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Yupeng Xiao, Zhao Li, Alberto Bianco, Baojin Ma. Recent Advances in Calcium-Based Anticancer Nanomaterials Exploiting Calcium Overload to Trigger Cell Apoptosis. *Advanced Functional Materials*, 2023, 33 (3), pp.2209291. 10.1002/adfm.202209291 . hal-04076618

**HAL Id: hal-04076618**

**<https://hal.science/hal-04076618v1>**

Submitted on 18 Oct 2023

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## **Recent Advances in Calcium-based Anticancer Nanomaterials Exploiting Calcium Overload to Trigger Cell Apoptosis**

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**Keywords:** calcium phosphate nanoparticles, cell uptake, oxidative stress, endoplasmic reticulum, Ca<sup>2+</sup> channels

## **Abstract**

Calcium ion is vital for the regulation of many cellular functions and serves as a second messenger in the signal transduction pathways. Once the intracellular  $\text{Ca}^{2+}$  level exceeds the tolerance of cells (called  $\text{Ca}^{2+}$  overload), oxidative stress, mitochondrial damage and cell/mitochondria apoptosis happen. Therefore,  $\text{Ca}^{2+}$  overload has started to be deeply exploited as a new strategy for cancer therapy due to its high efficiency and satisfactory safety. This review aims to highlight the recent development of  $\text{Ca}^{2+}$ -based nanomaterials (such as  $\text{Ca}_3(\text{PO}_4)_2$ ,  $\text{CaCO}_3$ ,  $\text{CaO}_2$ ,  $\text{CaH}_2$ ,  $\text{CaS}$ , and others) able to trigger intracellular  $\text{Ca}^{2+}$  overload and apoptosis in cancer therapy. The intracellular mechanisms of varied  $\text{Ca}^{2+}$ -based nanomaterials and the different types of strategies to enhance  $\text{Ca}^{2+}$  overload are discussed in detail. Moreover, the design of more efficient  $\text{Ca}^{2+}$  overload-mediated cancer therapies is prospected mainly based on (1) the enhanced cellular uptake by surface modification and morphology optimization of nanomaterials, (2) the accelerated  $\text{Ca}^{2+}$  release from nanomaterials by increasing the intracellular  $\text{H}^+$  level and by photothermal effect, and (3) the overload maintenance by  $\text{Ca}^{2+}$  efflux inhibition,  $\text{Ca}^{2+}$  influx promotion, or promoting  $\text{Ca}^{2+}$  release from the endoplasmic reticulum.

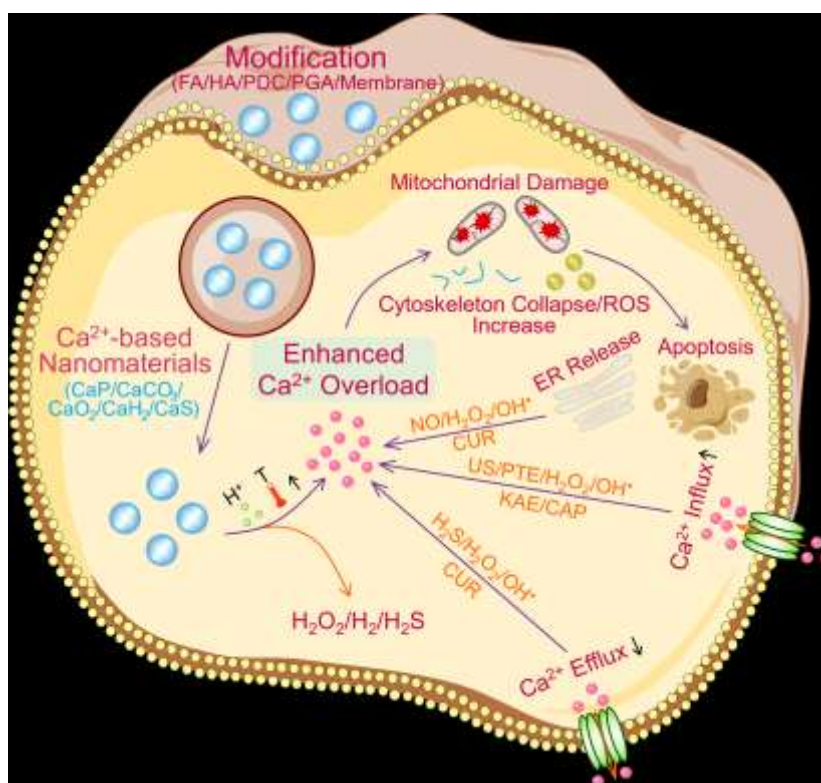
## 1. Introduction

Intracellular  $\text{Ca}^{2+}$  plays a pivotal role in many physiological processes, including proliferation, differentiation, apoptosis, the transmission of neural excitation, and contraction of the muscles.<sup>[1]</sup> In non-muscle cells (e.g., endothelial cells, fibroblasts, pericytes, and macrophages),  $\text{Ca}^{2+}$  is mainly stored in the endoplasmic reticulum (ER).<sup>[2-4]</sup> Owing to the efficient storage, intracellular  $\text{Ca}^{2+}$  can serve as a second messenger in the signal transduction pathways including  $\text{IP}_3$  (inositol triphosphate)-triggered  $\text{Ca}^{2+}$  release for regulation of the activity of numerous downstream targets (e.g., protein kinase C).<sup>[5-7]</sup>  $\text{Ca}^{2+}$  signaling generated by ER can be regulated by the mitochondria, and the cytoplasmic  $\text{Ca}^{2+}$  released from ER can be rapidly restored into ER with the assistance of mitochondria.<sup>[8-10]</sup> These processes constitute the intracellular  $\text{Ca}^{2+}$  signal networks.<sup>[11-13]</sup> Meanwhile, the contraction and relaxation behaviors of the muscles are controlled by  $\text{Ca}^{2+}$  through three major mechanisms, including the troponin-tropomyosin system, the calmodulin (CaM) system, and the direct binding of  $\text{Ca}^{2+}$  to myosin.<sup>[1,14,15]</sup>  $\text{Ca}^{2+}$  is also indispensable in the generation of electrical signals and signal transmission between neurons on nerve fibers.<sup>[16,17]</sup> Therefore,  $\text{Ca}^{2+}$  is vital for the regulation of cellular behavior and fate.

