomaterial dispersion. The ion release profiles were

plotted as mean $\pm$ standard deviation (SD) to evaluate the ultrasound responsiveness of $\text{Ca}_{19}\text{Cu}_2(\text{PO}_4)_{14}$ nanomaterials.

2.3. Cell Apoptosis Assay and Necrosis Analysis

The apoptotic and necrotic effects of different treatments on 4T1 murine breast cancer cells (ATCC, Manassas, VA, USA) were systematically evaluated using Annexin V-FITC/PI Double Staining Kit (BD Biosciences, San Jose, CA, USA) combined with flow cytometry, which enables the precise discrimination of viable, early apoptotic, late apoptotic, and necrotic cells. Prior to the assay, 4T1 cells were maintained in RPMI 1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin-streptomycin (100 U/mL penicillin, 100 $\mu\text{g}/\text{mL}$ streptomycin; Gibco) at 37 °C in a humidified atmosphere containing 5% CO_2 . All cell cultures were passaged every 2–3 days to ensure logarithmic growth phase, and cells with passage numbers 3–8 were used for experiments to maintain consistent biological characteristics. For the apoptosis assay, logarithmically growing 4T1 cells were seeded into 6-well plates at a density of 5×10^5 cells/well in 2 mL complete medium and incubated for 24 h to reach approximately 80% confluence. This confluence density was selected to ensure sufficient cell-cell interactions while avoiding overcrowding-induced contact inhibition, which may interfere with apoptotic signaling. After removing the spent culture medium via gentle aspiration, cells were assigned to four experimental groups with three biological replicates per group to ensure statistical robustness, i.e., PBS control, US alone, $\text{Ca}_{19}\text{Cu}_2(\text{PO}_4)_{14}$ alone, and $\text{Ca}_{19}\text{Cu}_2(\text{PO}_4)_{14}$ + US.

After the treatments, all groups were further incubated for 24 h at 37 °C with 5% CO_2 to allow the full activation of apoptotic signaling pathways. Cells were then harvested by digestion with 0.25% trypsin-EDTA (Gibco) for 2 min at 37 °C, and the digestion was immediately terminated by adding 2 mL complete medium to avoid overdigestion-induced nonspecific cell death. The cell suspensions were transferred to 15 mL centrifuge tubes and centrifuged at 1000 rpm for 5 min at 4 °C. The supernatants were discarded, and the cell pellets were washed twice with precooled PBS (4 °C) to remove residual medium components and nanomaterials. For staining, each cell pellet was resuspended in 100 μL Annexin V binding buffer (provided in the kit) to adjust the cell concentration to 1×10^6 cells/mL. According to the kit protocol, 5 μL Annexin V-FITC (fluorescent probe for phosphatidylserine externalization) and 5 μL propidium iodide (PI, fluorescent probe for nuclear membrane integrity) were added to the cell suspension, which was then incubated for 15 min at room temperature (25 ± 2 °C) in the dark to prevent fluorescence quenching. After incubation, 400 μL Annexin V binding buffer was added to each sample to dilute the staining solution, and flow cytometry analysis was performed immediately within 1 h using a BD FACSCanto II flow cytometer (BD Biosciences, San Jose, CA, USA). The flow cytometry acquisition parameters were set as follows: excitation wavelength = 488 nm (argon laser), emission filters = 530/30 nm (for Annexin V-FITC, green fluorescence) and 585/42 nm (for PI, red fluorescence). A total of 10,000 events were recorded per sample, and the data were analyzed using FlowJo software (Version 10.8.1, BD Biosciences). The total apoptosis rate was calculated as the sum of early and late apoptotic cells, and the necrosis rate was defined as the percentage of Annexin V[−]/PI⁺ cells. Statistical significance between groups was determined using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test with GraphPad Prism 9 software (GraphPad Software, San Diego, CA, USA). A p -value 5 was considered statistically significant, and all data were presented as mean $\pm$ standard deviation (SD) of three biological replicates.

