Intracellular Ca\textsuperscript{2+} Cascade Guided by NIR-II Photothermal Switch for Specific Tumor Therapy

HIGHLIGHTS
The Ca\textsuperscript{2+} cascade is selectively constructed in vivo without being limited to hypoxic tumor
Ca\textsuperscript{2+} overload leads to mitochondrial dysfunction and cancer death
The released CuS NPs provide an enhanced 3D photoacoustic imaging
Ca\textsuperscript{2+}-interference therapy avoids the obstacles of traditional treatment
Intracellular Ca\(^{2+}\) Cascade Guided by NIR-II Photothermal Switch for Specific Tumor Therapy

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SUMMARY
Currently, patients receiving cancer treatments routinely suffer from distressing toxic effects, most originating from premature drug leakage, poor biocompatibility, and off-targeting. For tackling this challenge, we construct an intracellular Ca\(^{2+}\) cascade for tumor therapy via photothermal activation of TRPV1 channels. The nanoplatform creates an artificial calcium overloading stress in specific tumor cells, which is responsible for efficient cell death. Notably, this efficient treatment is activated by mild acidity and TRPV1 channels simultaneously, which contributes to precise tumor therapy and is not limited to hypoxic tumor. In addition, Ca\(^{2+}\) possesses inherent unique biological effect and normal cells are more tolerant of the undesirable destructive influence than tumor cells. The Ca\(^{2+}\) overload leads to cell death due to mitochondrial dysfunction (upregulation of Caspase-3, cytochrome c, and downregulation of Bcl-2 and ATP), and in vivo, the released photothermal CuS nanoparticles allow an enhanced 3D photoacoustic imaging and provide instant diagnosis.

INTRODUCTION
At present, for metastatic tumor or unresectable lesion, chemotherapy is usually the preferred option despite the emergence of many advanced treatment technologies (Nam et al., 2018; Chen et al., 2017). However, traditional chemotherapy, where the drug toxicity is always “ON,” leads to severe systemic toxicity, including nausea, vomiting, diarrhea, kidney problems, and neuropathic pain, which often compromises patients’ quality of life (Milosavljevic et al., 2010; Wallace et al., 2010; Zhao et al., 2018). Although many smart carriers have been cunningly designed to target drug delivery to tumor sites (Cao et al., 2019; Wang et al., 2018; Dai et al., 2019), recent meta-analysis suggests that only 0.7% (median) of the administered dose is delivered to the tumor, whereas the rest is accumulated in healthy organs, which will cause some chronic diseases resulting from the toxic drugs (Wilhelm et al., 2016; Li et al., 2017). Therefore, developing a therapeutic model that can be biocompatible and keep the drug inert even if the rest accumulated in healthy organs, while it exerts its therapeutic efficacy only at the tumor site via specific transformation is necessary and also highly challenging owing to the tumor inhomogeneity.

Metal ions with diverse cellular biological effects are playing more important roles in cell metabolism and proliferation than expected (Gao et al., 2019; Zhang et al., 2019). Any adjustment of ion balance may induce a series of intracellular reactions, even cell death (Park et al., 2019). Such a dramatic ion interference technology is expected to avoid systemic toxicity through ingenious construction. In view of this, more attention will be paid to Ca\(^{2+}\), as it is an essential component of tissues such as bone and is involved in a host of vital cell survival processes (Sang et al., 2018; Chen et al., 2019). Calcium overload, characterized by an abnormal accumulation of free calcium ions (Ca\(^{2+}\)), is a widely recognized cause of damage in numerous cell types and even of cell death (Duan et al., 2019; Zhang et al., 2019). Tumor cells are more sensitive to the persistent Ca\(^{2+}\) overload than normal cells (Pesakhov et al., 2016; Xu et al., 2018). In particular, transient receptor potential cation channel subfamily V member 1 (TRPV1), a nonselective cation channel that prefers Ca\(^{2+}\) over other cations, has been revealed to be highly over-expressed in many malignancies (Zhen et al., 2018; Wu et al., 2014). TRPV1 can be activated by external stimuli such as heat, low pH, capsaicin, and vanilloids (Rodrigues et al., 2016). Being able to manipulate TRPV1 signaling at precise times and spaces in living systems remains a challenge.

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Keeping all these issues in mind, we proposed to construct a Ca\(^{2+}\) cascade via photothermal activation of the TRPV1 signaling pathways to specifically inhibit the growth of tumor with minimum systemic toxicity. In order to realize enhanced Ca\(^{2+}\) interference therapy, continuous supply of Ca\(^{2+}\) is essential. It is known that natural organisms CaCO\(_3\) with excellent biocompatibility and biodegradability is stable at neutral pH and can decompose in tumor acidic microenvironment, so it is mostly used as a smart carrier (Dong et al., 2018; Wan et al., 2019; Zhao et al., 2010, 2015). In addition, it could be used as a reservoir to keep the tumor supplied with plentiful Ca\(^{2+}\) (Li et al., 2020; Zhao et al., 2010). Considering to activate TRPV1 signaling using near-infrared (NIR) light, we turned our attention to copper sulfide (CuS) nanoparticles (NPs) as the photothermal switch owing to the excellent photothermal efficiency (Zhou et al., 2019), photothermal combination therapy (Shao et al., 2019), and photoacoustic (PA) imaging (Zhou et al., 2020). As shown in Scheme 1, based on enhanced permeability and retention (EPR) effect (Kobayashi et al., 2013), the CaCO\(_3\) nanocarrier could remain and decompose at tumor site to produce abundant Ca\(^{2+}\) and release photothermal CuS NPs. Irradiation of CuS NPs by NIR results in strong PA signal and local heating, which contribute to guide and open TRPV1 channels allowing an influx of Ca\(^{2+}\). After the intracellular Ca\(^{2+}\) concentration rose, much larger amounts of Ca\(^{2+}\) could accumulate in mitochondria, leading to disrupting mitochondria Ca\(^{2+}\) homeostasis and dysfunction followed by cell apoptosis. However, in normal physiological environment, CaCO\(_3\) was so stable that generous Ca\(^{2+}\) could not be supplied, and the absence of over-expression of TRPV1 and mild acidity stimulation ensured that Ca\(^{2+}\) interference therapy was ineffective in normal tissues. Besides, CaCO\(_3\) was biocompatible and degradable, which ensured the worry of systemic toxicity could be completely eliminated. More meaningfully, the intracellular Ca\(^{2+}\) cascade for specific tumor therapy may hold promise as an effective cancer therapeutic tool complementary to traditional clinical tumor treatment.