However, the dysfunction of calcium homeostasis would cause severe cell stress and sometimes cell death. Indeed, treating cells with apoptotic stimuli, like  $\text{C}_2$ -ceramide, can induce  $\text{Ca}^{2+}$  release from ER, increasing the cytoplasmic  $\text{Ca}^{2+}$  level and changing the morphological characteristics of mitochondria.<sup>[18-20]</sup> Once the intracellular  $\text{Ca}^{2+}$  level exceeds the tolerance of cells (identified as  $\text{Ca}^{2+}$  overload), particularly combined with pathological conditions of oxidative stress, cell apoptosis is occurring.<sup>[21,22]</sup> For example, it has been demonstrated that the increase of intracellular  $\text{Ca}^{2+}$  is required for the activation of mitochondrial calpain to release apoptosis-inducing factor (AIF) to trigger cell death.<sup>[23-25]</sup> AIF can translocate into the nucleus, and participate in chromatin condensation and large-scale DNA fragmentation, causing cell apoptosis. The sustained  $\text{Ca}^{2+}$  overload also could cause the opening of cyclophilin D-dependent mitochondrial membrane pores and the loss of oxidative phosphorylation due to the formation of calcium phosphate precipitation, inducing energy exhaustion.<sup>[26,27]</sup>

With the development of nanotechnology, cell apoptosis caused by  $\text{Ca}^{2+}$  overload can be regulated by functional nanomaterials, developed as a new cancer strategy (called  $\text{Ca}^{2+}$  interference therapy).<sup>[28]</sup>  $\text{Ca}^{2+}$ -based nanomaterials (such as  $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ ,  $\text{CaCO}_3$ , and  $\text{CaO}_2$ ) as  $\text{Ca}^{2+}$  overload regulators have an acid-response property, meaning that they can release abundant  $\text{Ca}^{2+}$  by responding to the tumor microenvironment (TME), thus increasing the intracellular  $\text{Ca}^{2+}$  level and inducing cell apoptosis and death. Due to these characteristics,

Ca<sup>2+</sup>-based nanomaterials have been recently widely applied in cancer therapy by triggering intracellular Ca<sup>2+</sup> overload. Moreover, Ca<sup>2+</sup>-based nanomaterials are usually low cost, due to the simple preparation methods and cheap raw materials, and possess relatively high biosecurity owing to the biocompatible components. Furthermore, Ca<sup>2+</sup> overload-mediated cancer therapy does not need to require external stimulations like photothermal therapy (PTT), photodynamic therapy (PDT), and sonodynamic therapy (SDT), which is beneficial for wide biomedical applications and clinical translation. Therefore, Ca<sup>2+</sup> overload-mediated cancer therapy has attracted more and more attention. Although two reviews of calcium-based biomaterials for diagnosis and therapy have very recently appeared, they mainly reported on various therapeutic strategies and calcification-mediated computed tomography imaging, rather than focusing on and discussing the opportunity of Ca<sup>2+</sup> overload-mediated cancer therapy.<sup>[29,30]</sup>



**Figure 1.** Schematic illustration of enhanced Ca<sup>2+</sup> overload.

In this review, different types of Ca<sup>2+</sup>-based nanomaterials will be discussed in detail, especially focusing on the action mechanisms and synergistic effects (e.g., enhanced endocytosis, Ca<sup>2+</sup> efflux inhibition, Ca<sup>2+</sup> influx promotion, triggering Ca<sup>2+</sup> release from ER, accelerated Ca<sup>2+</sup> release from nanomaterials and so on) to further enhance intracellular Ca<sup>2+</sup> level and amplify Ca<sup>2+</sup> overload (**Figure 1**). We would like to underline that our review is also alternative and different from a very recent review that mainly focuses on the combination of Ca<sup>2+</sup> overload with other cancer therapeutic strategies rather than enlightening the potential to

enhance the self-efficiency of  $\text{Ca}^{2+}$  overload-mediated cancer therapy.<sup>[31]</sup> Therefore, our review provides a targeted discussion about this new cancer therapy modality, which could inspire new ideas in designing increased efficiency systems and broadening its biomedical applications.

## 2. $\text{Ca}^{2+}$ -based Anticancer Nanomaterials

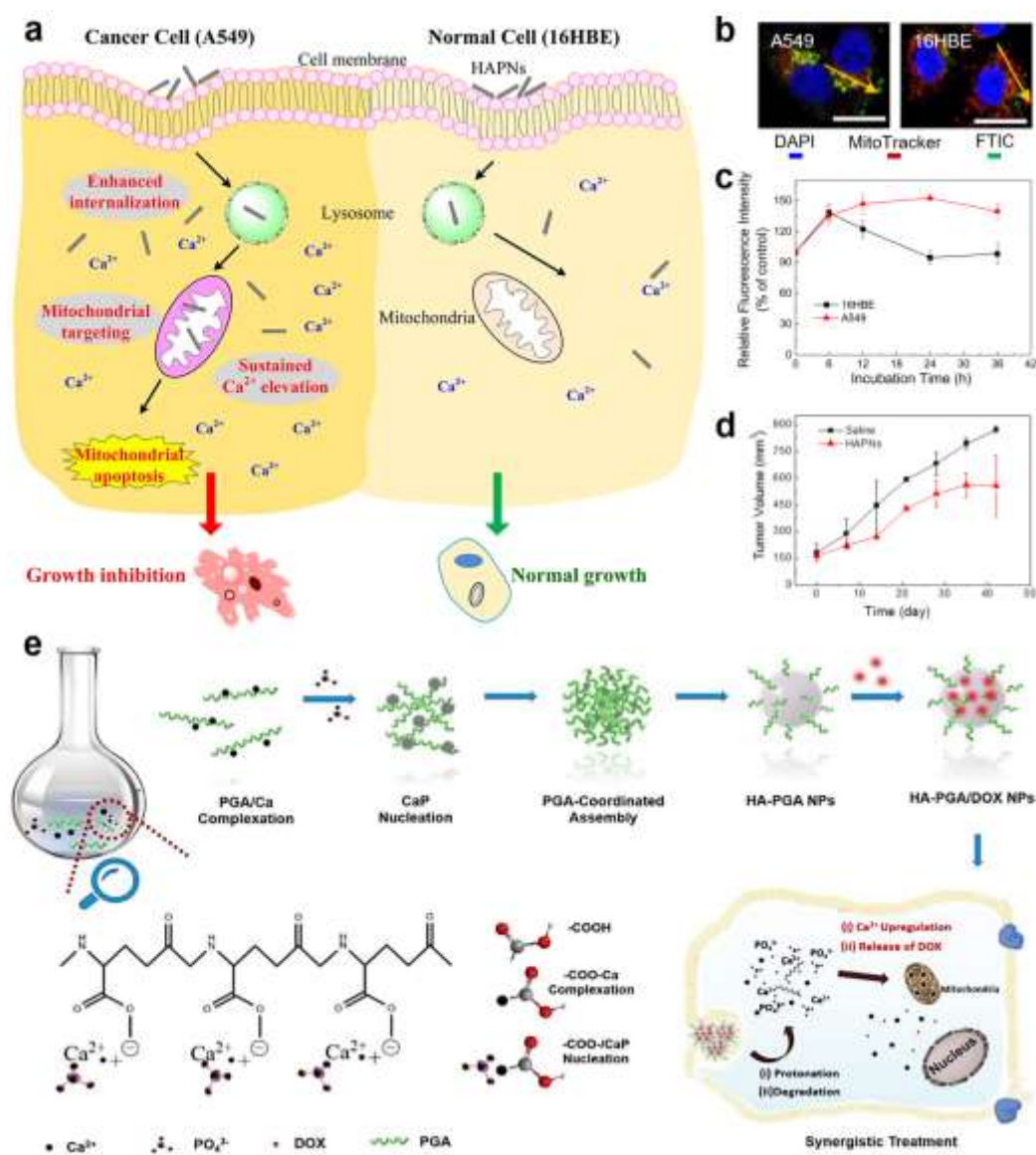
This section is divided into four parts based on the types of  $\text{Ca}^{2+}$ -based nanomaterials, describing conventional  $\text{Ca}_3(\text{PO}_4)_2$ ,  $\text{CaCO}_3$ ,  $\text{CaO}_2$ , and other new nanomaterials, respectively. The intracellular mechanisms of action and the unique advantages of each nanosystem are described.