2.4. Cell Viability Assay and Mitochondrial Membrane Potential Detection

Cell viability was evaluated using the Cell Counting Kit-8 (CCK-8, Dojindo, Tokyo, Japan) assay, which is based on the reduction of water-soluble tetrazolium salt (WST-8) to formazan by mitochondrial dehydrogenases. 4T1 cells were seeded into 96-well confocal plates at a density of 1×10^4 cells/well in 100 μ L complete medium and incubated for 24 h to allow cell adhesion. The cells were then divided into four experimental groups (PBS control, US alone, $\text{Ca}_{19}\text{Cu}_2(\text{PO}_4)_{14}$ alone, $\text{Ca}_{19}\text{Cu}_2(\text{PO}_4)_{14}$ + US) with five replicates per group. For the $\text{Ca}_{19}\text{Cu}_2(\text{PO}_4)_{14}$ -treated groups, nanomaterials were added at gradient concentrations (0, 2, 4, 6, 8, 10, 12 μ g/mL) to evaluate dose-dependent cytotoxicity. The ultrasound parameters and treatment timeline were consistent with those described in Section 2.3. After 24 h of treatment, the culture medium was carefully removed, and 100 μ L fresh complete medium containing 10 μ L CCK-8 solution (10% v/v) was added to each well. The plates were incubated for an additional 2 h at 37 °C in 5% CO_2 , and the optical density (OD) value at 450 nm was measured using a microplate reader (BioTek, Santa Clara, CA, USA). The cell viability was calculated using the formula: Cell viability (%) = (OD₄₅₀ of treatment group/OD₄₅₀ of control group) $\times$ 100%. Dose-response viability curves were plotted, and the half-maximal inhibitory concentration (IC₅₀) value of $\text{Ca}_{19}\text{Cu}_2(\text{PO}_4)_{14}$ nanomaterials (with/without US) was calculated using GraphPad Prism 9 software.

For the detection of mitochondrial membrane potential, mitochondrial membrane potential ($\Delta\Psi_m$) was detected using the JC-1 Mitochondrial Membrane Potential Assay Kit (Beyotime, Shanghai, China), based on the potential-dependent accumulation of JC-1 in mitochondria. Briefly, 4T1 cells were seeded into 6-well plates at a density of 5×10^5 cells/well and treated according to the four experimental groups described above. After 24 h of treatment, cells were harvested by trypsinization, washed twice with precooled PBS, and centrifuged at 1000 rpm for 5 min to collect cell pellets. The cell pellets were resuspended in 100 μ L JC-1 working solution (prepared according to the kit instructions) and incubated for 20 min at 37 °C in the dark. After incubation, 1 mL JC-1 staining buffer (provided in the kit) was added to each sample, and the cells were centrifuged at 1000 rpm for 5 min to remove unbound JC-1. The cell pellets were resuspended in 500 μ L JC-1 staining buffer, and flow cytometry analysis was performed using a BD FACSCanto II flow cytometer (BD Biosciences, San Jose, CA, USA). JC-1 monomers (low $\Delta\Psi_m$) emit green fluorescence (excitation: 488 nm, emission: 530 nm), while JC-1 aggregates (high $\Delta\Psi_m$) emit red fluorescence (excitation: 488 nm, emission: 590 nm). The ratio of red to green fluorescence intensity (R/G ratio) was used to quantify the mitochondrial membrane potential, with a decreased R/G ratio indicating mitochondrial depolarization. Each group was performed in triplicate, and the data were analyzed using FlowJo software. Additionally, confocal laser scanning microscopy (CLSM, Zeiss LSM 880, Berlin, Germany) was used for qualitative observation: after JC-1 staining, cells were seeded on glass coverslips, and fluorescence images were captured with excitation wavelengths of 488 nm (green) and 555 nm (red).

3. Results and Discussion

Morphology, Structure, and Physicochemical Characterization of $\text{Ca}_{19}\text{Cu}_2(\text{PO}_4)_{14}$ Nanomaterials

The $\text{Ca}_{19}\text{Cu}_2(\text{PO}_4)_{14}$ nanomaterials were synthesized via a modified co-precipitation method followed by high-temperature calcination, as schematically illustrated in Figure 1A. Transmission electron microscopy (TEM) images revealed that the synthesized $\text{Ca}_{19}\text{Cu}_2(\text{PO}_4)_{14}$ nanomaterials exhibited a uniform nanosheet-like morphology with good dispersion and no obvious aggregation (Figure 1B). Such favorable dispersibility is beneficial for reducing rapid clearance by the reticuloendothelial system during systemic circulation and facilitates efficient cellular internalization by 4T1 tumor cells. The nanomaterials displayed a dense internal structure without apparent hollow or porous

features, and the particle size was relatively uniform, predominantly distributed in the range of 100–150 nm. This size regime is generally considered suitable for passive tumor accumulation via the enhanced permeability and retention (EPR) effect, thereby enabling preferential enrichment in 4T1 tumor tissue.