Scheme 1. Schematic Illustration of Calcium-Overload-Mediated Tumor Therapy by CuS@CaCO\(_3\)-PEG
(A) The synthesis processes of CuS@CaCO\(_3\)-PEG.
(B) The photothermal activation of the TRPV1 Ca\(^{2+}\) channels on the cell membrane.
(C) The mechanism of tumor cell death induced by mitochondrial damage.
RESULTS
Preparation and Characterization of CuS@CaCO3-PEG

The preparation procedure of CuS@CaCO3-PEG was illustrated in Scheme 1A, and the detailed synthetic procedure was presented in the Experimental Section. Transmission electron microscopy (TEM) showed the morphology of CuS, CuS@CaCO3, and CuS@CaCO3-PEG and as-synthesized CuS@CaCO3-PEG NPs were monodispersed with an average diameter of around 100 nm. There were some
morphologically spherical pellets in the CuS@CaCO₃, indicating that CuS was successfully loaded into CuS@CaCO₃. The SEM image of CuS@CaCO₃ was shown in Figure S1. The corresponding size distributions were shown by dynamic light scattering in Figure 1C, in accordance with TEM results. Figure S2 further demonstrated that both CuS and CuS@CaCO₃ exhibited poor stability in 10% serum, whereas CuS@CaCO₃-PEG had better stability both in PBS and in10% serum suggesting the improvement of in vivo application potential. Besides, the ζ potential was reversed from 13.2 to 1.6 to −10.6 mV after loading and decorating, implying the successful encapsulation and PEGylation, which avoided nonspecific adsorption in vivo. The UV-visible (UV-vis) absorption spectrum of CuS@CaCO₃-PEG (Figure 1D) illustrated that there was a broad absorption between 700 and 1,100 nm, which was similar to the absorption of CuS NPs. X-ray diffraction results evidenced the presence of CuS NPs and CaCO₃ in the NPs. After PEGylation, the decrease of the peak intensity was due to the cover of phospholipid bilayer on the surface (Figure 1E). To substantially confirm the successful loading of CuS NPs in CaCO₃, energy-dispersive X-ray (EDX) mapping was conducted. As shown in Figure 1B, S and Cu elements were evenly dispersed in CuS@CaCO₃, which indicated that CuS was successfully encapsulated in CaCO₃.

**Acid Enhanced Ca²⁺ Release and Photothermal Effect of CuS@CaCO₃-PEG**

Owing to the successful loading of CuS NPs in CaCO₃, CuS@CaCO₃-PEG may disintegrate and release Ca²⁺ and CuS at low pH. First, the collapse of CuS@CaCO₃-PEG treated with different pH PBS was observed via TEM. As shown in Figure S4, with an increase of acidity, the CuS@CaCO₃-PEG NPs were gradually destroyed. At pH 7.4, all of them kept intact, indicating CuS@CaCO₃-PEG could remain stable in physiological environments. In particular, almost all of them disintegrated into smaller particles at pH 5.0. At the same time, the light transmittance of CuS@CaCO₃-PEG treated with different pH was shown in Figure 1F insert, and the light transmittance gradually increased with the decrease of pH, which reveals the pH-responsive decomposition of CuS@CaCO₃-PEG. After centrifugation and supernatant collection, the concentrations of Ca²⁺ and Cu²⁺ were, respectively, measured via inductively coupled plasma mass spectrometry (ICP-MS). As shown in Figure 1F, after interaction for 30 min, the concentration of Ca²⁺ rose to 82 mg L⁻¹ with decrease of solution pH, suggesting that the production of abundant Ca²⁺ was effective in tumor microenvironment. With disintegration of CuS@CaCO₃-PEG at lower pH, more CuS NPs will be released in the acid environment. As shown in Figure S5, less than 30% of Cu²⁺ is released after 8 h of treatment at pH 7.4, suggesting the CaCO₃ is a desired gatekeeper. On the contrary, within 1 h, the release of Cu²⁺ reached 66% and 52% at pH 5.0 and 6.5, respectively. All these results substantially demonstrated that CuS@CaCO₃-PEG could reactively decompose at low pH and rapidly release abundant Ca²⁺ and loaded CuS NPs.

The photothermal properties of CuS@CaCO₃-PEG were studied because of the influential NIR absorption band. Owing to a tumor acidic environment, we further evaluated the photothermal effect of CuS@CaCO₃-PEG at pH 6.5 and 5.0. As displayed in Figure 1G, the solution temperature quickly increased with prolonging of irradiation time. Notably, the lower the pH was, the faster and higher the temperature rose. It may be attributed to the release of CuS from the cracking of calcium carbonate, which had a stronger absorbance in NIR. The photothermal property of CuS@CaCO₃-PEG was also concentration and power dependent (Figure S6). Besides, CuS@CaCO₃-PEG showed excellent thermal stability in PBS (Figure S7).