### 2.1 Calcium Phosphate-based Nanomaterials

Calcium phosphate (CaP)-based nanomaterials widely exist in biological hard tissues such as teeth and bones. The common CaP-based nanomaterials include hydroxyapatite (Hap,  $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ ), tricalcium phosphate (TCP,  $\text{Ca}_3(\text{PO}_4)_2$ ), or monocalcium phosphate monohydrate ( $\text{Ca}(\text{H}_2\text{PO}_4)_2 \cdot \text{H}_2\text{O}$ ).<sup>[32]</sup> Synthetic CaP-based nanomaterials are similar to the natural form and possess high biocompatibility and good biodegradability.<sup>[33]</sup> Besides, some innate properties of CaP-based nanomaterials such as pH-sensitive solubility and easy methods of preparation make them useful in targeted tumor therapy.<sup>[34]</sup>

As the main inorganic component of bone, HAp has been widely used in the biomedical field.<sup>[35-37]</sup> Rod-like HAp nanoparticles (HAp NPs) were synthesized by coprecipitation and calcination and showed excellent selective anticancer activity (**Figure 2a**).<sup>[38]</sup> Cancer cells can internalize more HAp NPs than normal cells, leading to a sustained high  $\text{Ca}^{2+}$  concentration (Figure 2b and c). The different intracellular  $\text{Ca}^{2+}$  concentrations showed different effects on the diverse types of cells. HAp NPs had little effect on normal cells. Inversely, HAp NPs can cause cancer cell death by inducing mitochondrial damage and apoptosis. Besides, the distribution of HAp NPs in normal cells and cancer cells was investigated using fluorescent probes. In cancer cells, the HAp NPs signals were largely colocalized with the mitochondria, indicating that HAp NPs had the targeting ability to induce mitochondrial  $\text{Ca}^{2+}$  overload. The mitochondrial targeting of HAp NPs might be mainly triggered by cell-type specific mechanisms, due to the altered metabolic pattern in cancer cells. However, the detailed mechanism for this targeting ability still needs to be further studied. To complement the *in vitro* data, the *in vivo* antitumor efficacy and safety of HAp NPs were investigated. The growth rate of xenografted human lung tumors (using A549 cells) decreased after HAp NPs treatment (Figure 2d). Meanwhile, no obvious body weight changes and organ damage were observed

after being treated with HAP NPs three times a week for 26 days. Furthermore, to amplify the  $\text{Ca}^{2+}$  overload, ultrathin HAP nanosheets doped with pyrazine-2,3-dicarbonitrile (HAP-PDCNs) were prepared to enhance the internalization of HAP into cancer cells via clathrin-mediated endocytosis, and selectively concentrate it in the charged mitochondrial membrane.<sup>[39]</sup> The proton-triggered decomposition of HAP-PDCNs causes mtDNA damage by an instantaneous local  $\text{Ca}^{2+}$  overload in the mitochondria of cancer cells, leading to an inhibition of tumor growth. To endow HAP with targeting ability, polyacrylic acid (PAA)-coordinated HAP NPs chemically grafted with folic acid (HAP-PAA-FA) were prepared.<sup>[40]</sup> These spherical HAP-PAA NPs exhibited good tumor cell death. After linking FA, HAP-PAA-FA NPs can target cancer cells better, specifically killing these cells.



**Figure 2.** a) Schematic illustration of HAP NPs-mediated apoptosis and tumor growth inhibition; b) Selective accumulation of fluorescently labeled HAP NPs in cancer cells' mitochondria after 48 h incubation. The green and red fluorescence represent FITC-HAP NPs

and MitoTracker, respectively; c) Relative fluorescence intensity change of intracellular  $\text{Ca}^{2+}$  in normal and cancer cells with time. Reproduced with permission.<sup>[38]</sup> Copyright 2016, American Chemical Society; d) Volume change of xenografted A549 cell tumor; e) Schematic illustration of HA-PGA/DOX-mediated synergistic treatment. Reproduced with permission.<sup>[41]</sup> Copyright 2019, Royal Society of Chemistry.

Unfortunately, HAp alone can only inhibit tumor growth to a certain degree. To obtain more efficient cancer treatment, the combination of HAp NPs with antitumor drugs is a promising strategy. For this purpose, a so-called “calcium ion nanogenerator” (TCaNG) was designed by combining HAp with doxorubicin (DOX).<sup>[42]</sup> TCaNG was able to induce  $\text{Ca}^{2+}$  bursting release in lysosomes and then reverse drug resistance by suppressing cellular respiration and blocking intracellular ATP production. Interestingly, TCaNG can decrease the IC<sub>50</sub> of DOX to resistant MCF-7/ADR cells by ~30 times and reduce the proliferation of drug-resistant tumors by approximately 13 times without obvious side effects. To simultaneously achieve an enhanced  $\text{Ca}^{2+}$  overload and an efficient chemotherapeutic effect of DOX, a polyglutamic acid (PGA)-combined assembly of HAp NPs (HAp-PGA NPs) was designed (Figure 2e).<sup>[41]</sup> After being modified by PGA, more HAp NPs were internalized into cancer cells, bringing a highly sustained  $\text{Ca}^{2+}$  level. The dramatic elevation of intracellular  $\text{Ca}^{2+}$  induced a cascade of mitochondrial membrane damage and ATP content reduction, which activated apoptosis and upregulated the drug sensitivity to chemotherapy, respectively. Furthermore, it is known that DOX is released in a pH-responsive manner. With the synergistic effects of DOX and enhanced calcium overload, a selectively intensified toxicity to tumor cells was achieved. *In vivo* results further confirmed that the combined therapy mediated by HAp-PGA/DOX exhibited highly selective tumor inhibition and reduced heart toxicity.

Because the acid response of CaP is weak and the release of  $\text{Ca}^{2+}$  is not enough sufficient, the therapeutic efficacy is unsatisfactory due to a weak  $\text{Ca}^{2+}$  overload. To increase the level of intracellular  $\text{Ca}^{2+}$  and enhance  $\text{Ca}^{2+}$  overload, Xu et al. developed a doubly enhanced  $\text{Ca}^{2+}$  nanogenerator (DECaNG) based on TCP combined with curcumin (CUR) and photothermal effect (PTE) (**Figure 3a**).<sup>[43]</sup> CUR is a polyphenol extracted from the dietary spice turmeric and herb. As a bioactive agent, CUR can inhibit  $\text{Ca}^{2+}$  transfer from the cytoplasm to the extracellular compartment and promote  $\text{Ca}^{2+}$  release from the ER to the cytoplasm, thus increasing  $\text{Ca}^{2+}$  concentration in the mitochondria, resulting in a mitochondrial  $\text{Ca}^{2+}$  overload state.<sup>[44]</sup> The copper sulfide (CuS) was used to hybridize with TCP because of the hydrophilicity and the high drug loading capacity.<sup>[45]</sup> Besides, CuS NPs exert a PTE under near-infrared (NIR) light