The selected-area electron diffraction (SAED) pattern obtained from TEM analysis further confirmed the crystalline nature of the $\text{Ca}_{19}\text{Cu}_2(\text{PO}_4)_{14}$ nanomaterials (Figure 1C), with a characteristic lattice spacing of 0.18 nm. Consistently, X-ray diffraction (XRD) patterns (Figure 1D) showed that all diffraction peaks of $\text{Ca}_{19}\text{Cu}_2(\text{PO}_4)_{14}$ nanomaterials matched well with those of the standard copper-calcium phosphate crystal (JCPDS No. 97-005-0011). The sharp diffraction peaks and absence of impurity phases indicated high crystallinity and structural stability, which are essential for the stable loading of Cu^{2+} and Ca^{2+} ions and their controllable release under ultrasound stimulation. Thermogravimetric analysis (TGA) of the $\text{Ca}_{19}\text{Cu}_2(\text{PO}_4)_{14}$ nanopowder in Figure S1 from 28 to 600 °C shows an almost horizontal profile with only a slight, continuous mass decrease and no discernible stepwise weight-loss events, indicating minimal volatile content and high thermal stability over the investigated temperature window.

To further elucidate the elemental composition and chemical states, X-ray photoelectron spectroscopy (XPS) analysis was performed. As shown in Figure 2A–D and Figure S2, four characteristic elements—Cu, Ca, P, and O—were clearly detected on the surface of the nanomaterials. The Cu 2p spectrum exhibited characteristic peaks at binding energies of 934.3, 939.8, 943.5, 954.1, and 963.3 eV, with no obvious satellite peaks, corresponding to the Cu^{2+} oxidation state (Figure 2B). The Ca 2p spectrum displayed a typical doublet at 347.1 and 350.6 eV, confirming the presence of Ca^{2+} ions (Figure 2C). In addition, the P 2p spectrum showed characteristic peaks at 132.8 and 134.0 eV, attributable to P^{5+} species in phosphate groups (Figure 2D). These results collectively demonstrate that the nanomaterials possess a copper-calcium phosphate crystalline structure, in which Cu^{2+} and Ca^{2+} ions are stably incorporated through phosphate-mediated coordination. As shown in Figure S3, the FT-IR spectrum of $\text{Ca}_{19}\text{Cu}_2(\text{PO}_4)_{14}$ is dominated by orthophosphate vibrations, confirming a phosphate-based inorganic framework [44–46]. Specifically, the broad bands at 3442 and 1636 cm^{-1} are assigned to O–H stretching and H–O–H bending of surface-adsorbed water, which is typical for nanopowders. Intense absorptions at 1211–1157 and 1029–946 cm^{-1} correspond to P–O stretching modes of PO_4^{3-} ; their splitting indicates reduced local symmetry and multiple non-equivalent phosphate environments induced by Ca/Cu coordination. The features at 612/558 cm^{-1} and 496/454 cm^{-1} arise from O–P–O bending and coupled lattice modes. Dynamic light scattering (DLS) analysis further showed that $\text{Ca}_{19}\text{Cu}_2(\text{PO}_4)_{14}$ nanomaterials exhibited an average hydrodynamic diameter of 102.4 ± 8.3 nm and the PDI of 0.162 ± 0.021 in PBS (Figure 2E), which is consistent with the result in TEM image (Figure 1B), indicating a narrow size distribution and good particle uniformity. Meanwhile, the zeta potential was measured to be -14.8 ± 1.2 mV (Figure 2F). This moderately negative surface charge is advantageous for minimizing nonspecific adsorption of plasma proteins while avoiding excessive electrostatic repulsion between particles, thereby contributing to favorable colloidal stability under physiological conditions. Together, these physicochemical characteristics suggest that the $\text{Ca}_{19}\text{Cu}_2(\text{PO}_4)_{14}$ nanomaterials are well suited for in vivo circulation and passive accumulation at 4T1 tumor sites.

