Cascade-Responsive Nanobomb with Domino Effect for Anti-Tumor Synergistic Therapies

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ABSTRACT

The development of reactive oxygen species (ROS) generation agents that can selectively produce sufficient ROS at the tumor site without external energy stimulation is of great significance for the further clinical application of ROS-based therapies. Herein, we designed a cascade-responsive ROS nanobomb (ZnO₂@Ce₆/CaP@CPPO/BSA, designated as Z@Ce₆/CaP@CB) with domino effect and without external stimulation for the specific generation of multiple powerful ROS storms at the tumor site. The CaP shell and ZnO₂ core gradually degrade and release Ca²⁺, Zn²⁺, and hydrogen peroxide (H₂O₂) under acid stimulation. On the one hand, Zn²⁺ can enhance the generation of endogenous superoxide anions (·O₂⁻) and H₂O₂ through the inhibition of the mitochondrial electron transport chain (ETC). On the other hand, the generation of large amounts of exogenous H₂O₂ can cause oxidative damage to tumor cells and further activate bis[2,4,5-trichloro-6-(pentyloxycarbonyl)phenyl] oxalate (CPPO)-mediated chemiexcited photodynamic therapy. In addition, the oxidative stress caused by the generated ROS can lead to the uncontrolled accumulation of Ca²⁺ in cells and further result in Ca²⁺ overload-induced cell death. Therefore, the introduction of Z@Ce₆/CaP@CB nanobombs triggered the "domino effect" that caused multiple heavy ROS storms and Ca²⁺ overload in tumors and effectively activated anti-tumor immune response.
INTRODUCTION

Reactive oxygen species (ROS) can act as signal carriers during the evolution of malignant tumors.\textsuperscript{1} At the appropriate concentration, ROS (such as hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}), superoxide anions (\textbullet{}O\textsubscript{2} \textsuperscript{-}), and hydroxyl radicals (\textbullet{}OH)) mediate signal transduction and cell growth.\textsuperscript{2} However, ROS are a double-edged sword, as excessive ROS can oxidize proteins, damage the DNA structure, and induce cell apoptosis.\textsuperscript{3–6} Moreover, ROS can induce inflammation at the tumor site, which further improves tumor immunogenicity.\textsuperscript{7} Therefore, increasing the content of ROS in tumor sites has become an effective method for cancer therapy. At present, the ways to generate ROS through external stimulations, such as photodynamic reaction (photodynamic therapy (PDT)),\textsuperscript{8,9} sonodynamic reaction (sonodynamic therapy (SDT)),\textsuperscript{10,11} and radiation sensitization (radiotherapy (RT)),\textsuperscript{12,13} are greatly limited by the penetration depth of the laser, irradiation range of external excitation, and safety concerns of the radiation.

In response to these problems, chemodynamic therapy (CDT) has been developed, which has received widespread attention.\textsuperscript{14–16} CDT uses excess H\textsubscript{2}O\textsubscript{2} in the tumor microenvironment (TME) without external energy stimulation to generate ROS through the Fenton reaction. However, the current therapeutic effect of CDT is not satisfactory, because the initiation of an efficient Fenton reaction requires harsh acidic conditions (pH 3–4) and excess H\textsubscript{2}O\textsubscript{2}.\textsuperscript{17} In addition to exogenous ROS production strategies, increasing the generation of endogenous ROS to inhibit tumor growth is another promising approach. It is well known that inhibiting the mitochondrial-electron transport chain (ETC) can enhance the generation of \textbullet{}O\textsubscript{2} \textsuperscript{-} and H\textsubscript{2}O\textsubscript{2}.\textsuperscript{18} A variety of ETC inhibitors have been reported to effectively kill tumor cells.\textsuperscript{19} However, treating cancer only by increasing endogenous ROS is unsatisfactory, as it is difficult to effectively inhibit tumor growth with a low amount of produced endogenous ROS. Therefore, developing strategies for the selective generation of sufficient ROS without
external energy stimulation under mild in vivo conditions remains a huge challenge in the field of cancer therapy.