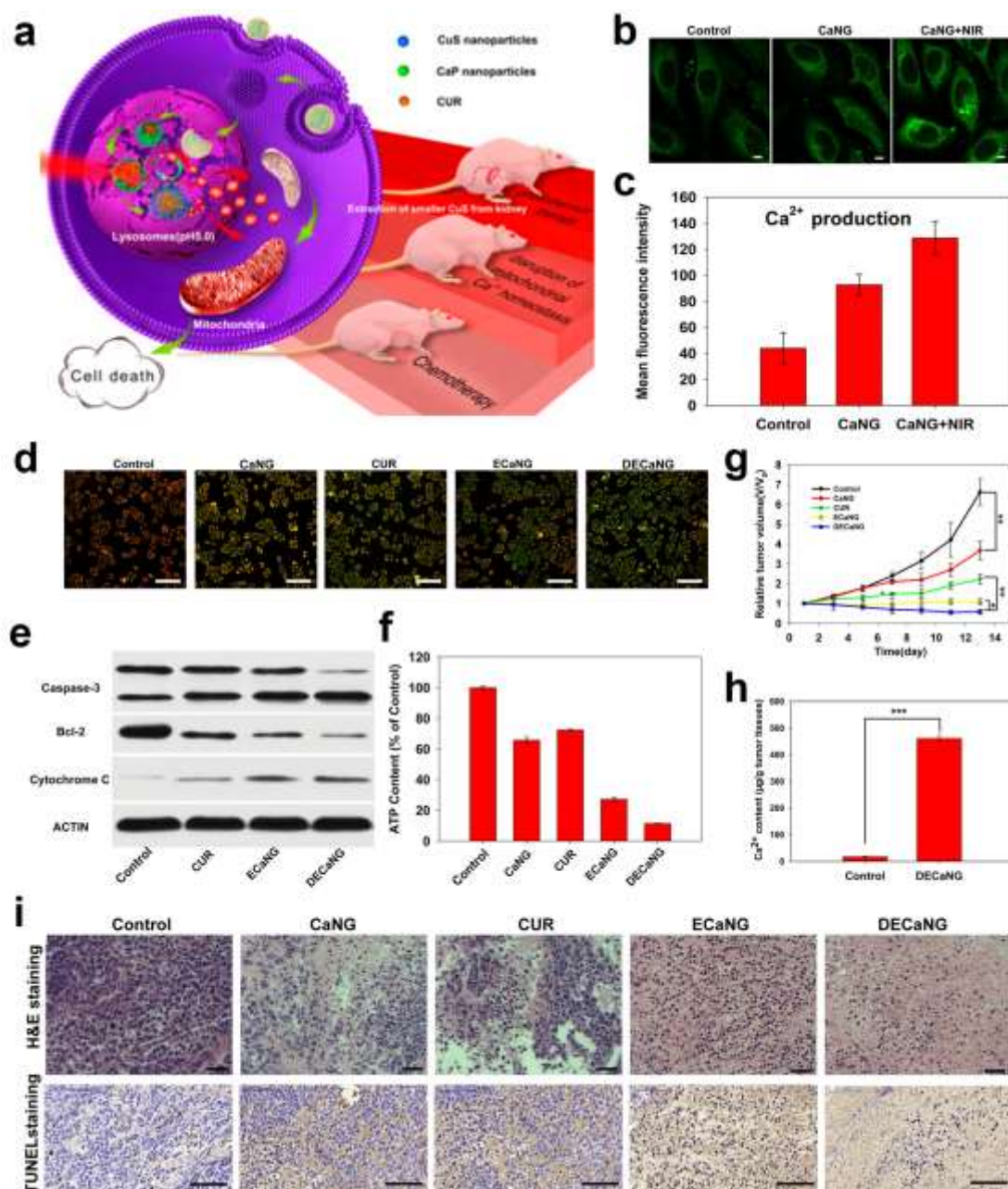


radiation, which not only showed great promise for PTT of tumors,<sup>[46]</sup> but also promoted the release of  $\text{Ca}^{2+}$ .<sup>[47]</sup> Based on the innate advantages of TCP and these ways to enhance the therapeutic effects, DECaNG could effectively induce cancer cell apoptosis. In the acidic environment of lysosomes,  $\text{Ca}^{2+}$  was released efficiently, being DECaNG pH-sensitive. The PTE of CuS could further promote  $\text{Ca}^{2+}$  release by causing a structural instability of TCP.<sup>[48]</sup> Due to the acid response in lysosomes, a lot of  $\text{Ca}^{2+}$  was released from DECaNG especially under NIR light irradiation, causing the intracellular  $\text{Ca}^{2+}$  level to remarkably increase (Figure 3b and c). Moreover, the abundant  $\text{Ca}^{2+}$  produced by DECaNG was observed to flow into mitochondria, leading to a decreased mitochondrial membrane potential and triggering cell apoptosis (Figure 3d). To confirm the detailed mechanism of apoptosis, the expressions of caspase-3, cytochrome c, and Bcl-2 were measured by Western blot (Figure 3e). Caspase-3 was activated in all different treatment groups, but most in the DECaNG group. In parallel, the amount of cytochrome c increased, and antiapoptotic protein Bcl-2 decreased, indicating the initiation of the mitochondria-mediated apoptosis pathway. Due to the structural damage and dysfunction of mitochondria, the content of ATP decreased especially in the DECaNG group (Figure 3f). Importantly, *in vivo* results showed that DECaNG possessed an efficient antitumor effect (Figure 3g).  $\text{Ca}^{2+}$  content in the tumor tissues of the DECaNG group was dramatically higher than that in the saline group (Figure 3h), with many cancer cells undergoing apoptosis (Figure 3i). Therefore, DECaNG showed great potential for cancer treatment via the synergy of chemotherapy, thermotherapy, and enhanced mitochondrial  $\text{Ca}^{2+}$  overload.

In an alternative approach, amorphous CaP nanosystems were also designed to treat cancer.<sup>[49-51]</sup> CaP-black phosphorus nanosheets (CaBPs) were synthesized by *in situ* mineralization strategy.<sup>[50]</sup> Compared to BPs alone, CaBPs exhibited enhanced and selective anticancer bioactivity due to the improved pH-responsive degradation behavior and intracellular  $\text{Ca}^{2+}$  overload in cancer cells. Interestingly, CaBPs specifically targeted and damaged mitochondria, causing mitochondria-mediated apoptosis in cancer cells. *In vivo* results demonstrated that CaBPs can target orthotopic breast cancer cells to inhibit tumor growth without obvious adverse side effects. Furthermore, amorphous CaP loaded with DOX and modified by RGD showed good efficiency for tumor inhibition by ROS-enhanced  $\text{Ca}^{2+}$  overload and chemotherapy.<sup>[52]</sup>

It has been reported in several papers that autophagy degrades dysfunctional organelles to sustain metabolism and homeostasis, participating in resistance to PDT.<sup>[53-55]</sup> In this context, complementary mitochondrial  $\text{Ca}^{2+}$  overload and autophagy inhibition were used to cover the shortage of PDT. Wang et al. designed biodegradable tumor-targeted inorganic/organic hybrid

nanocomposites (DPGC/OI) synchronously encapsulating IR780 and obatoclax (an autophagy inhibitor) by biomineralization of the nanofilms, which consist of pH-triggered CaP and long circulation phospholipid block copolymers 1,2-distearoyl-sn-glycero-3-phosphoethanolamine (DSPE)-poly(ethylene glycol) (PEG)2000-glucose (DPG).<sup>[56]</sup> In the presence of the hydrophilic PEG chain and the glucose transporter 1 (Glut-1) ligand, sufficient enrichment of DPGC/OI in tumor tissues was achieved. The combination of Ca<sup>2+</sup> overload, IR780 mediated PDT, and obatoclax-caused autophagy inhibition endowed DPGC/OI with a high therapeutic effect.



