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## H<sub>2</sub>O<sub>2</sub>-replenishable and GSH-deplete ROS 'bomb' for self-enhanced chemodynamic therapy†

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Chemodynamic therapy (CDT) is an emerging strategy of tumor therapy that utilizes the Fenton reagent to kill tumor cells by disproportionation of H<sub>2</sub>O<sub>2</sub> into hydroxyl radical (•OH). However, insufficient endogenous H<sub>2</sub>O<sub>2</sub> confines the antitumor efficacy of CDT. Additionally, the overexpressed glutathione (GSH) exhibits a potent scavenging effect on cytotoxic •OH, which further diminishes the efficacy of CDT. Though tremendous efforts have been done, engineering CDT agents with efficient and specific H<sub>2</sub>O<sub>2</sub> self-supplying and GSH-depletion is promising but remains a great challenge. Herein, Fe<sup>3+</sup>-chelated CaO<sub>2</sub> nanoparticles (CaO<sub>2</sub>-Fe NPs) are constructed as ROS 'bomb'. In the tumor microenvironment, CaO<sub>2</sub>-Fe NPs can release Fe<sup>2+</sup> by the reduction of GSH, and the remaining CaO<sub>2</sub> reacts with H<sup>+</sup> to selectively generate H<sub>2</sub>O<sub>2</sub>. The generated H<sub>2</sub>O<sub>2</sub> can produce •OH under the catalysis of Fe<sup>2+</sup> through the Fenton reaction, and re-oxidation from Fe<sup>2+</sup> to Fe<sup>3+</sup> endowing a long-lasting GSH-depletion, resulting in an improved CDT. These CaO<sub>2</sub>-Fe NPs supply H<sub>2</sub>O<sub>2</sub> and exhaust GSH simultaneously to achieve a self-enhanced CDT, and paves an emerging strategy to enhance the therapeutic efficacy of CDT by combining H<sub>2</sub>O<sub>2</sub>-replenishable and GSH-deplete together and realizing a self-enhanced Fenton reaction cycle.

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## Introduction

Reactive oxygen species (ROS) based tumor therapy is an emerging therapeutic strategy to effectively induce tumor-cell apoptosis.<sup>1–3</sup> Among various ROS, cytotoxic hydroxyl radical (•OH), which could be generated by H<sub>2</sub>O<sub>2</sub> *via* the Fenton reaction in an acidic environment is the most destructive and commonly applied type, which is defined as chemodynamic therapy (CDT).<sup>4–7</sup> Benefiting from the overproduction of H<sub>2</sub>O<sub>2</sub> in tumors (100 μM–1 mM) than normal tissues, and the mild acidity of the tumor microenvironment (TME), CDT is regarded

as a promising method for selective-tumor therapy with the help of catalysis by ferrous ions (Fe<sup>2+</sup>), manganese ions (Mn<sup>2+</sup>), or cuprous ions (Cu<sup>+</sup>).<sup>8–11</sup> However, some works recently suggested that the endogenous H<sub>2</sub>O<sub>2</sub> in the tumor site is still insufficient to support effective CDT, which restricts the clinical application.<sup>12–15</sup> Therefore, the introduction of the H<sub>2</sub>O<sub>2</sub>-replenishable agents should be taken into consideration.

A few signs of progress have been made to increase the intratumoral H<sub>2</sub>O<sub>2</sub> concentration by applying natural bio-enzymes such as glucose oxidase (GOx), nicotinamide adenine dinucleotide phosphate oxidase (NOX), and superoxide dismutase (SOD).<sup>16–18</sup> Nevertheless, these natural enzyme-based H<sub>2</sub>O<sub>2</sub> supplements suffer from some potential issues, such as instability of biological activity, high cost, and reliance on the exogenous H<sub>2</sub>O<sub>2</sub>-precursors such as glucose, superoxide anion (O<sub>2</sub><sup>•-</sup>) or O<sub>2</sub>.<sup>19,20</sup> Encouragingly, it has been reported that inexpensive calcium peroxide (CaO<sub>2</sub>) can steadily liberate a substantial amount of H<sub>2</sub>O<sub>2</sub> under acidic conditions due to the presence of peroxy bond (–O–O–), and the production of H<sub>2</sub>O<sub>2</sub> based on CaO<sub>2</sub> is independent of additional precursors.<sup>21–25</sup> Thus, based on the acidic environment of the tumor, CaO<sub>2</sub> is a practicable H<sub>2</sub>O<sub>2</sub>-replenishable 'bomb' for CDT to efficiently accumulate H<sub>2</sub>O<sub>2</sub> with tumor specificity.

Conceivably, if •OH generated is eliminated by ROS scavengers such as glutathione (GSH), it is worth nothing. For this reason, efficient ROS generation in the tumor runs into the

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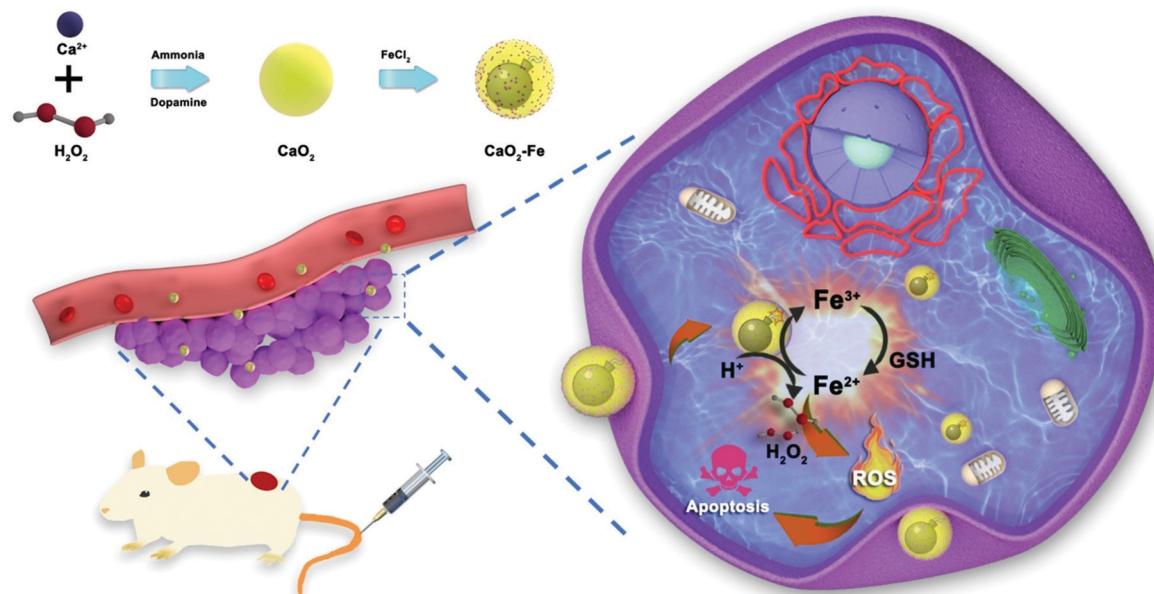