**Ca²⁺ Influx In Vitro by NIR-II Laser Irradiation**

Considering that CuS@CaCO₃-PEG can produce abundant Ca²⁺ and has excellent photothermal conversion characteristic in tumor acidic environment, the ability of CuS@CaCO₃-PEG to activate heat-sensitive TRPV1 Ca²⁺ channels was examined in two different cells. The U373 glioma cells are the experimental group, which provided a high expression of TRPV1 pathway, whereas HeLa cells are the TRPV1-negative cells (Lyu et al., 2016; Amantini et al., 2007). First, the intracellular Ca²⁺ influx was monitored by Ca²⁺ fluorescence probe (Fluo-4) (Figure 2A). Under laser irradiation at 1,064 nm (1.2 W cm⁻²) for 30 s, no fluorescence increase was observed in pH 7.4 + L group or pH 6.5 - L group of TRPV1-positive cells; in contrast, significant fluorescence increase was detected for 5 pH 6.5 + L group (Figure 2B). Therefore, Ca²⁺ influx was activated for U373 cells only when both acidic environment and NIR irradiation were satisfied, ensuring the high precision of CuS@CaCO₃-PEG to tumor cells. However, no fluorescence of Fluo-4 was observed in the TRPV1-negative cells (HeLa) even when the above conditions existed, validating that Ca²⁺ influx was specifically activated by PTT-induced TRPV1 opening. The quantitative fluorescence intensity of Fluo-4 was detected by flow cytometry in Figure 2C. The fluorescence intensity in U373 cells of pH 6.5 + L group was a high level when compared with the blank control group. In other groups, there was no significant
fluorescence enhancement, which was consistent with the results of CLSM, showing weak fluorescence of Fluo-4. In order to further verify that heat can induce the opening of calcium channels, the two kinds of cells treated with CuS@CaCO3-PEG were incubated in 43°C incubator for 10 min and the fluorescence of Fluo-4 was detected by CLSM. As shown in Figure S8, obvious fluorescence of Fluo-4 was displayed in the mitochondria of U373 cells but not in HeLa cells. This demonstrated that it was indeed heat-induced calcium ion influx in our work.

When intracellular Ca²⁺ concentrations rise, rapid mitochondrial Ca²⁺ uptake in living cells was shown (Thor et al., 1984). Because when temporary imbalance of intracellular Ca²⁺ occurs, mitochondria as sensors and regulators will take in Ca²⁺ from the cytoplasm to regulate intracellular calcium homeostasis (Rizzuto et al., 2012). Inspired by this, the colocalization of intracellular Ca²⁺ and mitochondria was then observed through CLSM, where mitochondria were marked by MitoRed indicator. Most of Ca²⁺ was well co-localized with mitochondria, which revealed that abundant Ca²⁺ finally arrived at mitochondria after flowing into the cells through TRPV1 channels.

Selective Toxic Activation of CuS@CaCO3-PEG

Mitochondria regulate intracellular calcium homeostasis (Williams et al., 2013); however, excessive Ca²⁺ in mitochondria triggers cell apoptosis (Giorgi et al., 2012). The calcium overload toxicity assays of CuS@CaCO3-PEG were executed for U373 cells and HeLa cells. As shown in Figure 2D, CuS@CaCO3-PEG would not initiate U373 cells death at pH 7.4 with/without light irradiation, and even when the concentration of Ca²⁺ was increased without light irradiation (Figure S9), suggesting the good biocompatibility of CuS@CaCO3-PEG under typical physiological pH environment. To evaluate the selective toxicity of CuS@CaCO3-PEG, the toxicities of CuS@CaCO3-PEG on normal cells (COS7 cells) and TRPV1-positive tumor cell lines (HEK293 cells) (Sanz-Salvador et al., 2012) were tested by MTT. As shown in Figure S10,
the CuS@CaCO3-PEG had no toxicity to normal cells, whereas it had obvious toxicity to TRPV1-positive cells (HEK 293 cells), which further confirmed the nanoplatform for specifically inhibiting cancer. When cells were cultured at pH 6.5 without irradiation, cells exhibited good survival, implying that the Ca2+ influx induced by endocytosis was not enough to induce cell death maybe due to its own mechanism of calcium regulation. Meanwhile, CuS exhibited little phototoxicity in the cell experiment, because the temperature of culture medium was strictly detected and controlled at around 43°C by thermal camera (Figure S11), in order to avoid the cell death caused by hyperthermia. In sharp contrast, CuS@CaCO3-PEG displayed apparent toxicity in U373 cells at pH 6.5 with laser irradiation. Combined with the results of Ca2+ influx and MTT, it was proved that Ca2+ overload in mitochondria could finally lead to cell death. However, there existed no toxicity in HeLa cell experiments (Figure 2E), which powerfully confirmed that selective cancer cell death caused by calcium overload was indeed guided by activation of TRPV1. In addition, the two kinds of cells treated with CuS@CaCO3-PEG were cultured in 43°C incubator for 10 min, and the same phenomenon of cell viability appeared. There existed obvious toxicity only in pH 6.5 group of U373 cells (Figure S12). All these results substantially indicated that CuS@CaCO3-PEG could selectively kill U373 cells (TRPV1+), because the opening of TRPV1 channels by heat was the fuse of calcium overload, which led to subsequent cell death.

To investigate the cell death pathway by calcium overload, cells were incubated with CuS@CaCO3-PEG in the presence or absence of light irradiation under different pH environment, and then cells were labeled with annexin VFITC/PI. Early apoptosis can be detected using annexin V by combining with the exposed phosphatidylserine on the cell surface, whereas PI can stain nuclear chromatin rapidly in membrane-compromised cells during late apoptosis or necrosis (Liang et al., 2018). As shown in Figure S13, cells treated with CuS@CaCO3-PEG at pH 6.5 with light irradiation exhibited the highest late apoptotic and the necrosis ratio of 20.17% compared with other groups. This was in good agreement with the published report that disruption of intracellular Ca2+ homeostasis will result in an increased rate of cell necrosis (Orrenius et al., 2003; Scorrano et al., 2003).

