Catalytic Core-Shell Nanoparticles With Self-Supplied Calcium And H2O2 To Enable Combinational Tumor Inhibition

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Research Article

Keywords: Self-supplied H2O2, CaO2@Co-ferrocene, Calcium overload, ROS generation, Tumor inhibition

DOI: https://doi.org/10.21203/rs.3.rs-685150/v1

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Abstract

Nanoparticles, presenting catalytic activity to induce intracellular oxidative species, have been extensively explored for tumor treatment, but suffer daunting challenges in the limited intracellular H$_2$O$_2$ and thus suppressed therapeutic efficacy. Here in this study, a type of composite nanoparticles, consisting CaO$_2$ core and Co-ferrocene shell, is designed and synthesized for combinational tumor treatment. The findings indicate that CaO$_2$ core can be hydrolyzed to produce large amounts of H$_2$O$_2$ and calcium ions at the acidic tumor sites. Meanwhile, Co-ferrocene shell acts as an excellent Fenton catalyst, inducing considerable ROS generation following its reaction with H$_2$O$_2$. Excessive cellular oxidative stress triggers agitated calcium accumulation in addition to the calcium ions released from the particles. The combined effect of intracellular ROS and calcium overload causes significant tumor inhibition both in vitro and in vivo.

Introduction

Metal peroxides, including CuO$_2$[1], ZnO$_2$[2], MgO$_2$[3], CaO$_2$[4], and BaO$_2$[5], have been extensively explored for biomedical applications, including tumor therapy. For instance, under an acidic aqueous condition, CaO$_2$ can be hydrolyzed to produce Ca$^{2+}$ ions and H$_2$O$_2$. The excessive content of intracellular Ca$^{2+}$ ions causes cell death via the induction of calcium-overload stress. The recent studies indicate that the uncontrollable accumulation of Ca$^{2+}$ ions in tumor cells may interfere with cell signaling, cause cytotoxicity and trigger apoptosis[6]. In addition, the enrichment of intracellular calcium can also be triggered by the abnormal variation of reactive oxygen species (ROS)[7]. The main mechanism is that ROS regulates the entry and exclusion of calcium ions by affecting proteins of calcium channels or pumps, such as transient receptor potential (TRP) channels[8] and plasma membrane Ca$^{2+}$-ATPase (PMCA)[9]. However, when utilizing CaO$_2$ as a potential therapeutic platform for tumor treatment, the strong alkalinity of Ca(OH)$_2$, the byproduct of CaO$_2$ hydrolyzation, results in severe toxicity to normal cells and tissue. An effective strategy, to lessen its intrinsic toxicity while maintaining its expected antitumoral properties, is therefore highly demanded.

In general, H$_2$O$_2$ is overexpressed in tumor cells[10], inducing its increased intracellular oxidative stress. Meanwhile, H$_2$O$_2$ can be converted into highly toxic hydroxyl radicals ($\cdot$OH) via Fenton reaction triggered by catalytic metal ions, leading to cell apoptosis[11]. This phenomenon, also known as chemodynamic therapy (CDT), has emerged as a highly potential approach for tumor-specific therapy[11,14]. Even so, the level of intratumoral H$_2$O$_2$ ($\sim$ 50$\times$10$^{-6}$ to $\sim$ 100$\times$10$^{-6}$ M) remains inadequate to maintain the sustained production of ROS for effective tumor inhibition. A comment strategy is to induce exogenous H$_2$O$_2$, for example utilizing the glycolysis reactions of glucose oxidase (GOx)[12]. As a biocatalyst, GOx can catalyze the oxidation of glucose to supply H$_2$O$_2$. However, the catalytic reaction is considerably restrained by tumor hypoxia. Thus, CaO$_2$ may potentially serve as an alternative H$_2$O$_2$ sources, due to its strong capability in hydrolysis, to incorporate with CDT agents for promoted tumor inhibition[13].
At present, ferric ion remains as the most comment and efficient catalyst in triggering Fenton reactions, and it is extensively used as an ingredient for the investigation of CDT agents\[^{15}\]. Ferrocene is an organic transition metallic compound, which is nontoxic under the neutral physiological condition. Centered on Fe \((\text{II})\) with electron donor-acceptor conjugated structure, ferrocene, as a potential heterogeneous Fenton catalyst, represents an excellent redox reversible characteristic\[^{16}\]. In addition, ferrocene can bridge various metal ions through functional groups modification. It has been recently reported that nanoscale Co-ferrocene MOF possesses enhanced Fenton reactivity, which effectively promote the conversion from \(\text{H}_2\text{O}_2\) to •OH\[^{17}\].