Fig. 1 The schematic illustration of  $\text{CaO}_2\text{-Fe}$  NPs as  $\text{H}_2\text{O}_2$ -replenishable and GSH-deplete ROS 'bomb' for self-enhanced CDT of the tumor.

bottleneck due to elevated GSH level intratumorally (up to 10 mM) compared with that in normal tissue.<sup>14,26-28</sup> This problem could be potentially solved by employing some transition metal ions with variable valences, such as ions of iron, copper, and manganese, as recyclable GSH consumers. The transformation from a high-valence ion to its low-valence form reduces the concentration of GSH, and the resulting low-valence ion is a good catalyst for the Fenton reaction.<sup>29-31</sup> Noteworthy that during the Fenton reaction, the low-valence ion is re-oxidized to its high-valence form, giving a sustained depletion of GSH and release of  $\cdot\text{OH}$  in GSH and  $\text{H}_2\text{O}_2$ -rich area.

Herein, a tumor-selective self-enhanced CDT 'bomb' is designed by using ferric ions ( $\text{Fe}^{3+}$ )-chelated  $\text{CaO}_2$  nanoparticles ( $\text{CaO}_2\text{-Fe}$  NPs). As elucidated in Fig. 1, under the tumor microenvironment,  $\text{CaO}_2$  NPs reacted with specific  $\text{H}^+$  to form  $\text{H}_2\text{O}_2$  *in situ* as ROS 'bomb', and the chelated  $\text{Fe}^{3+}$  is reduced to  $\text{Fe}^{2+}$  by high-leveled GSH as the trigger. Subsequently, the Fenton reaction is activated by the generated  $\text{H}_2\text{O}_2$  and  $\text{Fe}^{2+}$ . Finally,  $\cdot\text{OH}$  is produced for tumor therapy. Furthermore, the consumption of GSH enhances the CDT efficiency, and the re-oxidation of  $\text{Fe}^{2+}$  to  $\text{Fe}^{3+}$  endowing a long-lasting GSH-depletion. As a result,  $\text{CaO}_2\text{-Fe}$  NPs are able to selectively generate amounts of ROS to induce apoptosis of tumor cells with low systemic toxicity both *in vitro* and *in vivo*. These  $\text{CaO}_2\text{-Fe}$  NPs are good candidates for constructing ROS 'bomb' with endogenous replenishment of  $\text{H}_2\text{O}_2$  and depletion of GSH, providing a novel strategy for improving tumor-selective CDT.

## Results and discussion

To obtain  $\text{CaO}_2\text{-Fe}$  NPs,  $\text{CaO}_2$  NPs were first synthesized *via* a modified dopamine-assisted method, followed by mixing

ferrous chloride to load iron ions.<sup>21,32,33</sup> The chelated iron contents were adjusted by different feeding ratios between  $\text{CaO}_2$  and ferrous chloride (mass ratio). As shown in Table S1 (ESI†), contents of the chelated iron within  $\text{CaO}_2\text{-Fe}$  NPs were gradually improved with the increase of the feeding ratio. However,  $\text{CaO}_2\text{-Fe}$  NPs would not be formed when the feeding ratios were higher than 2:1. Thus, feeding ratios (4:1) were chosen to synthesize  $\text{CaO}_2\text{-Fe}$  NPs. The morphology of  $\text{CaO}_2\text{-Fe}$  NPs remained unchanged compared with the original  $\text{CaO}_2$  NPs, and their diameters increased from 91 nm to 122 nm (Fig. 2a and Fig. S1, S2, ESI†). Subsequently, as confirmed using X-ray diffraction (XRD), the introduction of iron ions could not influence the phase of  $\text{CaO}_2$  NPs (Fig. 2b). Strong and homogeneous iron signals were then observed from energy-dispersive X-ray spectroscopy (EDS) and EDS mapping, demonstrating the efficient binding of iron ions within  $\text{CaO}_2$  NPs (Fig. 2c and Fig. S3, ESI†). To explore the valence state of chelated iron ions, X-ray photoelectron spectrometry (XPS) was applied. The central peak at  $\sim 710.0$  eV ( $\text{Fe } 2\text{p}_{3/2}$ ) and the shakeup satellite peak at  $\sim 724.0$  eV ( $\text{Fe } 2\text{p}_{1/2}$ ) demonstrated that  $\text{Fe}^{2+}$  was transformed into  $\text{Fe}^{3+}$ , which might be oxidized by  $\text{CaO}_2$  (Fig. 2d and e). The photoelectron peak at 532.5 eV of O 1s could be assigned to O–O, indicating the presence of peroxy groups (Fig. 2f).<sup>24</sup>