After the confirmation of favorable physicochemical properties, the ultrasound-responsive release behaviors of Cu^{2+} and Ca^{2+} ions from $\text{Ca}_{19}\text{Cu}_2(\text{PO}_4)_{14}$ nanomaterials were systematically investigated in PBS (pH 7.4) (Figure 3A) and DMEM containing 10% FBS (Figure S4) to mimic the physiological microenvironment. As shown in Figure 3A, the $\text{Ca}_{19}\text{Cu}_2(\text{PO}_4)_{14}$ -alone group exhibited extremely low ion leakage over 12 h of incubation: the cumulative release ratios of Cu^{2+} and Ca^{2+} were only 0.05% and 0.04% in PBS,

corresponding to final concentrations of 10 ± 0.4 ng/mL and 8 ± 0.4 ng/mL, respectively. This negligible ion leakage is attributed to the high crystallinity and structural integrity of $\text{Ca}_{19}\text{Cu}_2(\text{PO}_4)_{14}$ nanomaterials (verified in Figure 1B–D), which prevents uncontrolled ion dissociation under physiological conditions. This feature is of great significance for in vivo applications, as it minimizes off-target toxicity to normal tissues by avoiding premature ion release during systemic circulation. In contrast, US irradiation triggered a dramatic enhancement in ion release from $\text{Ca}_{19}\text{Cu}_2(\text{PO}_4)_{14}$ nanomaterials. After 12 h of incubation with periodic US stimulation (1 MHz, 1 W/cm², 5 min at 2, 4, 8 h), the cumulative release ratios of Cu^{2+} and Ca^{2+} in the $\text{Ca}_{19}\text{Cu}_2(\text{PO}_4)_{14}$ + US group reached 0.87% and 1.27%, corresponding to concentrations of 174 ± 14.2 µg/mL and 254 ± 21.4 µg/mL, respectively, representing 17.4-fold and 31.8-fold increases compared with the non-US group. The trend of in vitro ultrasound-triggered ion release data from materials in DMEM containing 10% FBS (Figure S4) is consistent with that observed in PBS. These findings confirm that ultrasound irradiation can disrupt the crystalline structure of $\text{Ca}_{19}\text{Cu}_2(\text{PO}_4)_{14}$ nanomaterials through mechanical and cavitation effects, achieving spatiotemporally controllable release of Cu^{2+} and Ca^{2+} ions and laying the foundation for subsequent intracellular ion accumulation and synergistic antitumor activity in 4T1 cells.

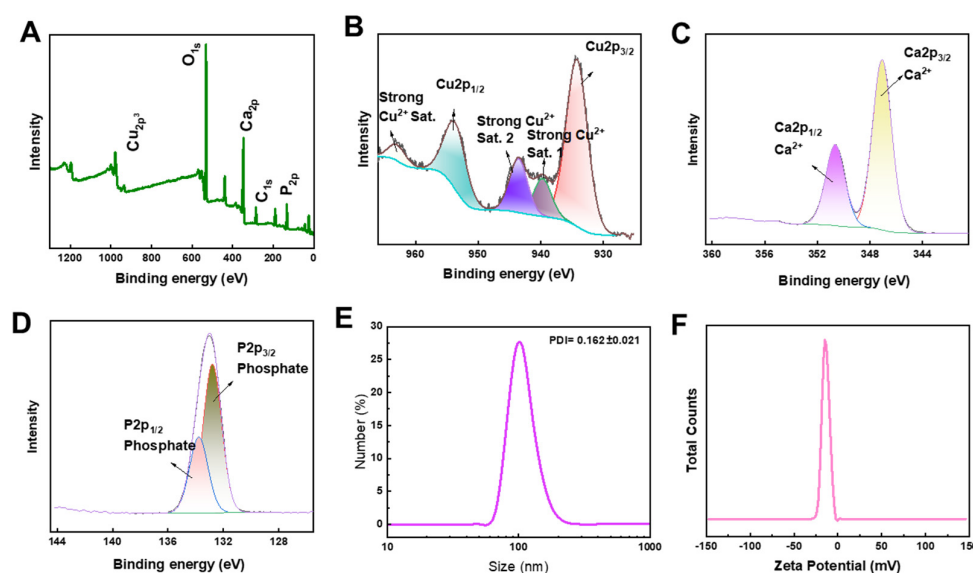


Figure 2. XPS survey spectrum of $\text{Ca}_{19}\text{Cu}_2(\text{PO}_4)_{14}$ (A), High-resolution XPS spectra for Cu 2p (B), Ca 2p (C), and P 2p (D), DLS (E) and zeta potential (F) of $\text{Ca}_{19}\text{Cu}_2(\text{PO}_4)_{14}$ in PBS solution.