**Figure 3.** a) Schematic illustration of DECaNG-mediated Ca<sup>2+</sup> overload and apoptosis; b,c) Increased Ca<sup>2+</sup> level after the different treatments; d) Mitochondria membrane potential changes recorded by JC-1 staining; e) Western blot assays of intracellular caspase-3, Bcl-2, and cytochrome c; f) Intracellular ATP content change; g) Relative tumor volume changes with

different treatments; h)  $\text{Ca}^{2+}$  content in tumor tissues after treatment with DECaNG; i) H&E and TUNEL staining. Reproduced with permission.<sup>[43]</sup> Copyright 2018, American Chemical Society.

## 2.2 $\text{CaCO}_3$ -based Nanomaterials

$\text{CaCO}_3$  is relatively stable in neutral and alkaline environments and is more easily decomposed into  $\text{Ca}^{2+}$  ions in acidic environments than CaP. Therefore,  $\text{CaCO}_3$  is a suitable candidate as a  $\text{Ca}^{2+}$  resource to trigger  $\text{Ca}^{2+}$  overload in cancer cells. However,  $\text{CaCO}_3$ -mediated  $\text{Ca}^{2+}$  overload alone was unable to inhibit tumor growth effectively. To improve the therapeutic efficacy, some combined therapies based on  $\text{CaCO}_3$ -mediated  $\text{Ca}^{2+}$  overload have been proposed.<sup>[57,58]</sup> For example, liquid metal-glucose oxidase- $\text{CaCO}_3$  NPs (LMGC) were constructed, showing excellent efficacy in inhibiting tumor growth by the synergistic effect of  $\text{Ca}^{2+}$  overload, ATP generation inhibition, and photothermal effect.<sup>[59]</sup>

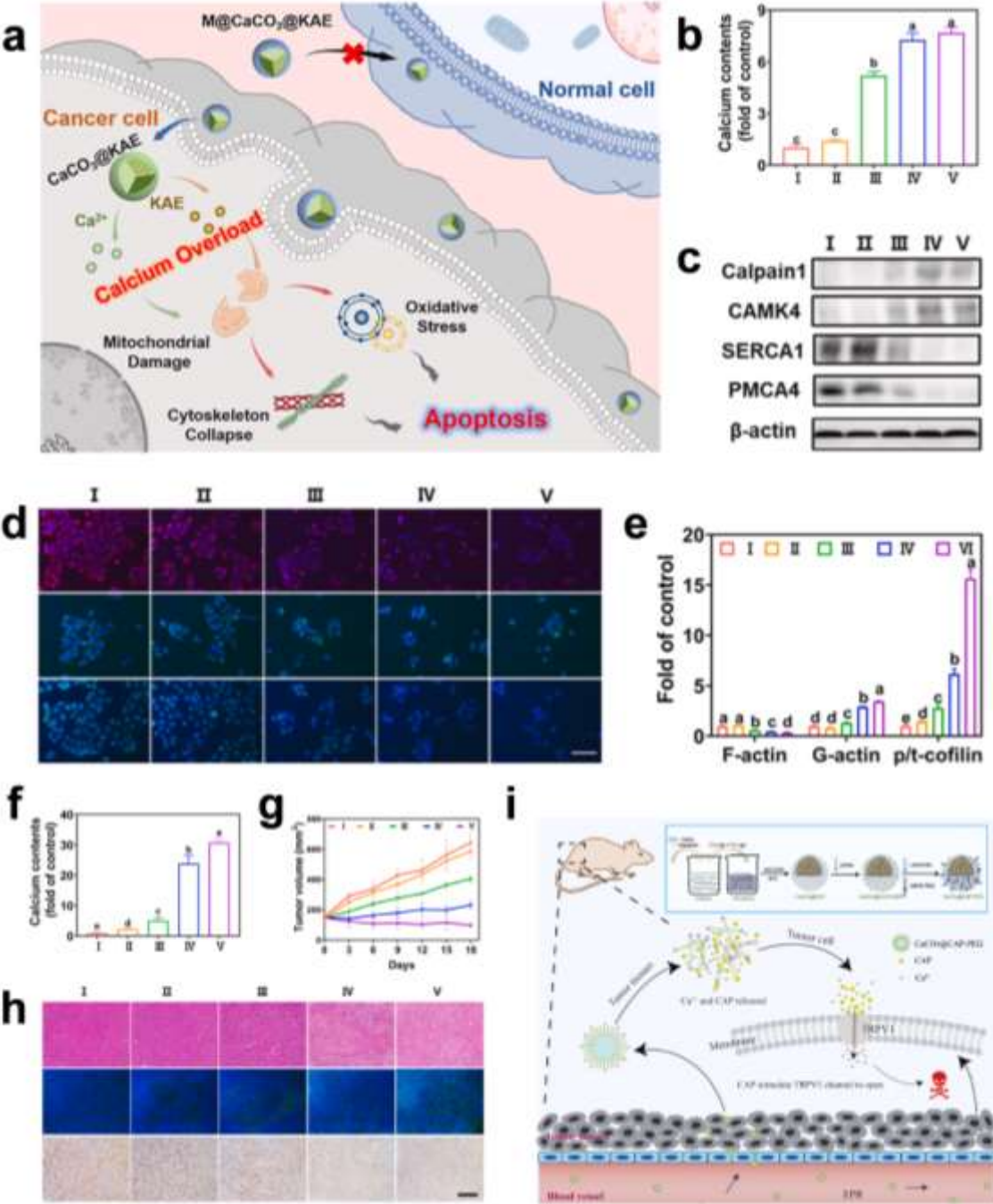
It is worth noting that the reason for the unsatisfied therapeutic effect on cancers treated with  $\text{CaCO}_3$  NPs alone should be attributed to cellular self-regulation, which leads to the difficulty to increase the level of  $\text{Ca}^{2+}$  efficiently. Therefore, alternative approaches to disrupt  $\text{Ca}^{2+}$  homeostasis will be of help to increase the therapeutic outcome of  $\text{Ca}^{2+}$  overload. Similar to CaP, a phospholipid-coated amorphous  $\text{CaCO}_3$  hybrid loaded with CUR (PL/ACC-CUR) was prepared to enhance  $\text{Ca}^{2+}$  overload by CUR-mediated  $\text{Ca}^{2+}$  efflux inhibition.<sup>[60]</sup> PL/ACC-CUR could specifically increase the intracellular  $\text{Ca}^{2+}$  level to cause  $\text{Ca}^{2+}$  overload and trigger mitochondria-related apoptosis in MCF-7 cells and inhibit tumor growth while sparing normal hepatocytes. In another study, anticancer cisplatin (CDDP) and CUR co-incorporated into  $\text{CaCO}_3$  NPs as a multichannel  $\text{Ca}^{2+}$  nanomodulator were designed and obtained by a facile one-pot strategy by inducing multilevel destruction of mitochondria, exploiting the combined effects of  $\text{Ca}^{2+}$  burst release,  $\text{Ca}^{2+}$  efflux inhibition by CUR, and chemotherapeutic CDDP.<sup>[61]</sup> Interestingly, Zheng et al. found that  $\text{CaCO}_3$ /CUR nanoparticles can quickly cause mitochondrial  $\text{Ca}^{2+}$  overload and result in ROS increase, cytochrome C release, caspase-3 activation, gasdermin E cleavage, finally leading to pyrolysis and subsequent immune activation for immune therapy.<sup>[62]</sup>