Disruption of Mitochondrial Ca2+ Homeostasis of CuS@CaCO3-PEG

Proposed apoptosis mechanism induced by CuS@CaCO3-PEG was illustrated in Figure 3A. For further investigating the mechanism of apoptosis of calcium overload in mitochondria, mitochondrial dysfunction resulting from disruption of mitochondrial Ca2+ homeostasis was studied by tetramethyl rhodamine dye (TMRM) staining, because the decrease in fluorescence of TMRM reflects an increase in the degree of depolarization of the membrane potential (Ma et al., 2018). As shown in Figure 3B, there was bright red fluorescence of TMRM when U373 cells treated with CuS@CaCO3-PEG were at pH 7.4 with light irradiation, suggesting that a little Ca2+ flowing through TRPV1 channels would not damage the mitochondria. When U373 cells treated with CuS@CaCO3-PEG were at pH 6.5 without light irradiation, the fluorescence changed little, indicating that the cell itself could maintain Ca2+ homeostasis and protect mitochondria, unless a specific regulating pathway was destroyed (Rizzuto and Pozzan, 2006). However, in pH 6.5 + L group of U373 cells, the red fluorescence dramatically decreased. This great difference suggested that Ca2+ overload in mitochondria by opening the TRPV1 channels seriously harassed the mitochondrial membrane potential of mitochondria in U373 cells (TRPV1+). In contrast, there was no difference in fluorescence intensity between pH 6.5 with and without light in HeLa cells. Simultaneously, the quantitative results of fluorescence intensity were further determined via flow cytometry in Figure S14. When compared with other groups, significant decrease of the fluorescence intensity of TMRM was observed in pH 6.5 + L group of U373 cells, in accordance with CLSM results. Once the mitochondria were damaged, there was the influx of various regulatory proteins including cytochrome c (Cyt c) and caspase-3 proteins into the cytoplasm (Li et al., 2019) and the decrease of antiapoptotic protein Bcl-2 (Tian et al., 2019). To confirm this, the applications of western blotting (WB) techniques were executed to study the expression of these proteins. As shown in Figure 3C, the protein levels of caspase-3 and cytochrome c were elevated when U373 cells treated by CuS@CaCO3-PEG were at pH 6.5 with light irradiation, whereas the protein levels in other groups were reduced. Besides, only when U373 cells were treated by CuS@CaCO3-PEG in pH 6.5 + L group, the expressions of Bcl-2 protein were significantly down-regulated. Detailed quantitative results of WB were gathered in Figure S15. Because mitochondria are the energy house, the damage of mitochondrial function might directly affect the production of ATP (Zhen et al., 2019). As shown in Figure 3D, the ATP content of U373 cells in 6.5 + L group decreased significantly compared with that of other groups, indicating that Ca2+ overload in mitochondria would damage the energy supply of cancer cells. All of the results revealed that mitochondrial-mediated apoptotic pathway was activated, after the mitochondrial calcium ion was overloaded.
Multimodal Imaging of Tumor

The therapeutic capability in vivo of CuS@CaCO₃-PEG NPs was evaluated on HepG2 (liver hepatocellular cells) xenograft tumor mouse models, but not on the U373 tumor-bearing mouse models, since U373 cells grew too slowly, which made it challenging to establish tumor-bearing mouse models, whereas HepG2 tumor-bearing mouse models were easy to establish and also possessed high expression of TRPV1 pathway (Waning et al., 2007; Vriens et al., 2004; Bort et al., 2019). Also, liver cancer is one of the most common and lethal cancers in the human digestive system. Prior to in vivo therapy, the biodistribution of the nanomedicine was tracked by Cy7 via fluorescence imaging in vivo. As shown in Figure 4A, the fluorescence gradually appeared in the tumor site over time. After 4 h of injection, more fluorescence signal of Cy7 was observed in the tumor than at other time points, so in the following in vivo antitumor activity studies, the NIR laser irradiation time was set at 4 h after injection. At 24 h post injection of Cy7-labeled CuS@CaCO₃-PEG, mice were killed and their tumors and organs were exfoliated for ex vivo imaging. As shown in Figure 4, except for the liver, tumor had the most robust fluorescence, since liver is the main metabolic organ, leading to the nonspecific liver uptake. The biodistribution of Cy7-labeled CuS@CaCO₃-PEG was further evaluated compared with CuS in HepG2 tumor-bearing BALB/c mice. The 3D high-resolution PAI of the whole mice were recorded in a time-dependent method after intravenous injection (Figures 4D and 4E). In the free CuS group, after injection of CuS for 1 h, the PA signal was little in the tumor, whereas the partial signal was in the tumor and kidney at 4 h, indicating that CuS NPs were cleared by the body after injection for 4 h. After injection for 24 h, there were few fluorescence signals in the tumor. Meanwhile, in the CuS@CaCO₃-PEG group, after injection for 1 h, the PA signal of CuS@CaCO₃-PEG was stronger compared with...
that of free CuS, whereas an obvious PA signal occurred in the tumor site at 4 h, and still was at a high level in tumor at 24 h. Comparisons showed that CuS@CaCO3-PEG had better tumor accumulation and 3D diagnosis than small size CuS in the blood circulation, which would contribute to the subsequent Ca2+-mediated therapy guided by 3D PA diagnosis.