To evaluate the antitumor efficacy of ultrasound-activated $\text{Ca}_{19}\text{Cu}_2(\text{PO}_4)_{14}$ nanomaterials, cell viability was first assessed using the CCK-8 assay. As shown in Figure 3B, the $\text{Ca}_{19}\text{Cu}_2(\text{PO}_4)_{14}$ -alone group exhibited negligible cytotoxicity even at the highest concentration tested (20 µg/mL), with cell viability remaining above 97% ($97.23\% \pm 1.92\%$), which is consistent with the low ion leakage observed in the release assay (Figure 3A). In contrast, the $\text{Ca}_{19}\text{Cu}_2(\text{PO}_4)_{14}$ + US group displayed a concentration-dependent cytotoxic effect: cell viability decreased gradually with increasing nanomaterial concentration, reaching $41.37\% \pm 2.17\%$ at 20 µg/mL. The half-maximal inhibitory concentration (IC_{50}) of $\text{Ca}_{19}\text{Cu}_2(\text{PO}_4)_{14}$ + US was calculated as 16.8 ± 1.2 µg/mL, whereas the IC_{50} of $\text{Ca}_{19}\text{Cu}_2(\text{PO}_4)_{14}$ alone exceeded 20 µg/mL (the maximum test concentration). On the other hand, the MCF-10A healthy cells maintained high cell viability after the treatment of materials and materials +US in Figure S5. These results confirm that ultrasound activation is indispensable for the antitumor activity of $\text{Ca}_{19}\text{Cu}_2(\text{PO}_4)_{14}$ nanomaterials, as it enables sufficient ion release to induce tumor cell death.

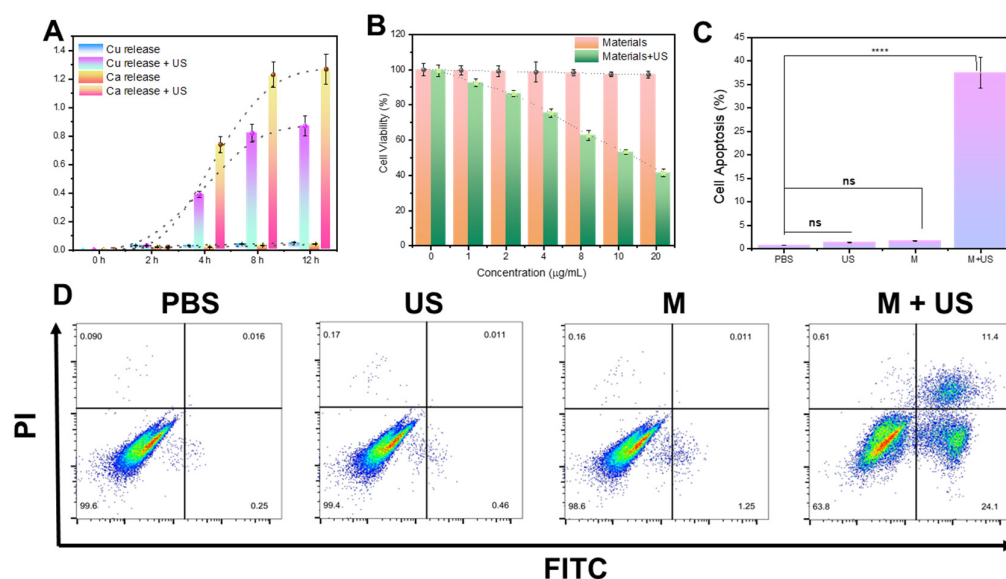


Figure 3. Results for in vitro ultrasound-triggered ion release from $\text{Ca}_{19}\text{Cu}_2(\text{PO}_4)_{14}$ nanomaterials in PBS (A), cell viability of 4T1 cells with the treatment of materials and materials +US (B) and the quantitative data of 4T1 cells apoptosis assay of $\text{Ca}_{19}\text{Cu}_2(\text{PO}_4)_{14}$ (C) using flow cytometry (D). Data represent the mean $\pm$ SD ($n = 3$). Statistical significance among groups: Not significant (ns), **** $p < 0.0001$.