Herein, we designed a cascade-responsive ROS generation device with domino effect and without external stimulation for the specific generation of multiple severe ROS storms at the tumor site. As shown in Scheme 1, zinc peroxide nanospheres (ZnO$_2$) were prepared, which were coated with a calcium phosphate (CaP) shell loaded with the photosensitizer chlorin e6 (Ce6) through an in situ template-assisted strategy (designated as Z@Ce6/CaP). Then, the surface of Z@Ce6/CaP (designated as Z@Ce6/CaP@CB) was further modified with bis[2,4,5-trichloro-6-(pentyloxy carbonyl)phenyl] oxalate (CPPO) and bovine serum albumin (BSA). Z@Ce6/CaP@CB gradually degraded and produced a variety of ROS through a cascade reaction under acid stimulation. The CaP shell on the surface will be degraded to release Ce6, Ca$^{2+}$, and CPPO. ZnO$_2$ can be further degraded to produce Zn$^{2+}$ and H$_2$O$_2$ under acid stimulation. On the one hand, Zn$^{2+}$ can enhance the generation of ·O$_2^-$ and H$_2$O$_2$ through the inhibition of the mitochondrial ETC, achieving a rapid increase in endogenous ROS. On the other hand, the large amount of H$_2$O$_2$ produced will quickly increase the ROS threshold at the tumor site, causing oxidative damage to tumor cells, but can also be used as another stimulus to activate CPPO-mediated chemiexcited PDT. The chemical energy generated by H$_2$O$_2$-triggered CPPO activation can further stimulate Ce6 to produce $^1$O$_2$ through chemiluminescence resonance energy transfer (CRET) without excitation by any external light source.$^{20,21}$ As the second messenger of intracellular signal transmission, Ca$^{2+}$ plays a vital role in the process of regulating various physiological functions. Under normal circumstances, cells have extremely strict regulatory mechanisms for Ca$^{2+}$. However, under oxidative stress, the ability of cells to regulate Ca$^{2+}$ decreases gradually, leading to intracellular calcium overload. Therefore, the oxidative stress caused by the generated ROS (H$_2$O$_2$, $^1$O$_2$, and ·O$_2^-$) can cause an uncontrolled accumulation of Ca$^{2+}$ in cells. Obstruction of
the accurate transmission of Ca signals will further induce cell death.$^{22,23}$ Therefore, the simple introduction of the prepared Z@Ce6/CaP@CB nanobomb into the tumor would cause a “domino effect”, which could trigger the production of multiple ROS storms and Ca$^{2+}$ overload, as well as effectively activate the systemic immune response while inhibiting the growth of primary tumors. Moreover, tumor metastasis can be effectively prevented by adjuvant treatment with anti-CTLA4 checkpoint blockers.

**Scheme 1.** (a) Synthesis process of Z@Ce6/CaP@CB. (b) Schematic illustration of the mechanism of Z@Ce6/CaP@CB-based synergistic therapy. Z@Ce6/CaP@CB nanobombs can trigger multiple ROS storms ($\text{H}_2\text{O}_2$, $\text{O}_2^-$, and $\cdot\text{O}_2^-$) and Ca$^{2+}$ overload-induced cell death through a cascade reaction without external energy activation and effectively activate the systemic immune response while inhibiting the growth of primary tumors.
RESULTS AND DISCUSSION