**Ca2+-Mediated Tumor Therapy In Vivo**

Subsequently, the in vivo Ca2+-interference therapy was conducted, as shown in Figure 5A. The HepG2 tumor-bearing female BALB/c nude mice were randomly divided into six groups for the different treatments: (1) PBS, (2) CuS + 1,064 nm light irradiation, (3) CaCO3-PEG + 1,064 nm light irradiation, (4) CuS@CaCO3-PEG, (5) CuS@CaCO3-PEG + 1,064 nm light irradiation, and (6) CuS@CaCO3-PEG 43°C. After 4 h injection, the irradiation was performed, and the laser irradiation time was controlled to be 10 min in an intermittent manner (30 s break after 30 s of irradiation) so that the temperature of tumor areas was controlled below 43°C by FLIR infrared camera (Figure 5B), which was favorable to open the TRPV1 channels while avoiding the burn of tumors by hyperthermia. As shown in Figure 5C, evident inhibition of tumors was observed in group 5 (CuS@CaCO3-PEG with light exposure) and group 6 (CuS@CaCO3-PEG 43°C). However, there was no therapeutic effect in other groups compared with the PBS group. During treatment, there was no change in body weight in the six groups (Figure S16A), suggesting the negligible side effect. After 10 days treatment, it was found that the harvested tumor displayed the lowest values in size and weight in group 5 and group 6 than those in the other groups (Figures 5D and S16B), suggesting that the nanoplastform had specific therapeutic effect in vivo. The blood circulation half-lives of CuS@CaCO3-PEG (3.0 h) could reach about 2-fold that of CuS@CaCO3 (1.6 h) and 4-fold that of the bare CuS (0.8 h) by ICP-MS.
in Figure S17, revealing that CuS@CaCO3-PEG exhibited enhanced blood circulation after PEGylation in vivo. To check whether a large amount of Ca$^{2+}$ accumulated in the tumors, organs and tumors of mice in those groups were collected, digested, and then examined by ICP-MS. As shown in Figure SE, it could be found that, after treatment by CuS@CaCO3-PEG with light irradiation, the Ca$^{2+}$ content in tumors significantly increased compared with that of the control groups, confirming abundant Ca$^{2+}$ production and
retention in tumors from CuS@CaCO3-PEG after light irradiation. Yet, Ca^{2+} in the control groups were mainly concentrated in spleen, lung, and kidney, since these are the main metabolic organs (Wu and Tang, 2018). The desired therapeutic effects were also confirmed by physiological pathological staining in Figure 5F. For H&E staining, a small portion of purple-blue (normal nucleus) and large amounts of deformed nuclei (karyopyknosis, separation, and fragmentation) in tumor tissues were observed in the CuS@CaCO3-PEG + Laser and CuS@CaCO3-PEG 43°C groups. From the TUNEL staining, representative apoptosis-positive cells were marked by green nuclei in the last two groups. Simultaneously, there were large numbers apoptotic cells in caspase-3 staining assay. On the contrary, there were no significant physiological morphology changes in the staining of tumor tissue sections in other groups as compared with results in PBS group. Altogether, the photothermally induced opening of TRPV1 channels led to the overload of Ca^{2+} in tumor, which effectively realized Ca^{2+}-interference therapy and damaging against tumor survival in vivo.

Neglectable systemic toxicity should also be taken into account when effective cancer treatment was carried out. So, the minimal systemic toxicity had also been demonstrated to analyze the physiological pathology of major organs by H&E staining. Compared with the results of PBS, there were no physiological morphology changes in heart, liver, spleen, lung, and kidney in Figure 6A, suggesting biosafety of the nanoplatform in vivo. In addition, potential toxicity had been studied through liver function markers glutamic pyruvate transaminase (ALT) and aspartate aminotransferase (AST) and kidney function markers blood urea nitrogen (BUN) and creatinine (CR) in serum in Figure 6B. These results suggested that there were no dysfunctions of liver and kidney in the treatment of CuS@CaCO3-PEG in vivo. Thus, it can be concluded that the Ca^{2+}-interference therapy induced by photothermal activation of a TRPV1 signaling pathway to specifically inhibit tumor had negligible biotoxicity, owing to its excellent biocompatibility and biodegradability. More importantly, there were no toxic drugs in the whole treatment, which may cause the worry of systemic toxicity.

**DISCUSSION**

We developed an intracellular Ca^{2+} cascade that can be induced by photothermal activation of a TRPV1 signaling pathway to specifically inhibit tumor without any worry of systemic toxicity. CuS@CaCO3-PEG could specifically construct Ca^{2+} cascades in the presence of the overexpress TRPV1 channels and NIR-II light...
irradiation at tumor sites. Subsequently, the overflowing Ca\(^{2+}\) caused disruption of mitochondrial Ca\(^{2+}\) homeostasis and dysfunction, leading to efficient tumor inhibition. In addition, released photothermal CuS NPs could be used as an enhanced photoacoustic (PA) imaging agent simultaneously to provide instant diagnostic functions. More importantly, the Ca\(^{2+}\)-interference therapy demonstrated here avoided the obstacles of cancer treatments, i.e., premature drug leakage, off-targeting, and poor biocompatibility, which lightens the availability of other metal ions in oncotherapy and opens a new door for further higher-quality clinical cancer treatment.

**Limitations of the Study**

Even though we performed a Ca\(^{2+}\) cascade by NIR-II photothermal switch for specific tumor therapy *in vitro* and *in vivo*, we did not study the threshold of Ca\(^{2+}\) concentration in Ca\(^{2+}\)-interference therapy. Single calcium overload showed obvious antitumor effect; whether the nanoplatform combined with other treatments possessing the desired effect of “1 + 1 > 2” needs further investigation.

**METHODS**

All methods can be found in the accompanying Transparent Methods supplemental file.

**SUPPLEMENTAL INFORMATION**

Supplemental Information can be found online at https://doi.org/10.1016/j.isci.2020.101049.

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**AUTHOR CONTRIBUTIONS**

Conceptualization, Z.M.; Methodology, Z.M., J.Z., and W.Z.; Investigation, Z.M., J.Z., W.Z., Y.Z., and L.G.; Writing – Original Draft, Z.M.; Writing – Review & Editing, Z.M., M.F.F. and H.H.; Supervision, H.H.; Funding Acquisition, M.F.F. and H.H.. All authors edited and agreed on the final version.

**DECLARATION OF INTERESTS**

The authors declare no competing interests.

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Supplemental Information

Intracellular Ca\textsuperscript{2+} Cascade Guided
by NIR-II Photothermal Switch
for Specific Tumor Therapy

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Figure S1: The SEM images of CuS@CaCO_3. Related to Figure 1.

Figure S2: Dynamic light scattering of CuS, CuS@CaCO_3 and CuS@CaCO_3-PEG in PBS and 10% FBS respectively. Related to Figure 1.