Flow cytometry analysis of apoptosis further corroborated the pro-apoptotic effect of the $\text{Ca}_{19}\text{Cu}_2(\text{PO}_4)_{14}$ + US system (Figure 3C–D). The total apoptosis rates of 4T1 cells in the PBS control, US alone, and $\text{Ca}_{19}\text{Cu}_2(\text{PO}_4)_{14}$ alone groups were $0.74\% \pm 0.40\%$, $1.34\% \pm 0.09\%$, and $1.65\% \pm 0.12\%$, respectively, with no statistically significant differences among these groups ($p > 0.05$). This result is consistent with the intrinsic anti-apoptotic characteristics of 4T1 cells, which are known to resist single-modality stimulation by upregulating anti-apoptotic proteins (e.g., Bcl-2) and enhancing DNA damage repair capacity. Notably, the $\text{Ca}_{19}\text{Cu}_2(\text{PO}_4)_{14}$ + US group (abbreviated as M + US) exhibited a dramatic increase in apoptosis rate to $37.56\% \pm 3.26\%$, which was significantly higher than all other groups ($p < 0.0001$). Collectively, these results demonstrate that the synergistic release of Cu^{2+} and Ca^{2+} ions mediated by ultrasound-activated $\text{Ca}_{19}\text{Cu}_2(\text{PO}_4)_{14}$ nanomaterials exerts a potent pro-apoptotic effect on 4T1 tumor cells, effectively overcoming their intrinsic anti-apoptotic resistance.

To visually confirm the in vitro antitumor effect of the $\text{Ca}_{19}\text{Cu}_2(\text{PO}_4)_{14}$ + US system, confocal laser scanning microscopy (CLSM) was performed using Calcein-AM/PI (Beyotime, Shanghai, China) double staining, where Calcein-AM (green fluorescence) labels viable cells with intact cell membranes and PI (red fluorescence) stains dead cells with compromised membranes. As shown in Figure 4, cells in the PBS control group exhibited strong and uniform green fluorescence, with intact cellular morphology and no detectable red fluorescence, indicating high cell viability. In contrast, cells treated with US alone or $\text{Ca}_{19}\text{Cu}_2(\text{PO}_4)_{14}$ alone displayed only marginal red fluorescence, which is consistent with the negligible cytotoxicity observed in CCK-8 and flow cytometry assays above. Notably, the $\text{Ca}_{19}\text{Cu}_2(\text{PO}_4)_{14}$ + US group showed a dramatic increase in red fluorescence and a significant reduction in green fluorescence with enhanced cytotoxicity, indicating the ultrasound-triggered release of Cu^{2+} and Ca^{2+} ions from the $\text{Ca}_{19}\text{Cu}_2(\text{PO}_4)_{14}$ nanomaterials, which markedly amplifies intracellular ion accumulation and induces effective tumor cell killing. These confocal observations are highly consistent with the CCK-8 and flow cytometry results discussed above, collectively confirming that the synergistic combination

of $\text{Ca}_{19}\text{Cu}_2(\text{PO}_4)_{14}$ nanomaterials and ultrasound irradiation is essential for achieving efficient antitumor activity in 4T1 cells.