Promoting  $\text{Ca}^{2+}$  influx is another strategy to enhance  $\text{Ca}^{2+}$  overload. It has been proved that kaempferol-3-O-rutinoside (KAE) shows an excellent anticancer activity due to its ability to disrupt calcium homeostasis and promote  $\text{Ca}^{2+}$  influx to fulfill  $\text{Ca}^{2+}$  overload-mediated cell apoptosis.<sup>[63]</sup> Therefore, the combination of  $\text{CaCO}_3$  NPs and KAE is expected to display an efficiently synergistic  $\text{Ca}^{2+}$  overload effect.  $\text{CaCO}_3$  NPs loaded with KAE and coated with

cancer cell membranes (M@CaCO<sub>3</sub>@KAE NPs) were prepared to treat cancer by enhanced Ca<sup>2+</sup> overload (**Figure 4a**).<sup>[64]</sup> The efficient Ca<sup>2+</sup> overload-mediated by M@CaCO<sub>3</sub>@KAE NPs can cause mitochondrial damage, oxidative stress, cytoskeleton collapse, and final apoptosis. The coating with the cancer cell membrane ensured the ability of tumor-targeting via innate properties of immune escape and aggregation. Compared to the use of CaCO<sub>3</sub> NPs alone, CaCO<sub>3</sub>@KAE NPs could significantly improve the accumulation of Ca<sup>2+</sup> (Figure 4b). Meanwhile, SERCA1 (Sarco(endo)plasmic Reticulum Ca<sup>2+</sup>-ATPase Isoform 1) and PMCA4 (plasma membrane Ca<sup>2+</sup>-ATPase), which are proteins related to calcium outflow, were down-regulated, while the pro-apoptotic protein CAMK4 (Ca<sup>2+</sup>/calmodulin-dependent protein kinase) was up-regulated (Figure 4c).

Furthermore, M@CaCO<sub>3</sub>@KAE NPs caused a decrease in the number of mitochondria, a mitochondrial membrane potential change, and mitochondrial lipid peroxidation (Figure 4d). As mitochondria are organelles responsible for cell energy supply, they play a crucial part in cellular cytoskeleton formation. When mitochondria are damaged, the cytoskeleton cannot form correctly (Figure 4e). As a consequence, the lamellipodium collapses and the structure of filopodia is modified, thus repressing the migration and invasion abilities of cancer cells and finally causing apoptosis. The membrane modification is the critical step to ensure that the M@CaCO<sub>3</sub>@KAE NPs have the capacities for immune escape, thus avoiding the undesirable absorption at normal tissues.<sup>[65]</sup> By comparing the *in vivo* biodistribution of M@CaCO<sub>3</sub>@KAE NPs with CaCO<sub>3</sub>@KAE NPs, it was obvious that the membrane coating ensured enhanced targeting accumulation at the tumor sites. Therefore, M@CaCO<sub>3</sub>@KAE NPs significantly enhanced tumor tissue Ca<sup>2+</sup> overload *in vivo* (Figure 4f), and showed the highest efficacy of cancer therapy by causing a lot of cancer cell apoptosis and proliferation inhibition (Figure 4g and h). Besides, the expression of proliferation-related proteins (Ki67, PCNA, and BCL-2) was markedly restrained, while the apoptosis-related proteins (BAX, CAS3, and CAS9) were expressed at high levels. The expression of calcium outflow proteins (SERCA1 and PMCA4) decreased, and the level of the pro-apoptotic protein CAMK4 was significantly elevated. Meanwhile, Calpain1 was activated to achieve cytoskeleton degradation, similar to *in vitro* results. Interestingly, the natural non-toxic capsaicin (CAP), which is often used clinically to reduce inflammation and pain, has also been found to promote Ca<sup>2+</sup> influx. Recently, Xu et al. designed CaCO<sub>3</sub>@CAP NPs to achieve a highly therapeutic effect by simultaneously providing a TRPV1 channel activator (causing excessive Ca<sup>2+</sup> influx) and exogenous Ca<sup>2+</sup> to cause strong Ca<sup>2+</sup> overload and cancer cell death (Figure 4i).<sup>[66]</sup> Finally, ovalbumin (OVA)@CaCO<sub>3</sub> NPs mediated Ca<sup>2+</sup> overload can disrupt the autophagy inhibition condition in dendritic cells (DCs)

and improve DC maturation by enhanced DAMP (damage-associated molecular patterns) release from tumor cells, which offers an alternative strategy for improving cancer chemo-immunotherapy by the regulation of the intratumoral  $Ca^{2+}$ .<sup>[67]</sup>