Figure S3: The $\zeta$ potential of CuS, CuS@CaCO_3 and CuS@CaCO_3-PEG. Data are represented as mean ± SD. Related to Figure 1.
**Figure S4:** TEM images of CuS@CaCO₃-PEG at pH 7.4, 6.5 and 5.0. Related to Figure 1.

**Figure S5:** Release profile of Cu²⁺ from CuS@CaCO₃-PEG at different pH (n = 3). Data are represented as mean ± SD. Related to Figure 1.

**Figure S6:** (a) Temperature variation of CuS@CaCO₃-PEG with different concentrations under the laser of 1064 nm (1.2 W cm⁻²) illumination. (b) Temperature variation of CuS@CaCO₃-PEG (0.5 mg/mL) with different power with prolonging of irradiation time. Related to Figure 1.
**Figure S7:** Heat Stabilization Experiment. The concentration of CuS@CaCO₃-PEG was 1.0 mg mL⁻¹ and the time between one heating and cooling cycle was 15 min. Related to Figure 1.

**Figure S8:** CLSM images and colocation ratio analysis of biodistribution of Ca²⁺ released in mitochondria after treatment with CuS@CaCO₃-PEG in U373 and HeLa cells after incubated in 43 °C incubator for 10 min, scale bar: 10 μm. Related to Figure 2.
**Figure S9:** Cell viability of CuS@CaCO₃-PEG incubated with U373 cells without light irradiation. Data are represented as mean ± SD. Related to Figure 2.

**Figure S10:** Cell viability of CuS@CaCO₃-PEG incubated with (a) COS7 cells and (b) HEK293 cells with light irradiation (1.2 W cm⁻², 10 min). Data are represented as mean ± SD. Related to Figure 2.

**Figure S11:** Detection and control of temperature under illumination. Related to Figure 2.
**Figure S12:** Cytotoxicity of CuS@CaCO₃-PEG against U373 and HeLa cells in 43 ℃ incubator for 10 min. **p < 0.01, compared with the indicated group. Data are represented as mean ± SD. Related to Figure 2.

**Figure S13:** Flow cytometry detection of apoptotic/necrosis by annexin V-FITC/PI staining: when (a) U373 cells and (b) HeLa cells were treated with CuS@CaCO₃-PEG under different pH in the presence or absence of light irradiation, respectively. Related to Figure 2.
**Figure S14:** Quantitative determination of intracellular (U373 and HeLa cells) TMRM by flow cytometry. “Control” indicated that pure cells (U373 or Hela cells) without any treatment were incubated with dye. Related to Figure 3.

**Figure S15:** Quantitative analysis of caspase-3, Cyt c and Bcl-2 protein expression, as the ratio of protein to GAPDH from Western Blot results. Related to Figure 3.

**Figure S16:** (a) The body weight changes during the treatment. (b) The tumor weight after the treatment. **p < 0.01, compared with the indicated group. Data are represented as mean ± SD. Related to Figure 5.
Figure S17: Pharmacokinetics of CuS, CuS@CaCO$_3$ and CuS@CaCO$_3$-PEG after intravenous injection. Data are represented as mean ± SD. Related to Figure 5.
**Transparent Methods**

**Materials.** Calcium chloride dihydrate (CaCl₂·2H₂O), ammonia bicarbonate (NH₄HCO₃), copper chloride dihydrate (CuCl₂·2H₂O), sodium sulfide (Na₂S·9H₂O) and cetyltrimethylammonium chloride (CTAC) were purchased from Sinopharm Chemical Reagent CO, Ltd., China. Cholesterol, lecithin, 1, 2-dioleoyl-sn-glycero-3-phosphate (sodium salt) (DOPA), lecithin, cholesterol and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-(methoxy (polyethylene glycol)-2000) DSPE-PEG2k was purchased from Sigma-Aldrich. Fluo-4 AM Ca²⁺ fluorescent probe, Mito-Red probe, tetramethylrhodamine methyl ester (TMRM), Cyanine7 (Cy7) and ATP testing kits were obtained from Beyotime Biotec Company (Shanghai, China). Trypsin, Dulbecco’s modified Eagle’s medium (DMEM), penicillin-streptomycin, and FBS were purchased from GIBCO Invitrogen Corp. All chemicals were used as received without further purification. The human HepG2 cell line and U373 cell line were obtained from KeyGEN Biotechnology (Nanjing, China). Sartorius ultrapure water (18.2 MΩ cm) was used throughout the experiments.

**Preparation of CuS Nanoparticles.** The synthesis of aqueous CuS nanoparticles was achieved through a typical reaction previously reported (Li et al., 2018). Briefly, 80 μL of CTAC (333.3 mg mL⁻¹) was added to 100 mL CuCl₂ water solution (0.215 mg mL⁻¹) and stirred for 30 min at room temperature. Then, 50 μL Na₂S aqueous solution (242 mg mL⁻¹) was added and stirred. 5 min later, the mixture was heated at 90 °C and
reacted for 15 min until the solution turned to dark green. Then, the mixture was cooled to room temperature by an ice bath. Finally, CuS nanoparticles were obtained by washed and centrifuged for three times through an ultra-high speeds centrifuge (50000 rmp, 30 min), and stored at 4 °C.