Herein, a type of composite nanoparticles, presenting unique self-supplied \(\text{H}_2\text{O}_2\), calcium release and Fenton activity, is designed and synthesized for combinational tumor treatment with both tumor specificity and therapeutic efficacy (Fig. 1). In this system, \(\text{CaO}_2\) nanoparticles are covered and protected with a Co-ferrocene shell (denoted as \(\text{CaO}_2@\text{Co-Fc}\)). The findings indicate that \(\text{CaO}_2@\text{Co-Fc}\) may remain stable in the neutral aqueous condition, and hydrolyze to produce \(\text{H}_2\text{O}_2\) and release Ca ions due to the degradation of Co-Fc shell in the acidic TME. The \(\text{H}_2\text{O}_2\) reacts with Co-Fc molecules to generate considerable content of cytotoxic •OH. More interestingly, the induction of intracellular ROS further agitates the intracellular aggregation of \(\text{Ca}^{2+}\) ions, leading to calcium overload. The combined effects of strong CDT phenomenon mediated by self-supplied \(\text{H}_2\text{O}_2\) and calcium overload in cancer cells enable significant anticancer properties both \textit{in vitro} and \textit{in vivo}.

### Materials And Methods

**Chemicals and agents.** Calcium chloride anhydrous (\(\text{CaCl}_2, \text{AR}\)), hydrogen peroxide (\(\text{H}_2\text{O}_2, 30 \text{ wt}\%\)), ammonia solution (\(\text{NH}_3\cdot\text{H}_2\text{O}, 25 \text{ wt}\%\)), N, N-dimethylformamide (DMF, AR), and ethanol anhydrous were purchased from Sinopharm Chemical Reagent Co. Ltd. Cobalt (II) acetate tetrahydrate ((\(\text{CH}_3\text{COO})_2\text{Co} \cdot 4\text{H}_2\text{O}, 99.9\%\)) and 1,1′-Ferrocenedicarboxylic acid (\(\text{Fc(COOH)}_2, > 98\%\)) were purchased from Aladdin-Reagent Co. Ltd. Polyvinyl pyrrolidone (PVP, K30), Fluo-4 AM and Cell Count Kit-8 (CCK-8) were purchased from Meilunbio Co. Ltd. 3, 3′, 5, 5′-Tetramethylbenzidine (TMB), 5, 5-Dimethyl-1-pyrroline N-oxide (DMPO), 2′,7′-Dichlorofluorescin diacetate (DCFH-DA, \(\geq 97\%\)) were purchased from Sigma-Aldrich. Horseradish peroxidase (HRP) was purchased from Hefei Bomei Biotechnology. The ECL western blotting system was purchased from Beyotime Biotechnology. Primary and secondary antibodies were obtained from Abcam. Calcein AM and propidium iodide (PI) probes were obtained from Dojindo.

**Characterization.** Scanning electron microscopy (SEM) images was obtained by field-emission scanning electron microscopy (FESEM, Phenom Pharos). Transmission electron microscopy (TEM) images was obtained by transmission electron microscopy (TEM, HITACHI HT-7700; X-MAX\(^n\)65 T). The zeta potential and dynamic light scattering were measured by zetasizer (0.3-10000 nm, Zetasizer Nano-ZS Malvern). The X-ray diffraction pattern was obtained by X-ray diffractometer with Cu Ka radiation (XRD, X’pert PRO MPD). The chemical composition and valence states of different elements were measured by X-ray photoelectron spectroscopy (XPS, AXIS Supra). The UV-visible absorbance was measured by UV-vis
spectrophotometer (Shimadzu, UV-2600). Hydroxyl radical signal was monitored by electron spin resonance spectroscopy (EPR, Bruker A300). CCK-8 and intracellular H$_2$O$_2$ content were recorded using a microplate reader. The fluorescence images of Live and Dead, intracellular reactive oxygen species (ROS) and Ca$^{2+}$ ions were obtained by an inverted fluorescent microscope (Nexcope, The USA).