Considering the significance of  $\text{H}_2\text{O}_2$  for CDT, the  $\text{H}_2\text{O}_2$  generation ability of  $\text{CaO}_2\text{-Fe}$  NPs was investigated using potassium permanganate ( $\text{KMnO}_4$ ) as the indicator. As shown in Fig. 3a, the color of permanganate ( $\text{MnO}_4^-$ ) disappeared after adding  $\text{CaO}_2\text{-Fe}$  NPs to the acidic solution, suggesting the reduction of  $\text{MnO}_4^-$  to colorless  $\text{Mn}^{2+}$  by the generated  $\text{H}_2\text{O}_2$ . The dissociation of  $\text{CaO}_2\text{-Fe}$  NPs in acidic solution further verified the acid-activated  $\text{H}_2\text{O}_2$  generation (Fig. S4, ESI†). In comparison, less  $\text{H}_2\text{O}_2$  was generated from  $\text{CaO}_2\text{-Fe}$  NPs in a



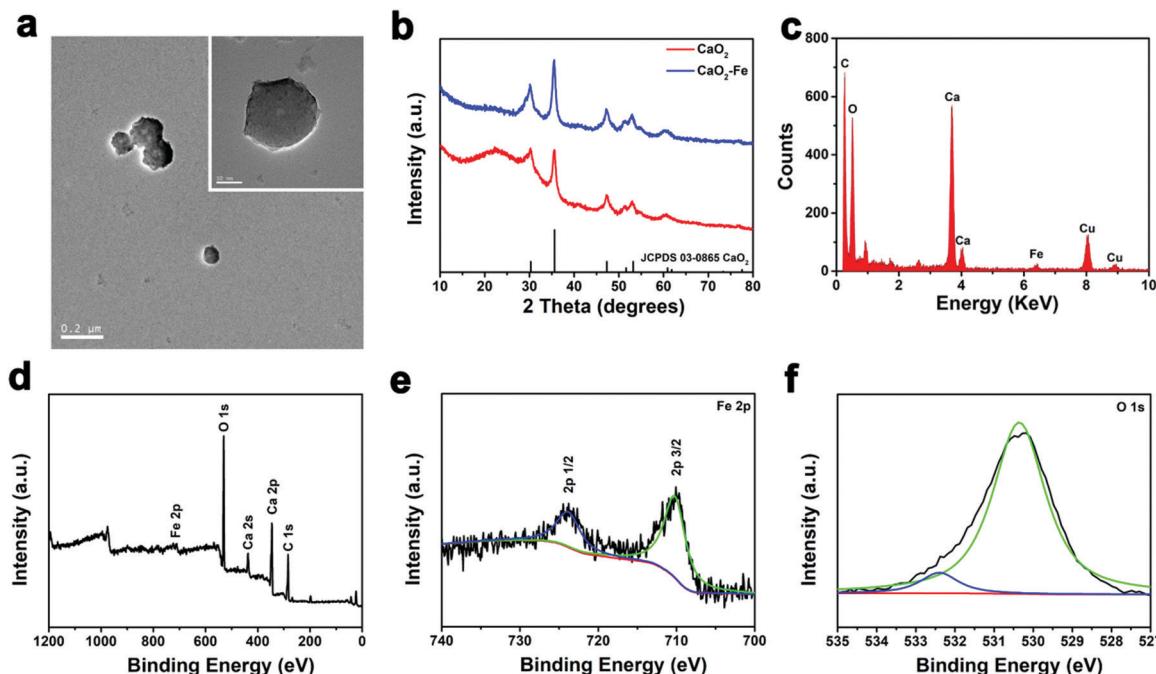


Fig. 2 (a) TEM image of  $\text{CaO}_2$ –Fe nanoparticles (inset: an image at a higher magnification of  $\text{CaO}_2$ –Fe NPs). (b) XRD pattern of  $\text{CaO}_2$  and  $\text{CaO}_2$ –Fe NPs. (c) EDS spectrum of  $\text{CaO}_2$ –Fe NPs. (d) Survey XPS spectra of  $\text{CaO}_2$ –Fe NPs. (e) High-resolution Fe 2p XPS spectra of  $\text{CaO}_2$ –Fe NPs. (f) High-resolution O 1s XPS spectra of  $\text{CaO}_2$ –Fe NPs.

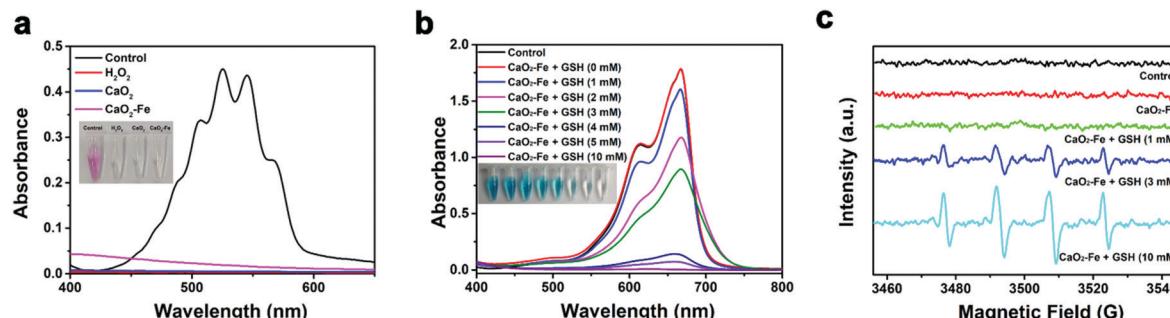


Fig. 3 (a) UV-Vis absorption spectra and photo (inset) of  $\text{KMnO}_4$  after treating with  $\text{H}_2\text{O}_2$ ,  $\text{CaO}_2$  NPs, and  $\text{CaO}_2$ –Fe NPs in an acidic environment. (b) UV-vis absorption spectra and photo (inset) of MB after degradation by  $\text{CaO}_2$ –Fe NPs treated with different amounts of GSH at pH 5.4. (c) ESR spectra of  $\text{CaO}_2$ –Fe NPs treated with different amounts of GSH at pH 5.4 (5,5-dimethyl-1-pyrroline N-oxide (DMPO) as the spin trap).

neutral environment (Fig. S5, ESI<sup>†</sup>).  $\text{CaO}_2$ –Fe NPs could maintain long-term stability in a neutral environment (Fig. S6, ESI<sup>†</sup>). It indicated that  $\text{CaO}_2$ –Fe NPs were good candidates for  $\text{H}_2\text{O}_2$  replenishment in the acidic environment. These  $\text{H}_2\text{O}_2$  suppliers could further release  $\cdot\text{OH}$  induced by the Fenton reaction.