**Synthesis of CuS@CaCO₃ and CuS@CaCO₃-PEG.** CuS@CaCO₃ nanoparticles were prepared by a typical gas diffusion reaction (Dong et al., 2016). In detail, 800 µl of concentrated CuS NPs was added dropwise to 500 mL ethanol solution containing 250 mg CaCl₂ under ultrasound. Next, the glass bottle was covered by the foil and placed in a vacuum drying oven (40 °C) containing 5 g of dry NH₄HCO₃. After 24 h of reaction, CuS@CaCO₃ were obtained by centrifugation (8000 rmp, 10 min) and drying. PEGylation of CuS@CaCO₃ nanoparticles was manipulated by a two-step approach (Duan et al., 2019). First, 2 mL CuS@CaCO₃ ethanol solution (10 mg mL⁻¹) and 1 mL DOPA chloroform solution (2 mg mL⁻¹) were mixed under ultrasonication for 20 min. Then the mixture was centrifugally washed to remove the unbound DOPA and dispersed in 2 mL chloroform. Secondly, the obtain mixture was added with 8 mg of lecithin, 8 mg of cholesterol and 4 mg of DSPE-PEG₂K under vigorous stirring overnight. Finally, after chloroform was removed by evaporation, and the CuS@CaCO₃-PEG was obtained by washed and dispersed in water.
**Characterization.** Morphologies of CuS, CuS@CaCO3 and CuS@CaCO3-PEG were observed via Transmission electron microscopy (TEM) (JEM-2100 microscope). The dynamic light scattering (DLS) and zeta potential of these nanoparticles were conducted on a Nano-ZS-ZEN3600 (Malvern Instruments) at 25 °C. Energy-dispersive X-ray EDS mapping images (Cu, S, Ca, O and C) were analyzed by Four-Detector designed Super X Energy Dispersive Spectrometry. The UV–vis absorptions were measured using a UV-2450 UV-vis spectrophotometer. μg/mL

**Ca²⁺ and CuS Release of CuS@CaCO₃-PEG at Acidic pH.** The CuS@CaCO₃-PEG (1 mg) was respectively dissolved in 2 mL different buffer (pH 7.4, 6.5 and 5.0) and placed for 2 h. The morphology changes of CuS@CaCO₃-PEG at different pH were observed via TEM. The pH-sensitive disintegration was verified by detecting Ca²⁺ and CuS release in solution at different pH. Simply, CuS@CaCO₃-PEG (0.5 mg mL⁻¹) treated with different pH were gentle shaking at 37 °C, and then, at a preset time, the supernatants were collected by centrifugation, then digested by aqua regia and detected by inductively coupled plasma mass spectrometry (ICP-MS) to test the concentration of Ca²⁺ and Cu²⁺.

**Photothermal Effect of CuS@CaCO₃-PEG.** In order to explore the photothermal effect of CuS@CaCO₃-PEG under different pH, CuS@CaCO₃-PEG (0.5 mg mL⁻¹) placed in different pH buffer for 30 min were exposed to the laser of 1064 nm (1.2 W
cm$^2$) for 10 min and the temperature values were recorded every two min via the thermal imager. The concentration-dependent thermal effect was observed by setting different concentrations at pH 6.5: 0.25 mg mL$^{-1}$, 0.35 mg mL$^{-1}$ and 0.5 mg mL$^{-1}$ of CuS@CaCO$_3$-PEG. These solutions were irradiated by 1064 nm laser for 10 min (1.2 W cm$^{-2}$), and real-time temperature was recorded every minute through the thermal imager. Similarly, the photothermal stability of CuS@CaCO$_3$-PEG was measured by recording temperature values during four continuous heating and cooling cycles.

**Cell Culture.** U373 cells were cultured in RPMI-1640 medium with 10% FBS and HeLa cells and HepG2 cells were cultured in DMEM medium with 10% FBS in a humidified atmosphere with 5% CO$_2$ at 37 °C.

**Intracellular Calcium Analysis by Fluo-4.** For CLSM observation, U373 cells or HeLa cells were seeded in small dish and incubated for 1 day. Firstly, the cells were incubated with Fluo-4 AM Ca$^{2+}$ fluorescent probe and Mito-Red probe for 1 h in the dark at room temperature. Then the cells were washed twice and added with 1 mL different pH medium (pH 7.4 and 6.5) with CuS@CaCO$_3$-PEG (0.5 mg mL$^{-1}$). Subsequently, these cells were exposed to 1064 nm light irradiation (1.2 W cm$^{-2}$) or incubated in a 43 °C incubator for 10 min, while the temperature was monitored and controlled at about 43 °C, and real-time distribution of intracellular Ca$^{2+}$ were observed via CLSM (Fluo-4 AM, Ex: 488 nm, Em: 495-550 nm; Mito-Red, Ex: 561 nm, Em: 570-620 nm). For flow cytometry, U373 cells, HeLa cells or HepG2 cells were seeded
in 6-well plates and incubated for 1 day. Similarly, the cells were incubated with Ca\(^{2+}\) fluorescent probe for 1 h and collected after washing. Then, the cells were resuspended in 1 mL different pH culture medium (pH 7.4 and 6.5) containing CuS@CaCO\(_3\)-PEG (final concentration: 0.5 mg ml\(^{-1}\)). After irradiation for 10 min, the fluorescence intensities were quantified in real time via a flow cytometer.

**Cytotoxicity In Vitro.** For MTT assay, U373 cells and HeLa cells were seeded in 96-well plates respectively. 24 h later, CuS and CuS@CaCO\(_3\)-PEG (CuS: 10 mg mL\(^{-1}\), or Ca\(^{2+}\):160 mg mL\(^{-1}\)) in different pH medium (pH 7.4 and 6.5) were added. Then the cells were treated with 1064 nm laser irradiation for 10 min by 30 s interrupt and 30 s radiation cycle. To design a positive control, the cells in 96-well plates with different samples were heated to 43 °C and cultured in a humidified atmosphere with 5% CO\(_2\) at 43 °C for 10 min. Similarly, HEK293 nuclear COS7 cells were treated with different concentrations of CuS@CaCO\(_3\)-PEG (0, 150, 300, 450, 600, 750 mg/L), and then irradiated for 10 min intermittently, and then passed the test. Then, all the cells in the plate further incubated for 24 h. Following, MTT was added to each well. 4 h later, the suspension was replaced by 150 µL DMSO. Finally, cell viability was calculated by recording the absorption of MTT at 490 nm. This formula was shown as following: cell viability (%) = OD\(_{(\text{sample})}\)/OD\(_{(\text{control})}\) × 100%. OD\(_{(\text{sample})}\) was the absorption value of MTT when the cells were incubated with different samples, and OD\(_{(\text{control})}\) was the absorption value of MTT when the cells were incubated with medium only.
**Apoptosis Evaluation.** U373 cells or HeLa cells were seeded in 6-well plates and cultured for 24 h. Then the cells were collected and washed twice with PBS and the dishes were added with 1 mL different pH medium (pH 7.4 and 6.5) with CuS@CaCO$_3$-PEG (0.5 mg mL$^{-1}$). Then 1064 nm laser irradiation was performed for 10 min (30 s interrupt and 30 s radiation cycle) in illumination groups. After another 24 h cultivation, the apoptosis was assessed using Apoptosis Kit with Annexin V FITC and propidium iodide by flow cytometer.