**Synthesis of CaO$_2$@Co-ferrocene.** CaO$_2$ nanoparticles were prepared according to previous procedures with the following protocol: CaCl$_2$ (0.1 g) and PVP (0.35 g) were dissolved in 15 mL ethanol. Subsequently, NH$_3$•H$_2$O (0.8 M, 1 mL) was added under continuously stirring. Then, H$_2$O$_2$ (1 M, 0.2 mL) was added dropwise to the mixture using a syringe pump. Finally, CaO$_2$ nanoparticles were collected by centrifugation at 12000 rpm for 10 minutes and washed three times with ethanol. The product was stored in 5 mL ethanol for further use. CaO$_2$@Co-ferrocene was prepared *via* in situ assembly. Typically, CaO$_2$ (5.3 mg) and PVP (160 mg) were dissolved into 18 mL DMF under ultrasound treatment. Afterwards, (CH$_3$COO)$_2$Co•4H$_2$O (0.1 M, 0.2 mL) and Fc(COOH)$_2$ (0.1 M, 0.2 mL) were added. After stirring at 80°C for 4 h, the product was obtained by centrifugation and washed several times with DMF and ethanol, respectively.

**H$_2$O$_2$ generation.** In brief, CaO$_2$@Co-ferrocene (0.5 mg, or CaO$_2$ containing the same amount of Ca$^{2+}$) was dissolved in 0.5 mL acetate buffer solution (ABS) at pH 5 or 7. Then, 20 µL supernatant was collected at the different time interval. HRP (1 U/mL, 300 µL) and phosphate buffer solution (pH = 5.8, 1.68 mL) were added. After 10 minutes' reaction, TMB (2 mM, 300 µL) was added and the characteristic absorbance of ox-TMB at 371 nm was recorded to quantify the H$_2$O$_2$ concentration according to the standard curve.

**Chemodynamic activity.** 3, 3', 5, 5'-Tetramethylbenzidine (TMB) can be oxidized to the oxidation state of TMB by •OH produced by CaO$_2$@Co-ferrocene under acidic condition, which shows a characteristic UV-vis absorbance at 655 nm. In brief, different concentrations of CaO$_2$@Co-ferrocene and TMB (8 mM, 300 µL) were added in ABS at different pH (5 and 7). The UV-vis absorption of various samples was recorded by UV-vis spectrophotometer. Subsequently, the reaction kinetic curve was monitored under a UV-vis spectrophotometer. The generation of •OH was further identified by electron spin resonance (ESR) spectroscopy with 5, 5-Dimethyl-1-pyrroline N-oxide (DMPO) as a spin trap. In addition, CaO$_2$@Co-ferrocene (1 mg, or CaO$_2$ with the same amount of Ca$^{2+}$) was dissolved in ABS (initial pH = 5, 2 mL). pH value was recorded with a real-time pH meter.

In vitro study. Human liver cell (7702), human umbilical vein endothelial cell (HUVEC) and murine breast cancer (4T1) cells were used for the in vitro study. The culture medium RPMI 1640 was supplemented with 10% fetal bovine serum (FBS) and the cells were cultured in an incubator with 5% CO$_2$ at 37°C.

**Cell viability assay.** 7702 and HUVEC cells were seeded in 96-well plates ($10^4$ cells per well) and incubated for 12 h. Subsequently, CaO$_2$@Co-ferrocene at desired concentrations were added and incubated for another 24 h. Finally, culture medium containing 10% CCK-8 was added to each well and the absorbance was detected by a microplate reader at 450 nm after incubation at 37 °C for another 1 h.
**Intracellular antitumor performance.** 4T1 cells were seeded in 96-well plates (10⁴ cells per well). After incubation for 12 h, the culture medium was replaced with fresh medium at different pH values. Then, CaO₂@Co-ferrocene at desired concentrations were added and incubated for another 24 h. Finally, culture medium containing 10% CCK-8 was added to each well and the absorbance was detected by a microplate reader at 450 nm after incubation at 37 °C for another 1 h.