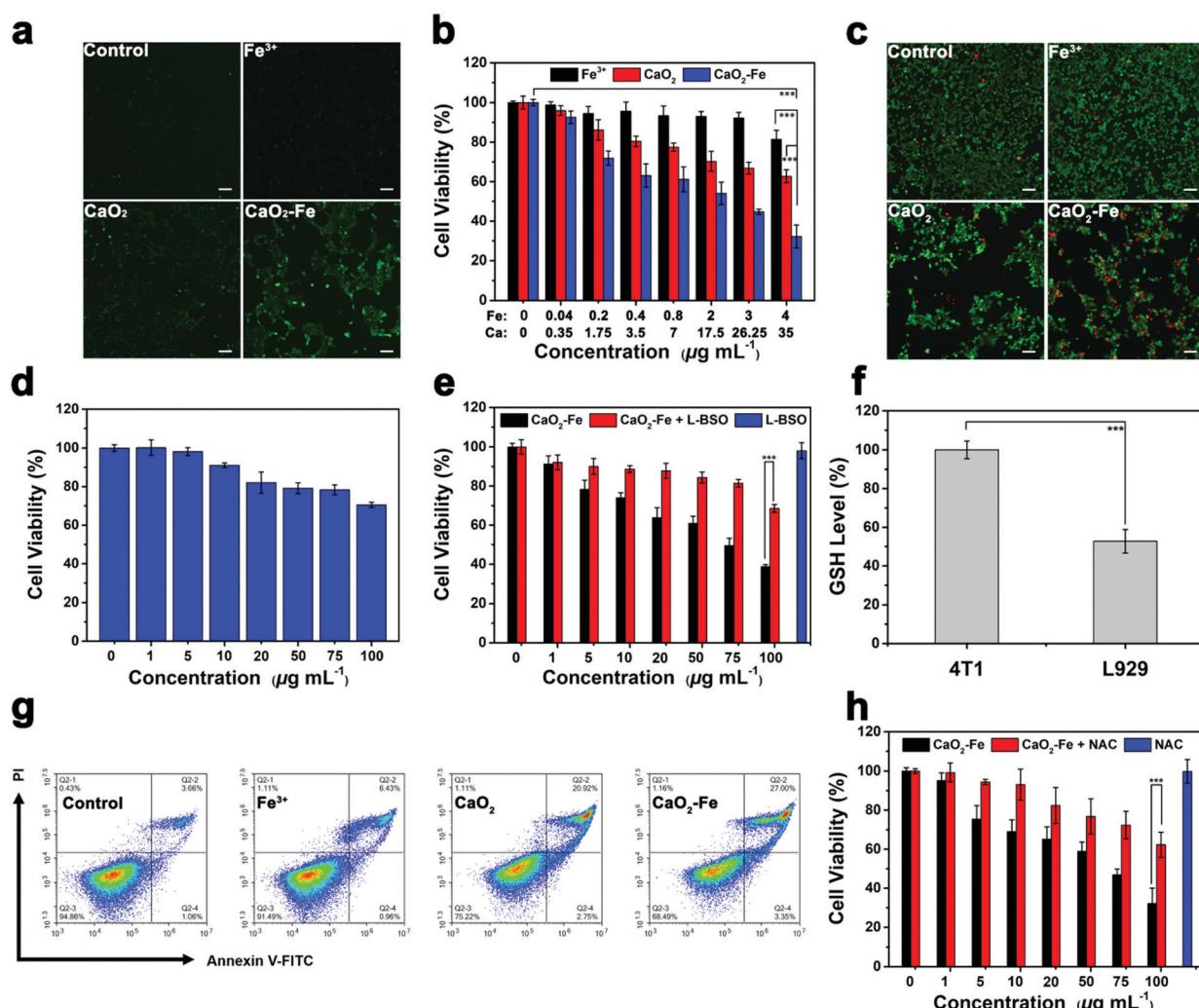
To evaluate the ROS triggered by  $\text{CaO}_2$ –Fe NPs, methylene blue (MB) was selected as the indicator. As can be seen in Fig. 3b and Fig. S7, S8 (ESI<sup>†</sup>), GSH is essential for ROS generation based on  $\text{CaO}_2$ –Fe NPs, due to the generation of Fenton-catalytic  $\text{Fe}^{2+}$  by the reduction of GSH. It is noteworthy that a high level of GSH was adverse for ROS generation in most reported cases due to the strong scavenging effect of GSH on ROS.<sup>14,26</sup> While  $\text{CaO}_2$ –Fe NPs exhibited an excellent ROS releasing capacity even when the concentration of GSH was at 10 mM,

with the MB degradation efficiency appeared to be 99%. This phenomenon could be ascribed to the continuous depletion of GSH under the Fenton reaction cycle based on  $\text{CaO}_2$ –Fe NPs (Fig. S9, ESI<sup>†</sup>). During the GSH depletion and Fenton reaction cycle,  $\text{Fe}^{3+}$  was indispensable. In comparison with bare  $\text{CaO}_2$  NPs without  $\text{Fe}^{3+}$  chelated,  $\text{CaO}_2$ –Fe NPs showed enhanced degradation of MB (Fig. S10, ESI<sup>†</sup>). Moreover, the ROS generation ability of  $\text{CaO}_2$ –Fe NPs was increased with the improvement of the chelated iron content (Fig. S11, ESI<sup>†</sup>). In addition,  $\text{CaO}_2$ –Fe NPs also showed a pH-dependent ROS due to the reliance on the generation of acidity of  $\text{H}_2\text{O}_2$ . These  $\text{CaO}_2$ –Fe NPs caused an apparent color degradation of MB under acidic conditions (pH 5.4) with the assistance of GSH, but no significant change was observed under neutral conditions

(pH 7.4) (Fig. S12, ESI†). The type of ROS produced by  $\text{CaO}_2$ –Fe NPs was further verified by the electron paramagnetic resonance (EPR) spin-trapping method. As shown in Fig. 3c, a characteristic 1:2:2:1 signal was obtained, indicating that the produced ROS by  $\text{CaO}_2$ –Fe NPs was  $\cdot\text{OH}$ . These results suggested that  $\text{CaO}_2$ –Fe NPs were promising candidates for pH/GSH dual stimuli-activated CDT agents by  $\text{H}_2\text{O}_2$  self-supplying and GSH-depletion.