**Mitochondrial Damage Determination.** Firstly, mitochondrial membrane potential was detected using TMRM staining via CLSM and flow cytometry. U373 cells and HeLa cells were seeded in small dish and incubated for 1 day. The culture medium at pH 7.4 or 6.5 containing CuS@CaCO$_3$-PEG (0.5 mg mL$^{-1}$) was added. Subsequently 1064 nm laser irradiation (1.2 W cm$^{-2}$) was executed for 10 min (30 s interrupt and 30 s radiation cycle). After incubation for another 24 h, TMRM (mitochondrial marker, final concentration: 800 nM) was added and the cells were stained for 30 min. Next, the cells were washed twice by PBS, and the fluorescence of TMRM was observed via CLSM (Ex: 514 nm, Em: 580-660 nm). For flow cytometry analysis, similarly, cells were collected, incubated with various samples, irradiated for 10 min and stained with TMRM for 30 min, and then fluorescence was detected by flow cytometry after washing. Secondly, WB testing of caspase-3, Cyt c and Bcl-2 was determined. Briefly, U373 cells or HeLa cells were seeded and incubated in 6-well plates for 1 day. Then
the cells were collected and washed twice with serum-free medium. Then the cells were
treated with CuS@CaCO₃-PEG (final concentration: 0.5 mg mL⁻¹) in different pH
medium (pH 7.4 and 6.5). The cells were irradiation at 1064 nm (1.2 W cm⁻²) for 10
min (30 s interrupt and 30 s radiation cycle). After that, the cells were washed with PBS
for three times and the protein was extracted through lysate, and finally detected by
SDS-PAGE. Finally, extracellular ATP levels were assessed. Cells were also treated
and collected according to the above procedures. Extracellular ATP level was tested by
the ATP assay kit according to the instructions.

**Mouse Experiment Model.** Our animal experiments were performed according to the
guidelines established by the Huazhong Agricultural University. The left flank of
BALB/c nude mice (6 weeks) were injected with 5 × 10⁶ HepG2 cells in 100 µL PBS.

**In Vivo Circulation and Biodistribution.** These mice were injected with CuS, CaCO₃-
PEG and CuS@CaCO₃-PEG (100 µL) *via* tail vein. At preset time (1 h, 3 h, 5 h, 8 h
and 24 h), the blood samples of mice were collected. Subsequently, the obtained blood
treated with concentrated nitric acid and H₂O₂ were digested for 30 min *via* microwave
digestion instrument. After a certain dilution, the contents of Cu²⁺ in the digestion
solution were detected *via* ICP-MS.

**In Vivo Fluorescence Imaging and PA Imaging.** Tumor-bearing mice were
intravenously injected with 200 µL Cy7 loaded CuS@CaCO₃-PEG nanoparticles. Then,
at preset time (2, 4, 6, 8 and 24 h), the mice were anesthetized and imaged by the Maestro small animal imaging system. 24 h later, the mice were sacrificed and the exfoliated tissues (liver, spleen, kidney, heart, lung and tumor) were imaged. PA imaging was performed on LOIS-3D, TomoWave Laboratories (USA). For in vitro PA imaging, the MOST images of CuS@CaCO₃-PEG aqueous solutions with different concentrations (0, 0.02, 0.04, 0.06, 0.08, 0.1 mg mL⁻¹) were recorded. For in vivo PA imaging, the tumor-bearing Balb/c nude mice were intravenously injected with 200 µL CuS or CuS@CaCO₃-PEG (CuS: 1.2 mg kg⁻¹). At preset time (1 h, 4 h and 24 h post-injection), the PA signals of the mice were carried out. The mice were anesthetized with 5% isofluorane, and fixed on the trestle. The mice were then brushed with a layer of glue to prevent bubbles from interfering with the photoacoustic coupling in the water. Rats receive 1064 nm laser scanning as they rotated and the photoacoustic signals were recorded in real time.

**In Vivo Antitumor Efficacy.** When the tumors reached 50 mm³ in size, the mice were randomly divided into six groups (4 mice in each group). Then the mice began to receive different formulation treatment at the same dosages based on CuS (1.2 mg kg⁻¹) or CaCO₃ (20 mg kg⁻¹) through tail vein on days 1, 3, 5 and 7 respectively. After 4 h of injection, the mice were exposed to 1064 nm laser (1.2 W cm⁻²) for 10 min, during which the temperature was controlled at 42-43 °C through the thermal camera. The volumes of tumor were calculated by measuring its diameters. The calculation formula
was as follows: \( V = a \times b^2 / 2 \) (a: the longest diameter of tumor, b: the shortest diameter of tumor). In addition, body weights of all mice were recorded. After treatment, the main organs (lung, liver, spleen, heart and kidneys) and tumor tissue from group 1, group 4, and group 5 were used for Ca\(^{2+}\) detection to verify abundant Ca\(^{2+}\) release from CuS@CaCO\(_3\)-PEG by ICP-MS.

**Systemic Toxicity In Vivo.** To evaluate the systemic toxicity, these mice were executed after therapy. Tissues (heart, liver, spleen, lung, kidney and tumor) were peeled and made into paraffin sections for H&E staining. Furthermore, the tumors were also assessed by TUNEL and caspase-3 staining. Meanwhile, liver and kidney function indices in blood samples were detected in Wuhan Hengyisai Biology.

**Supplemental References**

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