In addition, the live & dead cells staining was carried out using Calcein AM/PI staining. After seeded in 6-well plate and cultured for 12 h, 4T1 cells were cultured with CaO₂@Co-ferrocene at desired concentrations for 10 h. After treatments, cells were cultured with Calcein AM and PI for 30 minutes. After staining, cells were washed with PBS for three times and further observed at 480 nm and 525 nm, respectively.

**Colony efficiency assay.** 4T1 cells were seeded in 6-well plates (200 cells per well). After incubation for 12 h, the cells were cultured in fresh medium containing different concentrations of CaO₂@Co-ferrocene for 10 days. Then, 4T1 cells were fixed in 10% formaldehyde after washed with PBS. Finally, 4T1 cells were stained by crystal violet for 20 minutes.

**Examination of intracellular calcium, ROS and H₂O₂.** ROS was detected by a fluorescent probe, 2, 7-dichlorofluorescein diacetate (DCFH-DA). 4T1 cells were seeded in 6-well plates. After incubation for 12 h, the cells were cultured in fresh medium containing different concentrations of CaO₂@Co-ferrocene for another 24 h. Then, the medium was discarded, and the cells were washed three times with PBS before the addition of DCFH-DA (2 mg/mL, 10 µL). After staining, cells were washed with PBS for three times and DCF fluorescence images were observed by an inverted fluorescent microscope at an excitation wavelength of 480 nm. To detect intracellular Ca^{2+} ions, Fluo-4 AM, was applied as a green fluorescent probe according to the above steps. After treatments with CaO₂@Co-ferrocene (60 µg/mL), 4T1 cells were lysed by RIPA lysis buffer with 1% PMSF. Supernatant containing H₂O₂ was collected by centrifugation at 12000 rpm. Then, hydrogen peroxide assay kit was added and the absorbance at 560 nm was recorded with microplate reader.

**Western blot assay.** After treatments with CaO₂@Co-ferrocene, 4T1 cells were lysed by RIPA lysis buffer with 1% PMSF. The collected cells were further crushed with an ultrasonic probe. Supernatant containing proteins was collected by centrifugation at 12000 rpm. After quantification, protein (50 µg) was loaded onto SDS-PAGE, further transferred to the PVDF membrane and blocked by 5% skim milk for 2 h. Then, the PVDF membrane was incubated with primary antibodies and secondary antibodies successively. After washing three times in Tris-buffered saline Tween buffer (TBST), the band intensity was measured.

**In vivo study.** Four-week-old female Balb/c mice were purchased from Shanghai Slac Laboratory Animal Co. Ltd. and used in compliance with guidelines of the Biological Resource Centre of the Agency for Science, Technology and Research, Zhejiang University. The tumor-bearing mouse model was built via injecting 50 µL PBS with 4T1 cells (1 × 10⁶) into the right side back of each mouse. The mice were randomly distributed into four groups for in vivo experiments (5 mice per group) when the tumor volumes
reached about 80 mm\(^3\) and intratumorally injected with different formulations: Group 1: PBS, Group 2: Co-Fc MOF, Group 3: 5 mg/kg CaO\(_2\)@Co-Fc, Group 4: 15 mg/kg CaO\(_2\)@Co-Fc. The tumor size (V) was calculated as follows: \(V = \text{width}^2 \times \text{length}/2\). Body weight, tumor size and images were recorded every 2 days. After 2 weeks, tumors were collected for Immunohistochemistry analysis after sacrifice.

The paraffin-embedded slices were deparaffinized in xylene and subsequently hydrated in serially diluted grades of ethanol. Endogenous peroxidase was blocked with 3% hydrogen peroxide, followed by antigen retrieval using a microwave oven under at pH = 6.0 (citrate buffer). Slices were incubated with ki67 overnight at 4\(^\circ\)C and then overlaid with secondary antibody for 20 minutes at room temperature. Finally, a diaminobenzidine tetrahydrochloride (DAB) working solution was applied and the slices were counterstained with hematoxylin.