ZnO$_2$ nanospheres were prepared according to a previously reported method.$^{24}$ An aqueous solution of zinc acetate was mixed with H$_2$O$_2$ and immediately heated in a superheated plate at a temperature of 300 °C. Transmission electron microscope (TEM) images of ZnO$_2$ showed a spherical structure with a uniform size of about 80 nm (Fig. 1a and Fig. S1, Supporting Information). Then, ZnO$_2$ was coated with a CaP protective layer to form Z@CaP through an in situ template-assisted strategy.$^{25}$ We directly added Ce6 during the CaP coating process to achieve efficient loading and packaging of the photosensitizer (Z@Ce6/CaP). Scanning electron microscopy (SEM) and TEM images (Fig. 1b and Figs S2 and S3) showed a shell with a thickness of about 10 nm around ZnO$_2$ after coating with CaP. Elemental mapping images revealed that Zn, Ca, O, and P elements were homogenously distributed on the ZnO$_2$ surface (Fig. 1c), indicating the successful coating of CaP. These findings have been further confirmed by energy dispersive spectrometry (EDS) and X-ray photoelectron spectroscopy (XPS; Figs S4 and S5). In contrast to the absorption spectrum of Z@CaP, the absorption spectrum of Z@Ce6/CaP showed the characteristic absorption peaks of Ce6, indicating effective Ce6 loading during the coating process of CaP (Fig. S6). The N$_2$ adsorption–desorption isotherm and corresponding pore diameter distribution curve showed that the pores on the Z@Ce6/CaP surface had an aperture of 3.8 nm and were conducive to the further loading of CPPO molecules (Fig. S7). The characteristic peaks of CPPO in the absorption spectrum of Z@Ce6/CaP@CB proved the successful loading of CPPO (Fig. S6). Finally, the surface of the nanocomposites was further modified with BSA to improve their biocompatibility, and the successful modification with BSA was proved by Fourier transform infrared spectroscopy (FT-IR; Fig. S8). The modification with BSA also resulted in a good dispersibility of Z@Ce6/CaP@CB in water, phosphate-buffered saline (PBS), and RPMI 1640 medium with an average hydrodynamic diameter of 121 nm (Figs S9 and S10).
In order to simulate the degradation properties of Z@Ce6/CaP@CB in acidic conditions, we measured the release of Ca\(^{2+}\) and Zn\(^{2+}\) under neutral (pH 7.4) and acidic (pH 4.5 and 6.5) conditions (Fig. 1d, e). Z@Ce6/CaP@CB only exhibited a significant release of Ca\(^{2+}\) and Zn\(^{2+}\) in acidic environment, indicating effective degradation. The TEM image of Z@Ce6/CaP@CB after dispersion in acidic environment for 24 h showed that the nanostructure disappeared completely, which further proves the acidic degradation properties of Z@Ce6/CaP@CB (Fig. 1f). Then, we assessed the H\(_2\)O\(_2\) production ability of Z@CaP under acidic conditions. As shown in Fig. 1g, much more H\(_2\)O\(_2\) (nearly 17\%) was released from Z@CaP under acidic conditions than under neutral conditions after 2 h. All these results demonstrate that Z@Ce6/CaP@CB can be degraded under acidic conditions, resulting in the effective release of Ca\(^{2+}\), Zn\(^{2+}\), and H\(_2\)O\(_2\). Furthermore, the \(^1\)O\(_2\) generation ability of Z@Ce6/CaP@CB was measured through the ROS sensor agent 1,3-diphenylisobenzofuran (DPBF) in vitro. As shown in Fig. 1h, compared with the control and Z@Ce6/CaP groups, the Z@Ce6/CaP@CB group showed significant DPBF consumption, indicating that the presence of CPPO and H\(_2\)O\(_2\) enabled Ce6 to produce \(^1\)O\(_2\) without external energy stimulation. This is mainly because H\(_2\)O\(_2\) released by ZnO\(_2\) under acidic conditions reacted further with CPPO to produce chemical energy to trigger the production of \(^1\)O\(_2\) by Ce6 (Fig. S11).
Figure 1. TEM images of (a) ZnO and (b) Z@Ce6/CaP@CB. (c) HAADF-STEM image and elemental mapping of Zn, O, Ca, and P of Z@Ce6/CaP, (d) Ca$^{2+}$ and (e) Zn$^{2+}$ release in PBS solutions (pH = 4.5, 6.5, and 7.4) at different time points (1, 2, 3, 4, 5, 6, 12, and 24 h). (f) TEM images of Z@Ce6/CaP@CB in PBS solution (pH = 6.5) at 0 and 24 h. (g) H$_2$O$_2$ release in PBS solutions (pH = 6.5 and 7.4) at 2 h. (h) Depletion of DPBF due to CPPO-mediated chemiexcited PDT: (1) Control, (2) Z@Ce6/CaP, and (3) Z@Ce6/CaP@CB. ***p < 0.001 by Student’s two-tailed t test.