Encouraged by the efficient production of  $\cdot\text{OH}$  via  $\text{CaO}_2$ –Fe NPs with the assistance of GSH and  $\text{H}^+$ , *in vitro*  $\cdot\text{OH}$  generation was investigated due to the higher intracellular GSH concentration and lower pH value in tumor cells. By employing 2,7-dichlorofluorescein diacetate (DCFH-DA) as the  $\cdot\text{OH}$  indicator, fluorescence imaging was carried out on 4T1 cells, which showed that the fluorescence signal of  $\text{CaO}_2$ –Fe NPs was

dosage-dependent (Fig. S13, ESI†). Compared with  $\text{CaO}_2$  NPs and  $\text{FeCl}_3$  at the same dosage,  $\text{CaO}_2$ –Fe NPs exhibited significantly stronger green fluorescence, indicating the self-enhanced  $\cdot\text{OH}$  was generated from  $\text{CaO}_2$ –Fe NPs in tumor cells (Fig. 4a and Fig. S14, ESI†). Considering the therapeutic effect of  $\cdot\text{OH}$ , cell viability was then investigated by standard methyl thiazolyl tetrazolium (MTT) assay. As shown in Fig. 4b,  $\text{CaO}_2$ –Fe NPs induced greater cell death by increasing concentrations, and the cytotoxic effect of the  $\text{CaO}_2$ –Fe NPs treated group was greater than that of  $\text{FeCl}_3$  and  $\text{CaO}_2$  NPs at the same concentration. Results from live/dead cell staining assay further confirmed these results, which revealed that only a small number of 4T1 cells remained viable after treatment with  $\text{CaO}_2$ –Fe NPs, while only a few cells were dead after treatment with  $\text{FeCl}_3$  and  $\text{CaO}_2$  NPs for 24 h (Fig. 4c and Fig. S15, ESI†).



**Fig. 4** (a) Fluorescence images of DCFH-DA stained 4T1 cells after exposure to  $\text{FeCl}_3$ ,  $\text{CaO}_2$  NPs, and  $\text{CaO}_2$ –Fe NPs for 4 h. The scale bar represents 100  $\mu\text{m}$ . (b) Viability of 4T1 cells after 24 h of incubation with  $\text{Fe}^{3+}$ ,  $\text{CaO}_2$  NPs, and  $\text{CaO}_2$ –Fe NPs. ( $n = 6$ , mean  $\pm$  s.d., \*\*\* $p < 0.001$ ) (c) Fluorescence images of Calcein AM (green, live cells) and PI (red, dead cells) costained 4T1 cells after incubation with  $\text{Fe}^{3+}$ ,  $\text{CaO}_2$  NPs, and  $\text{CaO}_2$ –Fe NPs for 24 h. The scale bar represents 100  $\mu\text{m}$ . (d) Viability of L929 cells after 24 h of incubation with  $\text{CaO}_2$ –Fe NPs. ( $n = 6$ , mean  $\pm$  s.d.) (e) Viability of 4T1 cells after 24 h of incubation with  $\text{CaO}_2$ –Fe NPs plus or without L-BSO. ( $n = 6$ , mean  $\pm$  s.d., \*\*\* $p < 0.001$ ) (f) Intracellular GSH levels of 4T1 cells and L929 cells. ( $n = 3$ , mean  $\pm$  s.d., \*\*\* $p < 0.001$ ) (g) Flow cytometry analysis of 4T1 cells treated with  $\text{Fe}^{3+}$ ,  $\text{CaO}_2$  NPs, and  $\text{CaO}_2$ –Fe NPs for 24 h. The figure shows four flow cytometry plots for Control,  $\text{Fe}^{3+}$ ,  $\text{CaO}_2$ , and  $\text{CaO}_2$ –Fe. The x-axis is Annexin V-FITC and the y-axis is PI. Quadrant percentages are: Q2-1 (0.43%), Q2-2 (3.66%), Q2-3 (94.86%), Q2-4 (1.06%) for Control; Q2-1 (1.11%), Q2-2 (6.43%), Q2-3 (91.49%), Q2-4 (2.75%) for  $\text{Fe}^{3+}$ ; Q2-1 (1.11%), Q2-2 (20.92%), Q2-3 (75.22%), Q2-4 (2.75%) for  $\text{CaO}_2$ ; Q2-1 (1.16%), Q2-2 (27.00%), Q2-3 (68.49%), Q2-4 (3.35%) for  $\text{CaO}_2$ –Fe. (h) Viability of 4T1 cells after 24 h of incubation with  $\text{CaO}_2$ –Fe NPs plus or without NAC. ( $n = 6$ , mean  $\pm$  s.d., \*\*\* $p < 0.001$ )



Interestingly, the cell inhibition effect from  $\text{CaO}_2\text{-Fe}$  NPs was tumor cell-selective toxicity, which presented relatively low cytotoxicity toward normal cells (Fig. 4d and Fig. S16, ESI<sup>†</sup>). This phenomenon could be ascribed to the reliance of  $\cdot\text{OH}$  generation on GSH concentration (Fig. 4e and Fig. S17, ESI<sup>†</sup>). After treating with  $\text{CaO}_2\text{-Fe}$  NPs, the intracellular GSH level was decreased, and the cell viability was reversed by down-regulating GSH by using L-buthionine sulfoximine (L-BSO) as the GSH inhibitor. In addition, GSH concentration within normal cells (L929) was much lower than cancerous cells (4T1) (Fig. 4f). As a result, reduced GSH could hardly trigger the generation of enough  $\cdot\text{OH}$  on normal cells (Fig. S18, ESI<sup>†</sup>), and finally suppressed the side effect.