**Statistical analysis.** Data were expressed as mean ± SD. Comparison analysis between groups was conducted by student’s t test.

**Results And Discussion**

**Synthesis of CaO\(_2\)@Co-Fc.** As illustrated in Fig. 2a, CaO\(_2\) was firstly obtained through a typical wet-chemistry approach following the approach reported previously \(^{[18]}\). Subsequently, Co\(^{2+}\) ions and ferrocene molecules were absorbed at the surface of CaO\(_2\) nanoparticles. Under the heating at 80\(^\circ\)C, the Co-Fc coating was formed. As shown in the scanning electron microscopy (SEM, Fig. 2b) and transmission electron microscopy (TEM, **Figure S1**) images, CaO\(_2\) particles formed are of spherical morphology with rough surface and uniform dimension of ~ 80 nm. After the formation with Co-Fc shell, the composite nanoparticles possess a gently increased size, and the surface becomes smoother (Fig. 2c, d). The analysis using X-ray photoelectron spectroscopy (XPS) reveals that the composite particles present the peaks of Co and Fe elements, confirming the existence of Co-Fc coordination compound, and the particle solution turns from white into yellow colour (Fig. 2e, S2). The high resolution XPS spectra verify that the main valence state of iron element is divalent, and partial oxygen exists as peroxo groups (**Figure S3**). As shown in Fig. 2f, hydrodynamic particle size increases after being coated by Co-Fc, which is consistence with the findings from SEM and TEM examinations. Due to the negatively charged Co-Fc compound (Co-Fc MOF), the surface potential decreases from +17.1 mV to +13.8 mV (Fig. 2g). The coating of Co-Fc compound on CaO\(_2\) nanoparticles is also verified by the characterization using energy dispersive spectrometer (EDS, **Figure S4**). Co and Fe elements are evenly distributed at the surface of CaO\(_2\) nanoparticles. The X-ray diffraction (XRD) pattern of CaO\(_2\)@Co-Fc corresponds to the characteristic peaks belonged to CaO\(_2\) (PDF#03-0865, **Figure S5**). There are no characteristic peaks of Co-Fc MOF observed, indicating that the Co-Fc shell is in an amorphous nature.

**Functional characteristics of CaO\(_2\)@Co-Fc.** During the characterization, 3,3',5,5'-tetramethylbenzidine (TMB) chromogenic method was used to evaluate the Fenton activity of as-prepared CaO\(_2\)@Co-Fc, due to the fact that TMB can be oxidized by •OH to give an aquamarine blue color with characteristic
absorbance at about 650 nm\cite{12c, 19}. As shown in Fig. 3a, the characteristic absorption peaks of ox-TMB are observed, accompanying with the distinct color change under an acidic condition. In addition, the rate of the chromogenic reaction accelerates with the increased concentration of CaO$_2$@Co-Fc, showing a typical concentration-dependent manner. In contrast, under neutral condition, no clear absorption can be detected at the same peak position (Fig. 3b), indicating that the Fenton reaction of CaO$_2$@Co-Fc occurs in a selective manner corresponding to the acidic condition. The generation of H$_2$O$_2$ from the particles was examined at different pH values, as shown in Fig. 3c and S6. No clear sign regarding the H$_2$O$_2$ generation from CaO$_2$@Co-Fc is observed under the neutral condition, and in contrast H$_2$O$_2$ can be generated rapidly when the pH of solution is varied to 5. It is noteworthy that the content of H$_2$O$_2$ generated increases at the early stage and decreases after reaching the peak magnitude. The potential reason can be that the dissociation of CaO$_2$@Co-Fc under the acidic condition induces agitated production of H$_2$O$_2$ initially, and that is consumed by Co-Fc molecules, leading to a drop in the H$_2$O$_2$ content in the solution. During the consumption of H$_2$O$_2$, the absorbance of TMB solution at ~ 655 nm is enhanced over time, indicating that the ROS could be produced sustainably (Fig. 3d). It is clear that the ROS production is significantly promoted with the increased concentration of CaO$_2$@Co-Fc. As indicated by the analysis using electron paramagnetic resonance (EPR), when CaO$_2$@Co-Fc is immersed in an acidic solution for a certain period, a typical four-fold peak is observed, confirming the ROS generated belongs to hydroxyl radicals (•OH), and this is attributed to the Fenton catalytic reaction by the H$_2$O$_2$ generated and Co-Fc molecules (Fig. 3e). Overall, the as-prepared CaO$_2$@Co-Fc can effectively trigger the cascaded hydrolysis and catalytic reactions. As depicted in Fig. 3f, once CaO$_2$@Co-Fc is exposed to the acidic condition, the Co-Fc shell is disintegrated, and the CaO$_2$ core is hydrolyzed to produce a considerable amount of H$_2$O$_2$. The generated H$_2$O$_2$ serves as the substrate for the following Fenton reaction triggered by Co-Fc molecules, inducing the highly active hydroxyl radicals.