Inspired by the good ROS generation properties and on-demand Ca$^{2+}$, Zn$^{2+}$, and H$_2$O$_2$ release behaviors (Fig. 2a) of Z@Ce6/CaP@CB, we further evaluated its cytotoxicity and curative effect on mouse breast cancer cells (4T1) by the cell counting kit-8 (CCK-8) assay. Z@Ce6/CaP@CB did not show obvious cytotoxicity to normal cells (mouse fibroblast cells (L929)) after co-cultivation for 24 h (Fig. 2b), confirming the good biocompatibility of...
In contrast, Z@Ce6/CaP@CB showed significant killing ability to cancer cells after co-culture with 4T1 cells for 24 h (Fig. 2c). Compared with the Z@Ce6/CaP group, the Z@Ce6/CaP@CB group showed a more significant growth inhibitory effect on cancer cells due to the generation of \(^1\)O\(_2\) by the CPPO-mediated chemiexcited photodynamic reaction. The cancer cell killing mechanism of Z@Ce6/CaP@CB was further investigated by fluorescence staining experiments. The intracellular Ca\(^{2+}\) and Zn\(^{2+}\) contents after Z@Ce6/CaP@CB treatment were evaluated using cell-permeable fluorescent Ca\(^{2+}\) probes (Fluo-4 AM) and Zn\(^{2+}\) probes (zinquin ethyl ester), as shown in Fig. 2d, e.\(^{18,23}\) Compared with the control group, 4T1 cells showed significant enhancement of green and blue fluorescence after treatment with Z@Ce6/CaP@CB, indicating that Z@Ce6/CaP@CB was effectively endocytosed by 4T1 cells and further released Ca\(^{2+}\) and Zn\(^{2+}\). Furthermore, 2,7-dichlorofluorescin diacetate (DCFH-DA) fluorescence staining experiments showed strong green fluorescence of 4T1 cells after treatment with Z@CaP, indicating that ZnO\(_2\) effectively released H\(_2\)O\(_2\) in the cells (Fig. 2f). In addition, Zn\(^{2+}\) released by ZnO\(_2\) increased the generation of mitochondrial \(\cdot\)O\(_2\)\(^-\) and H\(_2\)O\(_2\) by inhibiting the ETC, resulting in a rapid increase in endogenous ROS. As shown in Fig. 2g, we used dihydroethidium (DHE, \(\cdot\)O\(_2\)\(^-\) probe) to verify the generation of endogenous \(\cdot\)O\(_2\)\(^-\) caused by Zn\(^{2+}\).\(^{26}\) Compared with the control group, 4T1 cells showed strong enhancement of red fluorescence after treatment with ZnO\(_2\), Z@CaP, and Z@CaP@CB, indicating that Zn\(^{2+}\) increased the content of endogenous ROS in the cells. Finally, the \(^1\)O\(_2\) generation capacity of Z@Ce6/CaP@CB was detected by singlet oxygen sensor green (SOSG, \(^1\)O\(_2\) probe).\(^{27}\) Compared with the control, ZnO\(_2\), and Z@Ce6/CaP groups, 4T1 cells showed strong enhancement of green fluorescence in the Z@Ce6/CaP@CB group, indicating that CPPO reacted with H\(_2\)O\(_2\) produced by the degradation of ZnO\(_2\) to form a high-energy intermediate and consequently excite Ce6 to generate \(^1\)O\(_2\) (Fig. 2h). Under the oxidative stress caused by the generated ROS (H\(_2\)O\(_2\), \(^1\)O\(_2\), and \(\cdot\)O\(_2\)\(^-\)), the ability of the cells to regulate Ca\(^{2+}\) declines gradually. Therefore, the large
amount of Ca\textsuperscript{2+} released by Z@Ce6/CaP@CB in cells under oxidative stress can further trigger Ca\textsuperscript{2+} overload-induced cell death.