Flow cytometry was further used to investigate the type of cell death using the annexin V-FITC/PI detection kit. As shown in Fig. 4g, cell death induced by all groups was apoptosis, and the  $\text{CaO}_2\text{-Fe}$  NPs treated group had higher ratios of apoptotic cells (27%) than other groups. As ROS-mediated cell killing is regarded as the major pathway for apoptosis, the influence of N-acetyl-cysteine (NAC), a kind of ROS scavenger, on cell viability was then investigated. With the addition of NAC, cell apoptosis induced by  $\text{CaO}_2\text{-Fe}$  NPs was obviously reversed,

indicating the cell inhibition was originating from the production of ROS in tumor cells (Fig. 4h). All these results suggested that  $\text{CaO}_2\text{-Fe}$  NPs was a GSH-enhanced CDT 'bomb' with self-supplied  $\text{H}_2\text{O}_2$  to induce tumor cell apoptosis efficiently and selectively by  $\cdot\text{OH}$ .

Tumor growth inhibition experiment was next performed by intravenous (*i.v.*) administration, inspired by outstanding treatment outcome of  $\text{CaO}_2\text{-Fe}$  NPs *in vitro*. *In vivo* biodistribution of  $\text{CaO}_2\text{-Fe}$  NPs was initially evaluated by labelling NIR dye (IR-783). As shown in Fig. S19 (ESI<sup>†</sup>), the  $\text{CaO}_2\text{-Fe}$  NPs could be accumulated in tumor tissue *via* the EPR effect, indicating that  $\text{CaO}_2\text{-Fe}$  NPs could serve as the ROS 'bomb' for self-enhanced chemodynamic therapy. The half-life of  $\text{CaO}_2\text{-Fe}$  NPs was  $1.17 \pm 0.45$  h (Fig. S20, ESI<sup>†</sup>). Mice treated with saline (as the control group),  $\text{FeCl}_3$ , and  $\text{CaO}_2$  NPs showed rapid tumor growth, while the size of tumors in  $\text{CaO}_2\text{-Fe}$  NPs injected mice was substantially inhibited (Fig. 5a and b). The therapeutic efficacy was also evidenced by hematoxylin and eosin (H&E) staining and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining, which revealed extensive tumor cell apoptosis after treatment with  $\text{CaO}_2\text{-Fe}$  NPs (Fig. 5c). The effective generation of toxic  $\cdot\text{OH}$  under TME