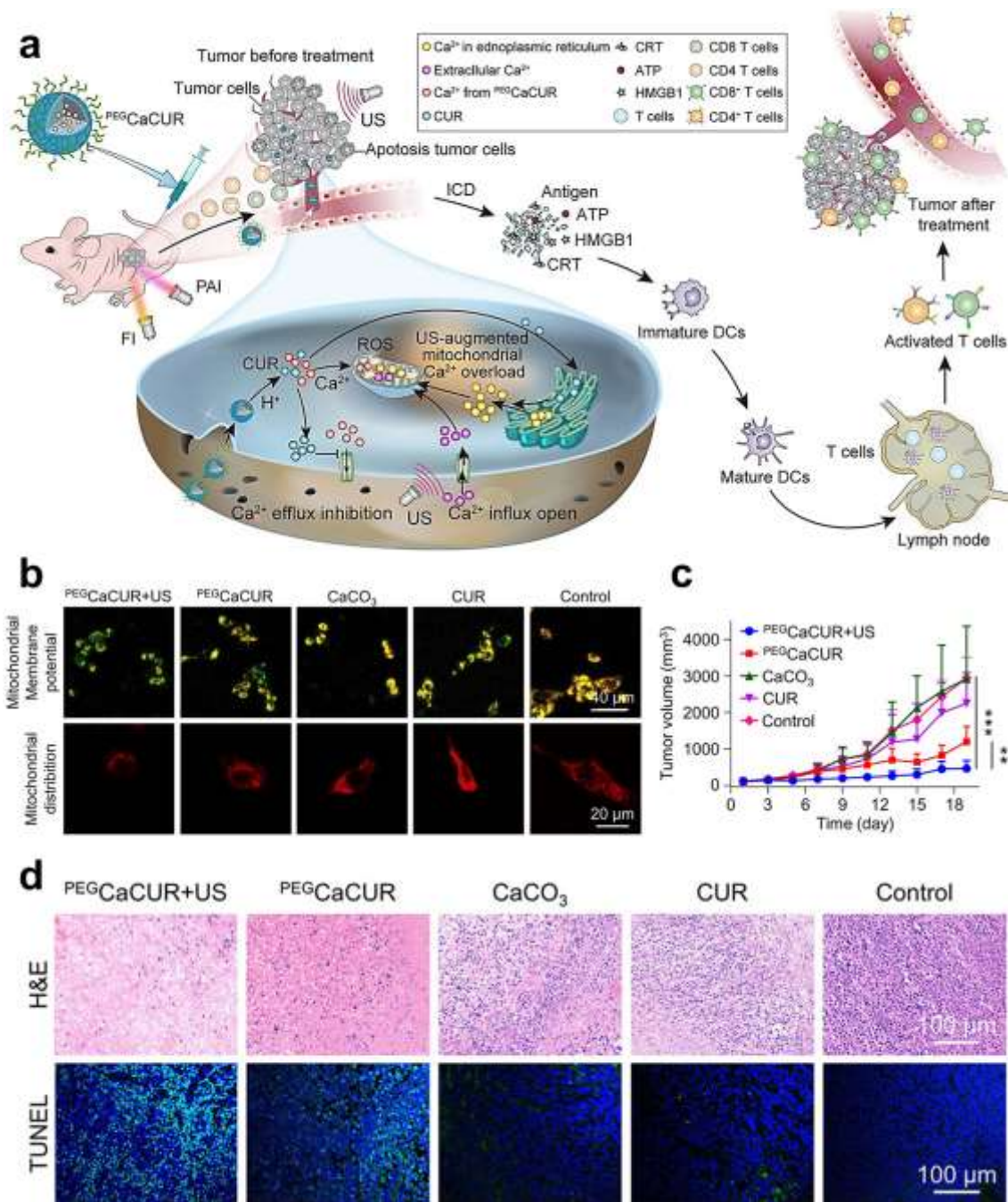


**Figure 4.** (a) Schematic illustration of M@CaCO<sub>3</sub>@KAE NPs-mediated cancer therapy. (b) Ca<sup>2+</sup> content changes. (c) Ca<sup>2+</sup> regulated protein expression levels. (d) Effects on mitochondria. Top: Mitotracker staining for mitochondria number visualization; Middle: rhodamine 123 for mitochondrial membrane potential visualization; Bottom: nonyl acridine orange for mitochondrial lipid peroxidation. (e) The intensity of F-actin, G-actin, p-cofilin, and t-cofilin. (f) Tumor volume changes. (g) Ca<sup>2+</sup> content change in tumor tissues. (h) H&E, TUNEL, and

Ki67 staining. I: Control; II: CaCO<sub>3</sub> NPs; III: KAE; IV: CaCO<sub>3</sub>@KAE NPs; V: M@CaCO<sub>3</sub>@KAE NPs. Reproduced with permission.<sup>[64]</sup> Copyright 2021, Elsevier Ltd. (i) Schematic illustration of CaCO<sub>3</sub>@CAP NPs-mediated cancer therapy. Reproduced with permission.<sup>[66]</sup> Copyright 2022, Elsevier Ltd.

A recent study has evidenced that ultrasounds (US) can serve as an exogenously physical stimulus inducing Ca<sup>2+</sup> overload through the upregulation of intracellular Ca<sup>2+</sup> concentration by promoting Ca<sup>2+</sup> influx from the extracellular fluid to the cytoplasm. Therefore, the US can enhance calcium-based nanomaterial-mediated Ca<sup>2+</sup> overload. Zheng et al. developed CUR-incorporated CaCO<sub>3</sub> (CaCUR) NPs modified with poly(ethylene glycol) (<sup>PEG</sup>CaCUR).<sup>[68]</sup> Dual US/CUR augmented mitochondrial Ca<sup>2+</sup> overload induced immunogenic cell death (ICD) leading to an efficient immune response (**Figure 5a**), which also was observed in postoperative filler gel containing Ca<sup>2+</sup> to prevent tumor recurrence and metastasis.<sup>[69]</sup> <sup>PEG</sup>CaCUR mediated Ca<sup>2+</sup> overload reduced the mitochondrial membrane potential, further decreased by US treatment. Meanwhile, fewer mitochondria were observed in the <sup>PEG</sup>CaCUR group than in the control group, with the fewest detected in the <sup>PEG</sup>CaCUR+US group (Figure 5b). Therefore, <sup>PEG</sup>CaCUR associated with US stimulation can cause obvious mitochondrial damage and decrease mitochondrial production by enhanced Ca<sup>2+</sup> overload. In addition, more calreticulin outside of the cell membrane appeared after <sup>PEG</sup>CaCUR+US treatment, and high-mobility group box 1 (HMGB1) and ATP showed significantly increased extracellular secretion. The above results confirmed that <sup>PEG</sup>CaCUR NPs with US treatment can induce stronger ICD. Efficient ICD promoted DC maturation and T cell activation. By the combination of Ca<sup>2+</sup> overload and immune response activation, <sup>PEG</sup>CaCUR with US treatment could significantly inhibit the growth of tumors (Figure 5c), and a large number of cancer cells underwent apoptosis (Figure 5d), showing the high cancer treatment efficiency.

The disruption of the calcium buffering capacity to enhance Ca<sup>2+</sup> overload by reactive oxygen species has been achieved recently.<sup>[70,71]</sup> A PEG-modified CaCO<sub>3</sub> nanoplatfrom loaded with a Ir(III) complex (IrCOOH-CaCO<sub>3</sub>@PEG) was constructed for combined calcium overload and two-photon photodynamic therapy.<sup>[72]</sup> The generated <sup>1</sup>O<sub>2</sub> during photodynamic reaction could cause calcium buffering capacity disruption, subsequently enhancing Ca<sup>2+</sup> concentration. Similarly, HO<sup>•</sup> from the Fenton-like reaction of Co<sup>2+/3+</sup> constituting a biodegradable chemodynamic therapeutic agent also showed inhibition on calcium buffering capacity enhancing Ca<sup>2+</sup> overload. The additional intracellular Ca<sup>2+</sup> promoted, in turn, hydroxyl radical accumulation.<sup>[73,74]</sup>



**Figure 5.** a) Schematic illustration of  $^{PEG}CaCUR$  mediated  $Ca^{2+}$  overload and immune response activation; b) Effect of  $^{PEG}CaCUR+US$  on mitochondria; c) Tumor volume changes; d) H&E and TUNEL staining. Reproduced with permission.<sup>[68]</sup> Copyright 2021, American Chemical Society.