**Figure 2.** (a) Schematic illustration of the mechanism of Z@Ce6/CaP@CB-based synergistic therapy in vitro. (b) CCK-8 assay of L929 cells after treatment with different concentrations of Z@Ce6/CaP@CB. (c) CCK-8 assay of 4T1 cells after treatment with different concentrations of Z@Ce6/CaP and Z@Ce6/CaP@CB in medium. Detection of intracellular (d) Ca\textsuperscript{2+} and (e) Zn\textsuperscript{2+} contents by Fluo-4-AM probe and zinquin ethyl ester probe, respectively. (f) Intracellular H\textsubscript{2}O\textsubscript{2} generation measured by DCFH-DA. (g) Intracellular \textperp{O}_2\textsuperscript{•} generation measured by the DHE probe: (1) Control, (2) ZnO\textsubscript{2}, (3) Z@CaP, and (4) Z@CaP@CB. (h) \textperp{O}_2 generation measured by the SOSG probe: (1) Control, (2) ZnO\textsubscript{2}, (3) Z@Ce6/CaP, and (4) Z@Ce6/CaP@CB. **p < 0.01 and ***p < 0.001 by Student’s two-tailed t test.
Since Z@Ce6/CaP@CB shows good anticancer ability at the cellular level, its therapeutic effect on solid breast cancer tumors was further investigated in vivo. Based on the 4T1 tumor model, we found that the solid tumors of mice were significantly inhibited after the intravenous injection of nanodrugs (Fig. S12). Then, the double tumor model was used to verify whether Z@Ce6/CaP@CB produces a significant anti-tumor immune response during the treatment of primary tumors. The tumor on the left side was used to evaluate the therapeutic effect of multiple ROS storms and Ca^{2+} overload, while the tumor on the right side was used to investigate the anti-tumor immunological effect triggered during the treatment (Fig. 3a). Tumor-bearing mice were randomly divided into four groups (n = 5): (1) Control group, (2) Z@Ce6/CaP group, (3) Z@Ce6/CaP@CB group, and (4) Z@Ce6/CaP@CB + anti-CTLA4 group. Compared with the control group, tumor-bearing mice treated with Z@Ce6/CaP or Z@Ce6/CaP@CB not only showed a good primary tumor growth inhibitory effect but also the growth of distant tumors without any treatment was significantly inhibited, indicating the activation of systemic anti-tumor immunity (Fig. 3b, c and Fig. S13). Although the activation of systemic anti-tumor immunity resulted in a good tumor growth inhibitory effect, the cytotoxic T-lymphocyte-associated protein 4 (CTLA4) immune checkpoint can prevent the activation and proliferation of T cells and seriously affect the effect of anti-tumor immunotherapy. Therefore, to further enhance the anti-tumor effect of Z@Ce6/CaP@CB, we investigated the therapeutic effect of the combination of anti-CTLA4 and Z@Ce6/CaP@CB on tumors in vivo. As shown in Fig. 3b, c, primary and distant tumors in mice were completely suppressed after the combined treatment with anti-CTLA4 and Z@Ce6/CaP@CB, indicating that anti-CTLA4 effectively blocked CTLA4 and hindered the activity of immunosuppressive regulatory T cells (Tregs). Hematoxylin and eosin (H&E) staining and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining assay further demonstrated that apoptosis of tumor cells was caused by the synergistic treatment with Z@Ce6/CaP@CB (Fig. 3d, e and Fig. S14).
addition, tumor-bearing mice also showed a significant survival rate after treatment with Z@Ce6/CaP@CB and anti-CTLA4 (Fig. 3f). Moreover, the weight of mice did not change significantly during the treatment period, indicating that the combined treatment with Z@Ce6/CaP@CB and anti-CTLA4 had no obvious side effects on the mice (Fig. S15). More importantly, Z@Ce6/CaP@CB was almost completely metabolized and cleared out of the mice 21 days after intravenous injection (Fig. S16), and it also did not show any obvious long-term toxic side effects on normal tissues (Fig. S17). The above results indicate that Z@Ce6/CaP@CB has good biocompatibility and application potential.