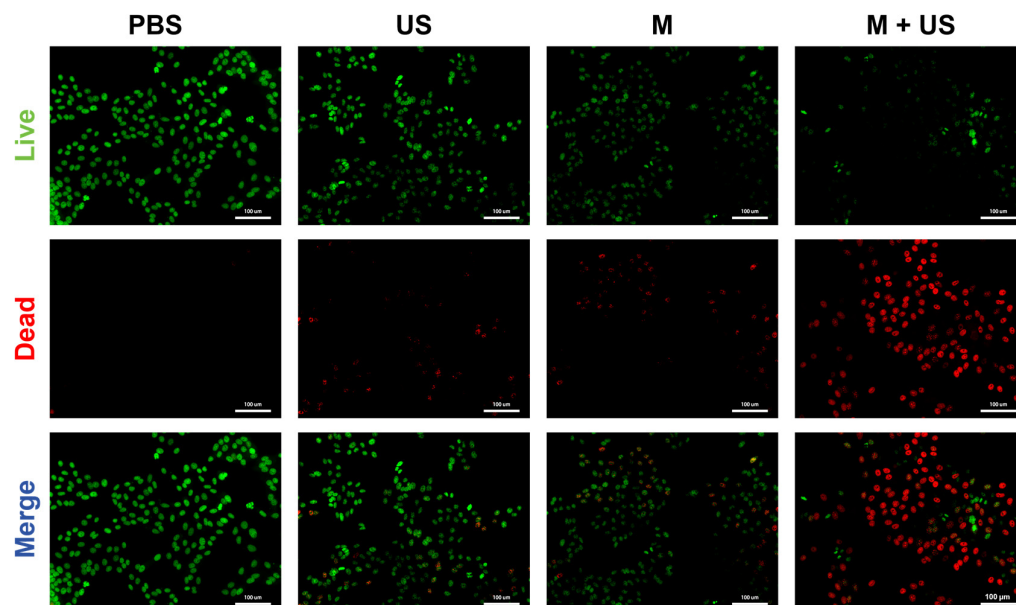


Figure 4. Confocal fluorescence micrographs of 4T1 cells stained with Calcein-AM/PI after different treatments. (Scale bar = 100 µm).

To evaluate the dysfunction of mitochondria after treatment with different methods, the fluorescent indicator JC-1 probe was applied to assess the mitochondrial membrane potential ($\Delta\Psi_m$) change, which exhibited red fluorescence (high $\Delta\Psi_m$) in healthy mitochondria and changed to green fluorescence (low $\Delta\Psi_m$) in damaged mitochondria. As shown in Figure 5 and Figure S6, compared to the cells in the PBS control group, US and $\text{Ca}_{19}\text{Cu}_2(\text{PO}_4)_{14}$ alone (mainly distributed in the Q2 region), the mitochondrial membrane potential of $\text{Ca}_{19}\text{Cu}_2(\text{PO}_4)_{14}$ -treated cells was largely depolarized, suggesting that $\text{Ca}_{19}\text{Cu}_2(\text{PO}_4)_{14}$ can induce apoptosis by inhibiting mitochondrial activity significantly through its mitochondrial targeting effect.

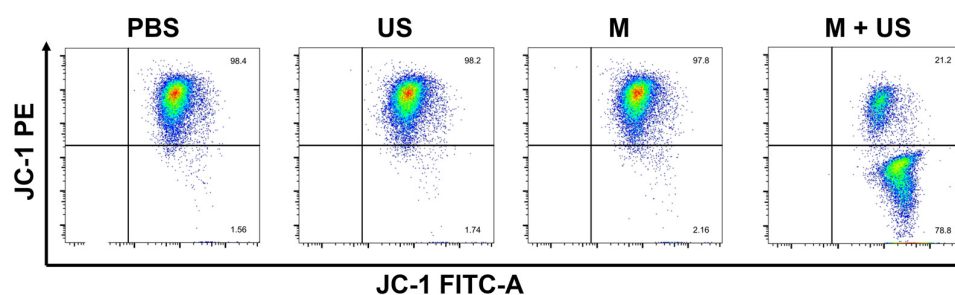


Figure 5. Mitochondrial membrane potential test after 8 h of 4T1 cells with PBS, US, $\text{Ca}_{19}\text{Cu}_2(\text{PO}_4)_{14}$, and $\text{Ca}_{19}\text{Cu}_2(\text{PO}_4)_{14}$ + US, respectively.