**In vitro study.** Before evaluating its anti-tumor effect, its cytocompatibility of CaO$_2$@Co-Fc was investigated using two types of normal cell lines. As shown in Fig. 4a, CaO$_2$@Co-Fc shows negligible cytotoxicity toward human liver cells (7702) and human umbilical vein endothelial cells (HUVEC) at the concentration range of 0–80 µg/mL. In contrast, pure CaO$_2$ nanoparticles cause clear negative effect to the viability of 7702 normal cells (Figure S7). After being incubated with breast cancer cells (4T1) for 24 h, 20 µg/mL CaO$_2$@Co-Fc could cause a certain magnitude of inhibition effect, and this is enhanced with the increase of particle concentration (Fig. 4b). It is noteworthy that the killing effect of CaO$_2$@Co-Fc is significantly enhanced under the acidic condition. This is mainly attributed to that acidic condition does not only promote the degradation of nanoparticles and the H$_2$O$_2$ production\cite{20}, but also provides a favoring condition for the •OH induction. The live & dead assay verifies that CaO$_2$@Co-Fc exhibits clear killing effect to tumor cells in both concentration- and pH-dependent manner (Fig. 4c and S8). In addition, the inhibitory effect of CaO$_2$@Co-Fc on the proliferation of tumor cells was confirmed by colony formation assay (Figure S9).
To explore the inhibition mechanism of CaO$_2$@Co-Fc to tumor cells, a variety of intracellular indices were characterized. As shown in Fig. 4d, the H$_2$O$_2$ content presents higher magnitude in the tumor cells incubated with CaO$_2$@Co-Fc, indicating the effective H$_2$O$_2$-supply by the particles. Compared with the control group (PBS), H$_2$O$_2$ content is ~ 26% higher in cells treated with CaO$_2$@Co-Fc. In addition, the intracellular ROS was also be detected using 2,7-Dichlorodi-hydrouorescein diacetate (DCFH-DA, an ROS probe). The intracellular ROS fluorescence images show that the CaO$_2$@Co-Fc-treated cells present bright fluorescence (Fig. 4e and S10), indicating that the nanoparticles can be effectively uptaken and produce •OH in the tumor cells. The fluorescent intensity increases with the increase of the CaO$_2$@Co-Fc concentration. Notably, the acidic condition can significantly promote the intracellular ROS fluorescence, suggesting tremendous ROS induction at a lower pH value. The content of intracellular Ca$^{2+}$ ions was examined by calcium fluorescent probe (Fluo-4 AM). As shown in Fig. 4f and S11, strong green fluorescence could be observed in CaO$_2$@Co-Fc-treated cells compared with the control group, indicating that CaO$_2$@Co-Fc could be effectively disintegrated in 4T1 cells and generate significant Ca$^{2+}$ aggregation. Western blot assay was used to investigate the underlying interaction behind these phenomena. In general, the surges of ROS and Ca$^{2+}$ ions are intrinsically avoided by tumor cells to prevent apoptosis[21]. Redox regulation of calcium homeostasis can occur via oxidation of Ca$^{2+}$ channels or its regulators. For example, high levels of oxidative stress can inhibit cytoplasmic Ca$^{2+}$ extrusion through the downregulation of plasma membrane Ca$^{2+}$ ATPase [22] (PMCA, a plasma membrane Ca$^{2+}$efflux pumps). Meanwhile, transient receptor potential (TRP) channels can enhance Ca$^{2+}$ entry[8]. As shown in Fig. 4g, the expression of PMCA4 is reduced whereas that of TRPA1 is strengthened after being incubated with CaO$_2$@Co-Fc, indicating that nanoparticles induce considerable influence on the activity of Ca$^{2+}$ channel-related protein by inducing oxidative stress. The enrichment of intracellular Ca$^{2+}$ ions, calcium overload, could further activate apoptosis-associated pathway, which is confirmed by the alterations in the expression of BAX, Bcl-2 and Caspase-3.