**Figure 3.** (a) Schematic illustration of the in vivo therapeutic mechanism. The tumor on the left side was used to evaluate the therapeutic effect of multiple ROS storms and Ca\(^{2+}\) overload, and the tumor on the right side was used to investigate the anti-tumor immunological effect activated during the treatment. Mice were injected intratumorally with Z@Ce6/CaP or Z@Ce6/CaP@CB on days 0, 2, and 4. Anti-CTLA4 was injected intravenously on days 1, 3, and 5. Volume curves of (b) primary tumor and (c) distant tumor: (1) Control, (2)
Z@Ce6/CaP, (3) Z@Ce6/CaP@CB, and (4) Z@Ce6/CaP@CB + anti-CTLA4. H&E staining images of (d) primary tumor and (e) distant tumor slides from different treatment groups. (f) Survival percentages of the mice. *p < 0.05 and **p < 0.01 by Student’s two-tailed t test.

In view of the significant distant tumor cure rate in the treated mice, the mechanism of the anti-tumor effect triggered by Z@Ce6/CaP@CB-based synergistic therapy in combination with anti-CTLA4 therapy was further studied. Dendritic cells (DCs) are the most powerful antigen-presenting cells (APCs), which can efficiently ingest, process, and present antigens. Mature DCs can effectively activate initial T cells, which is a key role in initiating, regulating, and activating the immune response.\textsuperscript{30,31} As shown in Fig. 4a, compared with the control group, the maturity of DCs was significantly improved after treatment with Z@Ce6/CaP@CB and anti-CTLA4. As the activation of T lymphocytes is the core of the immune response, we further evaluated the activation degree of T cells in different treatment groups. Compared with the control group, the content of cytotoxic T lymphocytes (CD8\textsuperscript{+} T cells) and helper T lymphocytes (CD4\textsuperscript{+} T cells) in Z@Ce6/CaP, Z@Ce6/CaP@CB, and Z@Ce6/CaP@CB + anti-CTLA4 groups was significantly increased, showing that CD8\textsuperscript{+} and CD4\textsuperscript{+} T cells were significantly activated under the synergistic treatment with Z@Ce6/CaP@CB (Fig. 4b, c and Fig. S18). At the same time, the significant enhancement of the content of CD8\textsuperscript{+} and CD4\textsuperscript{+} T cells in distant tumors after the treatment with Z@Ce6/CaP, Z@Ce6/CaP@CB, and Z@Ce6/CaP@CB + anti-CTLA4 was confirmed by immunofluorescence staining experiments (Fig. 4d). Perforin and Granzyme B (Gran B) are important natural immune effectors in the body, which are mainly released by CD8\textsuperscript{+} T cells after the contact with tumor cells.\textsuperscript{32,33} As shown in Fig. 4e, perforin and Gran B were highly expressed in the Z@Ce6/CaP, Z@Ce6/CaP@CB, and Z@Ce6/CaP@CB + anti-CTLA4 groups. In addition, the secretion of cytokines also plays an important role in anti-tumor immunity. Therefore, we further evaluated the secretion of pro-inflammatory cytokines (TNF-\textalpha, IL-6, and IL-12p70) in serum samples by ELISA. All treatment groups exhibited higher secretion levels of
pro-inflammatory factors than the control group (Fig. 4f–h), indicating that the combined effect of Z@Ce6/CaP@CB and anti-CTLA4 effectively activated the anti-tumor immunity.