Furthermore, in order to elucidate the molecular basis underlying the enhanced antitumor effect of $\text{Ca}_{19}\text{Cu}_2(\text{PO}_4)_{14}$ + US, Western blotting was performed to probe key proteins involved in mitochondrial homeostasis and apoptosis (Figure S7). Importantly, WB data further revealed that FDX1 and DLAT were markedly downregulated after $\text{Ca}_{19}\text{Cu}_2(\text{PO}_4)_{14}$ + US treatment. FDX1 is a mitochondria-associated redox protein closely linked to copper handling and mitochondrial metabolic regulation, whereas DLAT is a core E2 component of the pyruvate dehydrogenase (PDH) complex that connects glycolytic carbon influx to

the TCA cycle. The concurrent suppression of FDX1 and DLAT suggests that ultrasound-triggered Cu^{2+} release not only initiates apoptotic signaling but also imposes substantial mitochondrial metabolic stress, potentially impairing PDH/TCA flux and destabilizing mitochondrial proteostasis. This pattern is mechanistically consistent with copper-dependent mitochondrial toxicity, in which copper preferentially disrupts mitochondria-centered metabolic nodes and sensitizes cells to irreversible mitochondrial failure. Additionally, within this nanomaterials, Ca^{2+} released from $\text{Ca}_{19}\text{Cu}_2(\text{PO}_4)_{14}$ under ultrasound may further amplify mitochondrial damage by promoting calcium overload, facilitating mitochondrial permeability transition, and accelerating membrane depolarization and Cyt c mobilization. Therefore, the therapeutic outcome of $\text{Ca}_{19}\text{Cu}_2(\text{PO}_4)_{14}$ + US can be interpreted as an ion-synergized process: Cu^{2+} drives mitochondrial metabolic/proteostasis disruption (supported by FDX1 and DLAT downregulation), while Ca^{2+} exacerbates mitochondrial membrane dysfunction, together converging on robust activation of the intrinsic apoptotic pathway.

4. Conclusions

In this study, a copper-calcium phosphate ($\text{Ca}_{19}\text{Cu}_2(\text{PO}_4)_{14}$) nanomaterial with favorable physicochemical properties and pronounced ultrasound responsiveness was successfully designed and synthesized. The molecular mechanism by which $\text{Ca}_{19}\text{Cu}_2(\text{PO}_4)_{14}$ nanomaterials induce apoptosis in metastatic 4T1 breast cancer cells through synergistic $\text{Cu}^{2+}/\text{Ca}^{2+}$ ion release was systematically elucidated. Comprehensive material characterization confirmed that the $\text{Ca}_{19}\text{Cu}_2(\text{PO}_4)_{14}$ nanomaterials possess a uniform and stable crystalline structure, with a nanoscale size distribution that is suitable for passive accumulation in 4T1 tumor tissues via the enhanced permeability and retention (EPR) effect. Ion release studies demonstrated that ultrasound irradiation could specifically trigger the degradation of $\text{Ca}_{19}\text{Cu}_2(\text{PO}_4)_{14}$ nanomaterials through mechanical and cavitation effects, enabling spatiotemporally controlled release of Cu^{2+} and Ca^{2+} ions. Importantly, the ion release kinetics closely matched the proliferation dynamics of 4T1 cells, providing a critical prerequisite for effective synergistic cytotoxicity. In vitro cellular experiments further verified that the combination of $\text{Ca}_{19}\text{Cu}_2(\text{PO}_4)_{14}$ nanomaterials and ultrasound irradiation markedly enhanced antitumor efficacy against 4T1 cells, whereas $\text{Ca}_{19}\text{Cu}_2(\text{PO}_4)_{14}$ nanomaterials or ultrasound treatment alone exerted negligible effects on cell viability. Moreover, at the molecular level, Western blot analysis revealed that the released Cu^{2+} and Ca^{2+} ions synergistically targeted mitochondrial function in 4T1 cells, suppressing the mitochondria-associated copper/redox regulator FDX1 and the PDH complex E2 subunit DLAT, thereby impairing mitochondrial metabolic homeostasis and promoting mitochondrial dysfunction. Overall, this ultrasound-responsive $\text{Ca}_{19}\text{Cu}_2(\text{PO}_4)_{14}$ nanomaterial-based strategy, which leverages synergistic copper/calcium ion release, offers a promising and mechanistically well-defined approach for overcoming the aggressive and therapy-resistant characteristics of metastatic breast cancer mediated by 4T1 cells.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/ijms27042016/s1>.

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Conflicts of Interest: The authors declare that they have no financial and personal relationships with other people or organizations that may have inappropriately influenced our work; there is no professional or other personal interest of any nature in any product, service and/or company that could be construed as influencing the position presented in the article.

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