Therefore, the intracellular phenomena induced by CaO$_2$@Co-Fc is clear, As demonstrated in Fig. 4h, after being exposed to acidic tumor environment, the nanoparticles disintegrate, and the internal CaO$_2$ is hydrolyzed to produce a large amount of H$_2$O$_2$. In the presence of Co-Fc, H$_2$O$_2$, both endogenous and exogenous, reacts with Fe ions of Co-Fc molecules to induce considerable production of toxic •OH, intensifying the cellular oxidative stress. The Co-Fc shell does not only act as Fenton catalyst, but also served as the protection to the CaO$_2$ core. In addition, high level of oxidative stress induces intracellular calcium accumulation by regulating calcium channels in the cell membrane, especially TRPA and PMCA4. The excessive ROS and calcium overload occurred within cells eventually activate the apoptosis pathway and lead to cell death[23].

In vivo anti-tumor properties. The anti-tumor efficacy of CaO$_2$@Co-Fc was examined using 4T1 tumor-bearing Balb/c mice. The mice were randomly divided into four groups (n = 5) with different treatments (Group 1: PBS; Group 2: Co-Fc MOF; Group 3: 5 mg/kg CaO$_2$@Co-Fc; Group 4: 15 mg/kg CaO$_2$@Co-Fc), and treated via intratumoral injection (Fig. 5a). There is no significant variation in body weight in the
following 14 days, suggesting the negligible side effects of the treatments to mice (Fig. 5b). According to the variation tendency of tumor volume between different groups of mice, CaO₂@Co-Fc nanoparticles has induced the most intense tumor inhibition (Fig. 5c). In contrast, Co-Fc MOF manifests the limited inhibitory effect on tumor growth, which is potentially attributed to its sole CDT effect. Compared with Co-Fc MOF, the integration of CaO₂ dramatically improves its tumor inhibitory effect, as expected. With the increase of injection dose, the antitumor effect of the CaO₂@Co-Fc nanoparticles is enhanced, suggesting a dose-dependent tumor inhibition manner. The tumor weight (Fig. 4d) and tumor photograph (Fig. 5e) on day-14 also verifies the tumor regression effect of CaO₂@Co-Fc nanoparticles. The corresponding H&E staining images of tumor slices visually show the tumor damage between different groups. As shown in Fig. 5f, the tumor tissues treated with CaO₂@Co-Fc nanoparticles possess typical apoptotic characteristics such as vacuolation, nuclear shrinkage and cell membrane rupture. Meanwhile, Ki67 stained images present similar results with the decreasing positive cells, confirming the proliferation inhibition of CaO₂@Co-Fc nanoparticles (Fig. 5g). The findings indicate that CaO₂@Co-Fc nanoparticles present cascaded Fenton activity and enable the excellent in vivo anti-tumor efficacy.