**Figure 4.** (a) CD80 and CD86 contents in lymph nodes of mice quantitatively detected by flow cytometry (gated on CD11c+ DC cells) on day 6 after different treatments: (1) Control, (2) Z@Ce6/CaP, (3) Z@Ce6/CaP@CB, and (4) Z@Ce6/CaP@CB + anti-CTLA4. Content of b) CD4+ and c) CD8+ T cells in splenocytes of mice (gated on CD3+ T cells) on day 6 after different treatments. (d) Immunofluorescence staining of distant tumor tissues of CD8 and CD4 cells on day 6 after different treatments. (e) Perforin and Gran B immunofluorescence staining of the distant tumor on day 6 after different treatments. (f–h) Secretion levels of pro-inflammatory cytokines (TNF-α, IL-6, and IL-12p70) in sera on day 6 after different treatments. ***p < 0.001 by Student’s two-tailed t test.
We further verified the synergistic effect of Z@Ce6/CaP@CB and anti-CTLA4 adjuvant immunotherapy by evaluating tumor metastasis in the lungs (Fig. 5a). Compared with the control group, the number of metastatic nodules in the lungs of mice after treatment with Z@Ce6/CaP or Z@Ce6/CaP@CB was significantly reduced, and almost no cancer cell metastasis was found in the lungs due to the synergistic effect of Z@Ce6/CaP@CB and CTLA4 blocking adjuvant immunotherapy (Fig. 5b, c). H&E staining experiments showed that almost no tumor tissue was present in the lung tissue after treatment with Z@Ce6CaP@CB and CTLA4 (Fig. 5b). All the above results indicate that the synergistic treatment with Z@Ce6/CaP@CB and anti-CTLA4 adjuvant immunotherapy can effectively inhibit the metastasis of malignant tumors by activating the body's anti-tumor immune response.

![Figure 5](https://academic.oup.com/nsr/advance-article-doi/10.1093/nsr/nwab139/6346575)

**Figure 5.** (a) Scheme of the synergistic effect of Z@Ce6/CaP@CB and anti-CTLA4 adjuvant immunotherapy on the inhibition of lung metastasis. (b) Representative photographs of lung metastasis nodules and H&E-stained images of lung tissues in different treatment groups: (1) Control, (2) Z@Ce6/CaP, (3) Z@Ce6/CaP@CB, and (4) Z@Ce6/CaP@CB + anti-CTLA4. (c)
Calculated lung metastasis nodules of the mice after different treatments. **p < 0.01 and ***p < 0.001 by Student’s two-tailed t test.

CONCLUSION

In summary, we designed a cascaded ROS nanobomb (Z@Ce6/CaP@CB), which can effectively treat breast cancer tumors without external energy excitation. Z@Ce6/CaP@CB can effectively release Ca\(^{2+}\), Zn\(^{2+}\), and H\(_2\)O\(_2\) through gradual degradation in the specific acidic TME. Released Zn\(^{2+}\) could increase the generation of mitochondrial ·O\(_2^-\) and H\(_2\)O\(_2\) by inhibiting the ETC, achieving a rapid increase in endogenous ROS. At the same time, the release of large amounts of H\(_2\)O\(_2\) can cause oxidative damage to cancer cells and further activate CPPO-mediated chemiexcited PDT. In addition, the generated H\(_2\)O\(_2\), ·O\(_2^-\), and O\(_2\) can further trigger Ca\(^{2+}\) overload. Therefore, the introduction of Z@Ce6/CaP@CB nanobombs triggered a “domino effect” and effectively inhibited primary tumors through the synergistic effect of multiple ROS storm and Ca\(^{2+}\) overload but also activated the systemic antitumor immune response to effectively inhibit distant tumors and metastases.

METHODS

Preparation of ZnO\(_2\), Z@Ce6/CaP, and Z@Ce6/CaP@CB; cytotoxicity test; detection of intracellular Ca\(^{2+}\) and Zn\(^{2+}\) concentrations; intracellular H\(_2\)O\(_2\), ·O\(_2^-\), and \(^1\)O\(_2\) production; in vivo anti-tumor effect; lung metastasis model; ex vivo analysis of different groups of immune cells; Granzyme B and perforin detection; and cytokine detection are shown in the Supplementary Information.

SUPPLEMENTARY DATA

Supplementary data are available at NSR online.

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AUTHOR CONTRIBUTIONS

H. Z. proposed and supervised the project. H.Z. and Y.W. conceived and designed the experiments. Y.L., Y.W., and S.S. carried out the synthesis and experiments. Y.L., Y.W., and H.Z. co-wrote the manuscript. All authors discussed the results and participated in analyzing the experimental results.

Conflict of interest statement. None declared.

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