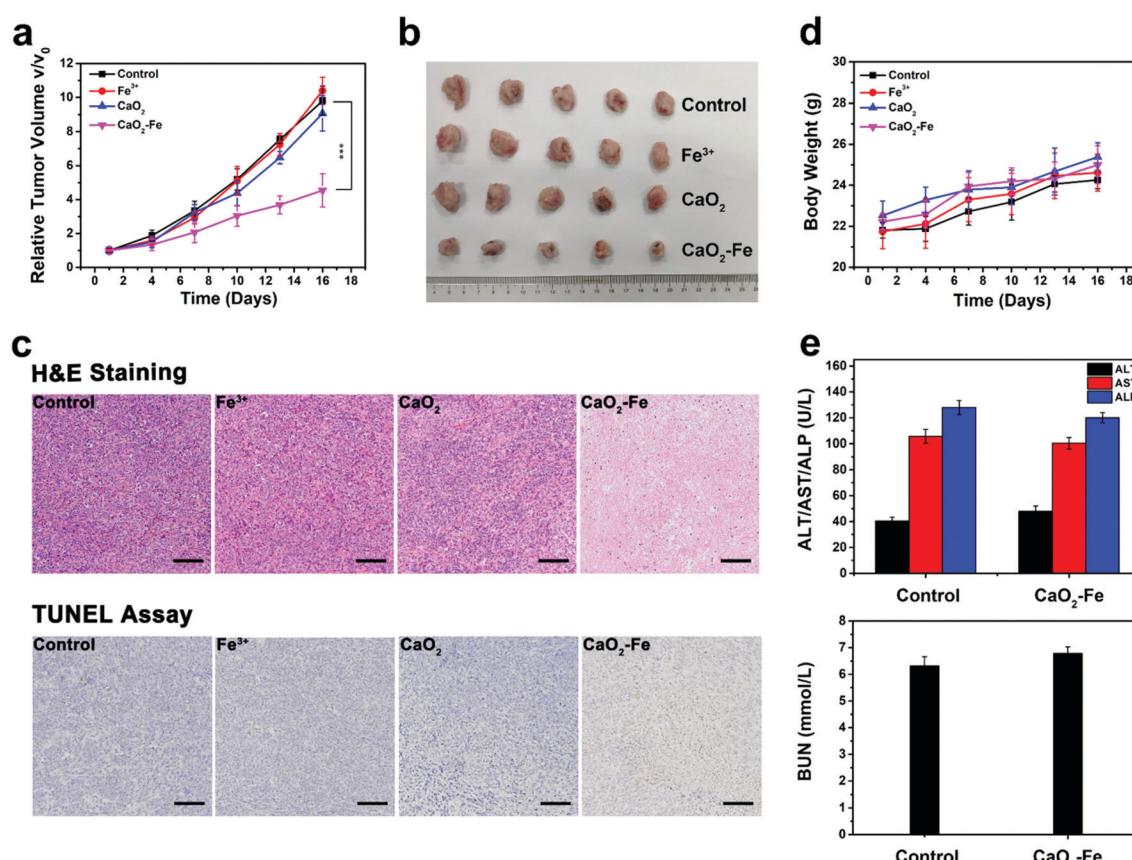


Fig. 5 (a) Relative tumor growth curves of 4T1 tumor-bearing mice after treatment with saline (control group),  $\text{FeCl}_3$ ,  $\text{CaO}_2$  NPs, and  $\text{CaO}_2\text{-Fe}$  NPs. ( $n = 5$ , mean  $\pm$  s.d., \*\*\* $p < 0.001$ ) (b) A representative photo of dissected tumors from the different groups on day 17 after administration. (c) Images of H&E and TUNEL stained sections of tumors from the different groups on day 17 after administration. The scale bar represents 100  $\mu\text{m}$ . (d) Time-dependent body-weight curves of mice in different groups. ( $n = 5$ , mean  $\pm$  s.d.) (e) Blood biochemistry analysis of healthy mice after intravenously injected with saline or  $\text{CaO}_2\text{-Fe}$  NPs for 17 days. ( $n = 3$ , mean  $\pm$  s.d.).



with acidic pH and overexpressed GSH from  $\text{CaO}_2$ -Fe NPs, leading to a remarkable tumor growth inhibition effect. Moreover, no apparent body weight changes were observed in mice injected with  $\text{CaO}_2$ -Fe NPs during the whole period (Fig. 5d), and there was no obvious histological alteration in the major organs (Fig. S21, ESI<sup>†</sup>). No obvious physiological damages were observed in  $\text{CaO}_2$ -Fe NPs treated group through blood biochemistry and hematology analysis (Fig. 5e and Fig. S22, ESI<sup>†</sup>). Therefore, it is feasible to use  $\text{CaO}_2$ -Fe NPs as a smart CDT agent for effective tumor therapy with low toxicity.

## Conclusions

In summary, an  $\text{H}_2\text{O}_2$ -replenishable and GSH-deplete ROS 'bomb' was successfully constructed for self-enhanced chemodynamic tumor therapy. After reaching tumor tissues, these ROS 'bomb',  $\text{CaO}_2$ -Fe NPs, could be triggered due to the generation of  $\text{Fe}^{2+}$  ions by GSH. Meanwhile, amounts of  $\text{H}_2\text{O}_2$  were generated by the reaction between  $\text{CaO}_2$  and  $\text{H}^+$ . Eventually, with the accumulation of  $\text{H}_2\text{O}_2$  as well as  $\text{Fe}^{2+}$  locally, a Fenton reaction cycle was achieved by continuously consuming GSH to output massive ROS, resulting in the improvement of the CDT efficacy by  $\text{H}_2\text{O}_2$ -supplementing and GSH-depletion. Both, *in vitro* and *in vivo* results demonstrated that  $\text{CaO}_2$ -Fe NP presented an inspiring antitumor performance as well as low systemic toxicity. Therefore, these  $\text{CaO}_2$ -Fe NPs could be regarded as a promising candidate for combining pH/GSH-responsive and GSH-depletion for CDT.

## Experimental section

### Materials

Calcium chloride ( $\text{CaCl}_2$ , 96%), ammonium hydroxide ( $\text{NH}_3 \cdot \text{H}_2\text{O}$ , 28%), dopamine (99%), ferrous chloride ( $\text{FeCl}_2$ , 98%), methylene blue (MB, 70%), *N*-acetyl cysteine (NAC), and glutathione (GSH, 99%) were obtained from J&K Scientific Ltd (Beijing, China). Ethanol ( $\text{C}_2\text{H}_5\text{OH}$ , 99.7%) and hydrogen peroxide ( $\text{H}_2\text{O}_2$ , 30%) were purchased from the Juhua Group Corporation.

### Characterization

X-Ray diffraction patterns (XRD) was recorded using the X'Pert PRO X-ray diffractometer with  $\text{Cu K}\alpha$  ( $\lambda = 1.54 \text{ \AA}$ ). Transmission electron microscopy (TEM) was performed using a FEI Tecnai G2 F30 microscope. X-Ray photoelectron spectroscopy (XPS) was performed using the Axis Ultra imaging photoelectron spectrometer (Kratos Analytical Ltd). Dynamic light scattering (DLS) measurements were conducted using the Zetasizer Nano ZS (Malvern). The concentrations of Fe and Ca were quantified using an inductively coupled plasma-atomic emission spectrometer (ICP-AES, NexION 350, PerkinElmer).

### Synthesis of $\text{CaO}_2$ NPs and $\text{CaO}_2$ -Fe NPs

$\text{CaCl}_2$  (0.1 g) and dopamine (0.003 g) were first dissolved in ethanol (15 mL) with the help of ultrasound. Subsequently,

$\text{NH}_4\text{OH}$  (1 mL) was added under magnetic stirring. Afterwards,  $\text{H}_2\text{O}_2$  solution (0.2 mL) was injected slowly. The product ( $\text{CaO}_2$  NPs) was finally collected by centrifugation (8000 rpm), washed with ethanol three times, and redispersed in ethanol.  $\text{CaO}_2$  NPs were then reacted with  $\text{FeCl}_2$  to form  $\text{CaO}_2$ -Fe NPs.

### Colorimetric assay of peroxy groups

An aqueous solution containing  $\text{KMnO}_4$  (50  $\mu\text{g mL}^{-1}$ ) and  $\text{HCl}$  (0.1 M) was first prepared. Subsequently, a certain amount of  $\text{CaO}_2$  NPs,  $\text{CaO}_2$ -Fe NPs, or  $\text{H}_2\text{O}_2$  was added into the mixture for 5 min. Finally, the mixture was measured by UV-vis spectra.

### Chemodynamic activity of $\text{CaO}_2$ -Fe NPs

The degradation of methylene blue (MB) was used for quantitative analysis of ROS production based on  $\text{CaO}_2$ -Fe NPs. In particular, the absorbance at  $\lambda = 644 \text{ nm}$  of MB solution (25  $\text{mg L}^{-1}$ ) in pH 7.4 or pH 5.4 with or without different concentrations of GSH (0, 1, 2, 3, 4, 5, and 10 mM) was measured before and after adding of 20  $\mu\text{g}$   $\text{CaO}_2$  NPs or  $\text{CaO}_2$ -Fe NPs for 3 hours.