**Conclusions**

In this study, a type of nanoparticles with core-shell microstructure, presenting unique self-supplied H₂O₂, calcium release and Fenton activity, is designed and synthesized for combinational tumor treatment with both therapeutic specificity and efficacy. In this system, fine CaO₂ nanoparticles are covered and protected with a Co-ferrocene shell. The findings indicate that CaO₂@Co-Fc remains stable in the neutral aqueous condition, and hydrolyzes to produce H₂O₂ and release Ca ions due to the degradation of Co-Fc shell in the acidic TME. The H₂O₂ reacts with Co-Fc molecules to generate considerable content of cytotoxic •OH. More interestingly, despite the calcium ions released from the particles intracellularly, the induction of intracellular ROS further agitates the intracellular aggregation of Ca²⁺ ions, leading to considerable cellular calcium overload. The combined effects of strong •OH induction mediated by self-supplied H₂O₂ and calcium overload in cells enable significant tumor inhibition both in vitro and in vivo. Overall, this study appears to offer a therapeutic platform with alternative concept, featuring self-supplied H₂O₂ and calcium overload, for effective combinational tumor treatment.

**Declarations**

**Ethics approval and consent to participate**

All animal experiments were approved by the Animal Ethics Committee of Zhejiang University.

**Consent for publication**

All authors agreed to publish this manuscript.

**Availability of data and materials**
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

**Competing interests**

The authors declare that they have no competing interests

**Funding**

National Natural Science Foundation of China (51672247); Provincial Key research program of Zhejiang Province (2020C04005); ‘111’ Program funded by Education Ministry of China and State Bureau of Foreign Experts Affairs (B16043); Fundamental Research Funds for the Central Universities.

**Authors’ contributions**

CF and HJK developed methods, analyzed data, organized figures, wrote the manuscript, and performed the experiments. QC and YZ participated in the animal experiments. ZFH drew the scheme and processed the data. GRH and XL designed and supervise the study. All authors read and approved the final manuscript.

**Acknowledgements**

This work was financially supported by National Natural Science Foundation of China (51672247), Provincial Key research program of Zhejiang Province (2020C04005), ‘111’ Program funded by Education Ministry of China and State Bureau of Foreign Experts Affairs (B16043) and Fundamental Research Funds for the Central Universities.

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**Figures**

![Schematic illustration for the functioning mechanism of CaO2@Co-Fc.](image)

**Figure 1**

Schematic illustration for the functioning mechanism of CaO2@Co-Fc.
Figure 2

(a) Schematic illustration of the synthesis procedure of CaO2@Co-Fc. (b) SEM image of CaO2. (c) SEM image of CaO2@Co-Fc. (d) TEM image of CaO2@Co-Fc. (e) XPS survey spectra of CaO2@Co-Fc. (f) Size distribution of CaO2 and CaO2@Co-Fc. (g) Zeta potential of CaO2 and CaO2@Co-Fc.
Figure 3

UV-vis spectra of CaO2@Co-Fc with varied concentrations in TMB solutions of (a) pH 5 and (b) pH 7. (c) H2O2 release profile of CaO2@Co-Fc at different pH values. (d) Time-course absorbance at 655 nm with different concentrations of CaO2@Co-Fc in TMB solution. (e) EPR analysis of •OH production of CaO2@Co-Fc at different pH values. (f) Schematic illustration of the in vitro performance of CaO2@Co-Fc.
Figure 4

(a) The viability of 7702 and HUVEC cells incubated with CaO2@Co-Fc with varied concentrations. (b) Viability of 4T1 tumor cells treated with different concentrations of CaO2@Co-Fc. (c) Fluorescence images of calcein-AM and propidium iodide (PI) stained 4T1 cells treated with different concentrations of CaO2@Co-Fc. (d) Intracellular H2O2 content in 4T1 cells after being treated with CaO2@Co-Fc. Fluorescence images of (e) ROS level and (f) Ca2+ ions in 4T1 cells with different treatments. (g) The
expression of PMCA4, TRPA1, BAX, BCI-2 and caspace-3 in 4T1 cells after treated with CaO2@Co-Fc. (h) Schematic illustration for the intracellular functioning mechanism of CaO2@Co-Fc.

Figure 5

(a) Experimental procedures of injection treatment. (b) Body weight and (c) relative cancer volume variations of 4T1 tumor-bearing mice after different treatments within 14 days. (d) Weight and (e) representative photos of the tumors collected from different groups at 14th day. (f) H&E and (g) ki67
stained tumor slices collected from different groups at 14th day. (Group 1: PBS, Group 2: Co-Fc MOF, Group 3: 5 mg/kg CaO2@Co-Fc, Group 4)

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