To confirm the ROS type from  $\text{CaO}_2$ -Fe NPs, ESR spectroscopy was used. 5,5-Dimethyl-1-pyrroline N-oxide (DMPO) was utilized as the spin trap. DMPO solution (40  $\mu\text{L}$ , 100  $\text{mmol L}^{-1}$ ) was added into  $\text{CaO}_2$ -Fe NP solution at different concentrations of GSH (0, 1, 3, and 10 mM) at pH 5.4. Subsequently, the above mixture (20  $\mu\text{L}$ ) was injected into a capillary, the results were recorded using a Bruker A300.

### Cell culture

4T1 and L929 cell lines were obtained from Zhejiang Provincial People's Hospital. All biological reagents were purchased from Biological Industries. DMEM or 1640 with 10% FBS and 1% penicillin/streptomycin were treated as the cell culture medium. All cells were cultured in a cell incubator at 37 °C, 5%  $\text{CO}_2$  and 100% humidity.

### Cytotoxicity assays

MTT assay was tested to evaluate the *in vitro* cytotoxicity. First,  $5 \times 10^3$  per well 4T1 and L929 cells were seeded into 96-well plates and incubated overnight. Subsequently, various amounts of  $\text{FeCl}_3$ ,  $\text{CaO}_2$  NPs, and  $\text{CaO}_2$ -Fe NPs at the same Ca or Fe concentrations were added. After further incubation for 24 h, a fresh cell culture medium with 5% 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was used to replace the culture medium with nanoparticles. Finally, dimethyl sulfoxide (DMSO, 100  $\mu\text{L}$ ) was used to replace the MTT solution and co-incubation for 4 h. Cell viability was measured using a Tecan m200. Furthermore, after 4T1 cells were treated after the above conditions, Calcein-AM and propidium iodide (PI) live/dead cell staining was used to further verify the cytotoxicity of  $\text{CaO}_2$ -Fe NPs.

### Intracellular ROS levels detection

$5 \times 10^4$  per well 4T1 and L929 cells were plated into a 24-well plate and incubated overnight. Then, cells were incubated with  $\text{FeCl}_3$ ,  $\text{CaO}_2$  NPs, and  $\text{CaO}_2$ -Fe NPs at the same Ca or Fe



concentrations for 4 h. After washing with PBS, cells were stained with DCFH-DA (10  $\mu$ M) for 30 min. Later, PBS was used to remove the free DCFH-DA. Finally, the fluorescence images were obtained using a Nikon ECLIPSE Ti.

### Assessment of apoptosis

Annexin-V/PI assay kit (Sony) was used to determine the apoptosis of 4T1 cells treated with  $\text{CaO}_2$ -Fe NPs using flow cytometry. In particular,  $2 \times 10^5$  per well 4T1 cells were plated into a 6-well plate and incubated for 12 h. Afterwards, cells were treated with  $\text{FeCl}_3$ ,  $\text{CaO}_2$  NPs, and  $\text{CaO}_2$ -Fe NPs at the same Ca or Fe concentrations for a further 24 h. After washing with PBS, cells were detached by trypsin. Finally, apoptosis was detected using flow cytometry (ACEA NovoCyte) using PI vs. Annexin V plots.

### Animal model

All animal experiments were performed abiding by the guidelines of the Institutional Animal Care and Use Committee (IACUC) of Zhejiang Provincial People's Hospital, Hangzhou, China. 6-Week female Balb/c mice were provided by Shanghai Sippe-Bk Lab Animal Co., Ltd, Shanghai, China.  $1 \times 10^6$  4T1 cells solution (0.1 mL cells in PBS) was subcutaneously injected into the right axillary of all mice to construct the tumor model.

### Tumor inhibition and *in vivo* toxicity assay

After tumor volume reached  $100 \text{ mm}^3$ , 20 mice were randomly divided into 4 groups ( $n = 5$ ): mice were intravenously injected with saline (as the control group),  $\text{FeCl}_3$ ,  $\text{CaO}_2$  NPs, and  $\text{CaO}_2$ -Fe NPs at the same Ca or Fe concentrations. The whole experiment period was 15 days. All treatments were performed every three days and tumor volume and body weight were recorded 1-day after injection. Tumor volume was calculated using the formula as  $0.5 \times (\text{length} \times \text{width}^2)$ .

On the 17th day, all mice were executed. Subsequently, tumors and major organs (heart, liver, spleen, lung, and kidney) were removed and stored in formalin. After the section of the tumors and major organs, hematoxylin and eosin (H&E) staining and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining were then detected. A Nikon ECLIPSE Ni-U was used to observe the slides.

### Statistical analysis

The test data are shown as mean  $\pm$  s.d. The student's two-tailed *t*-test was used to calculate the statistical comparisons. \* means  $p < 0.05$ , \*\* means  $p < 0.01$ , \*\*\* means  $p < 0.001$ .  $p < 0.05$  was regarded as statistically significant.

## Author contributions

Fan Zhao: carried out all experiments and performed the statistical analysis, contributed to discussion, writing - original draft. Jiayu Yao: participated in animal studies and molecular biology experiments. Yu Tong: participated in animal studies and molecular biology experiments. Qing Xu: participated in

molecular biology experiments. Dan Su: participated in molecular biology experiments. Juan Li: supervision. Yao Ying: supervision. Wangchang Li: supervision. Liang Qiao: supervision. Jingwu Zheng: supervision. Wei Cai: supervision. Xiaozhou Mou: conceptualization, supervision, writing - reviewing and editing. Shenglei Che: conceptualization, supervision, writing - reviewing and editing. Jing Yu: carried out all experiments and performed the statistical analysis, conceptualization, writing - review & editing. Yanglong Hou: conceptualization, supervision, writing - reviewing and editing.

## Conflicts of interest

There are no conflicts to declare.

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