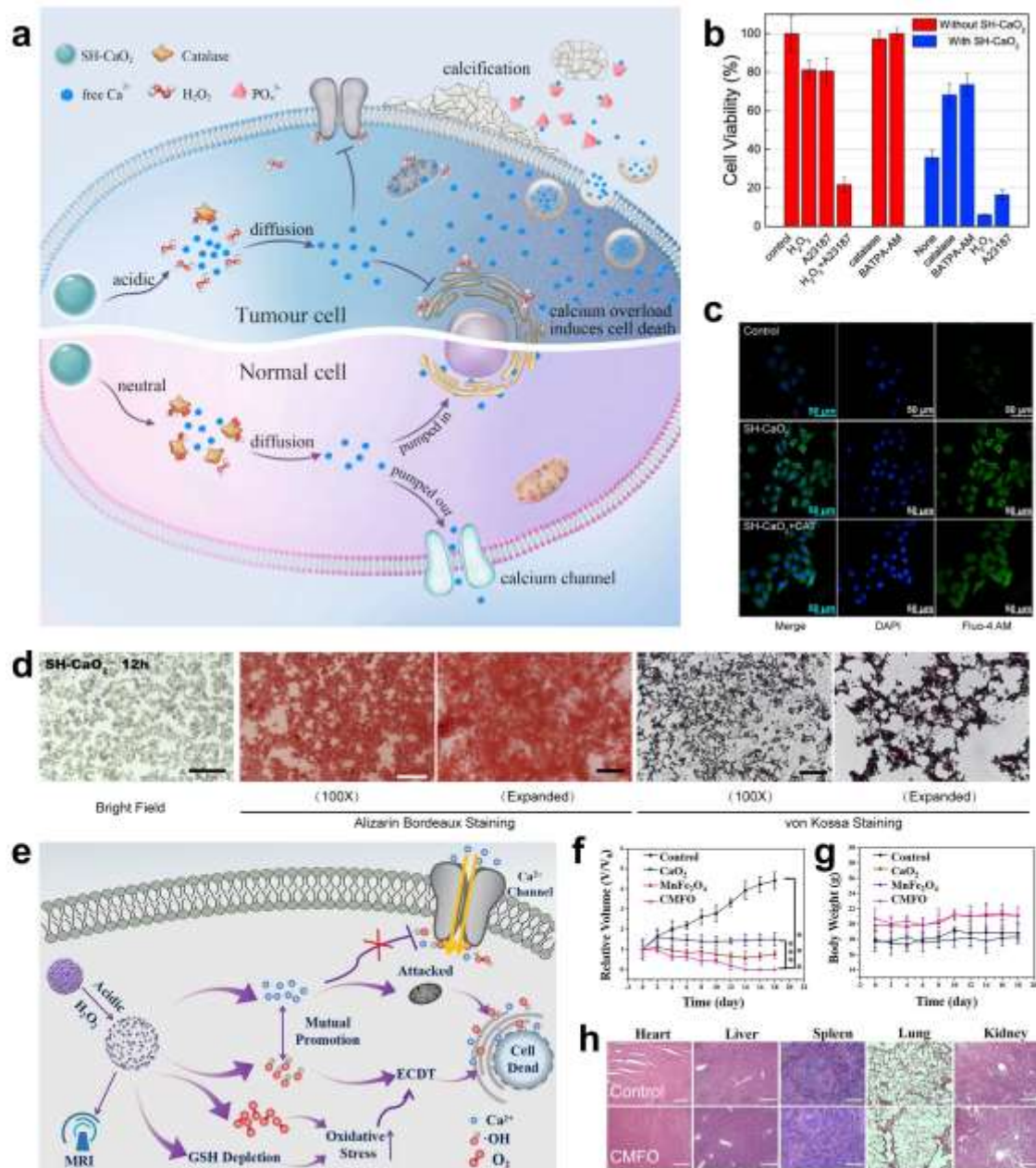
### 2.3 $CaO_2$ -based Nanomaterials

Compared with  $CaP$  and  $CaCO_3$ ,  $CaO_2$  NPs with lower stability cannot only generate  $Ca^{2+}$  but also  $H_2O_2$  in the TME. leading to a direct increase of oxidative stress and higher mortality of cancer cells. Oxidative stress can cause  $Ca^{2+}$  influx into the cytoplasm from the extracellular

environment and ER or sarcoplasmic reticulum (SR) through the cell membrane and ER/SR channels, respectively.<sup>[75]</sup> Therefore, the increase of intracellular oxidative stress level can further disrupt the homeostasis of  $\text{Ca}^{2+}$  and enhance the  $\text{Ca}^{2+}$  overload, leading to intense cancer cell apoptosis.

Due to quick hydrolysis, surface modifications are necessary to endow  $\text{CaO}_2$  with relatively high stability in body fluid and blood. To overcome this issue, sodium hyaluronate-modified  $\text{CaO}_2$  NPs (SH- $\text{CaO}_2$ ) were prepared. SH- $\text{CaO}_2$  NPs can be easily and fast decomposed in an acid environment to produce abundant  $\text{Ca}^{2+}$  and  $\text{H}_2\text{O}_2$ , following to efficiently induce  $\text{Ca}^{2+}$  overload and cell death (**Figure 6a** and b).<sup>[76]</sup> The generated  $\text{H}_2\text{O}_2$  can inhibit  $\text{Ca}^{2+}$  efflux, enhancing  $\text{Ca}^{2+}$  overload. After being treated with SH- $\text{CaO}_2$  NPs, ROS level and intracellular  $\text{Ca}^{2+}$  content remarkably increased. Moreover, the low expression of catalase in tumor cells can cause abnormal cellular  $\text{H}_2\text{O}_2$  accumulation and an imbalanced calcium transport pathway, which are of help to guarantee an efficient  $\text{Ca}^{2+}$  overload (Figure 6c). Meanwhile, after the treatment with SH- $\text{CaO}_2$  NPs, the cell nucleus condensed and the mitochondrial activity drastically reduced. Furthermore, the enriched local concentration of  $\text{Ca}^{2+}$  increased the calcification around cancer cells, leading to tumor growth inhibition (Figure 6d). The *in vivo* results demonstrated that SH- $\text{CaO}_2$  NPs alone were very efficient against cancer, at a degree that was difficult to reach with CaP and  $\text{CaCO}_3$ , confirming the intrinsic advantage of using  $\text{CaO}_2$ . Interestingly, interference with  $\text{Ca}^{2+}$  overload can mediate M2-like tumor-associated macrophages (TAM) re-education and local  $\text{Ca}^{2+}$ -activated PD-L1 depletion for immunotherapy. In another study, An et al. designed DSPE-PEG2000 modified  $\text{CaO}_2$  NPs loaded with a circular aptamer-DNAzyme complex ( $\text{CaNP@cAD-PEG}$ ) to boost tumor immunotherapy.<sup>[77]</sup>  $\text{CaNP@cAD-PEG}$  were able to induce a significant  $\text{Ca}^{2+}$  interference effect and simultaneously reset TAM toward the M1 phenotype, by activating multiple inflammation-related signaling pathways and promoting *in situ* tumor-associated antigen release. The catalytic shear activity of cAD can be specifically activated by  $\text{Ca}^{2+}$ , reducing potential autoimmune disorders triggered by PD-L1 antibody. By synergistic effect,  $\text{CaNP@cAD-PEG}$  inhibited primary tumor growth and lung metastasis and showed a long-term immunological memory to prevent tumor recurrence. However, the direct effects of  $\text{Ca}^{2+}$  overload on NK cells and T cells still need further investigation. Alternatively, DOX was used in combination with ZIF-8 coated  $\text{CaO}_2$  to treat cancer with higher efficacy exploiting the synergetic effect of mitochondrial  $\text{Ca}^{2+}$  overload-induced cell death and chemotherapy.<sup>[78]</sup>





**Figure 6.** a) Schematic illustration of SH-CaO<sub>2</sub>-mediated apoptosis; b) Cell viability assay. (c) Ca<sup>2+</sup> content change after SH-CaO<sub>2</sub> NP treatment; d) Calcium mineralization. Reproduced with permission.<sup>[76]</sup> Copyright 2019, Elsevier Ltd. e) Synthesis process and spatially selective self-cascade catalyst mechanism of CMFO; f) Tumor volume changes after CMFO treatment; g) Body weight changes and h) H&E staining of the main organs. Reproduced with permission.<sup>[79]</sup> Copyright 2022, Elsevier Ltd.

Additionally, due to the high level of H<sub>2</sub>O<sub>2</sub> produced by CaO<sub>2</sub>, it is promising to combine Ca<sup>2+</sup> overload with chemodynamic therapy (CDT), which utilizes Fenton or Fenton-like reactions to catalyze H<sub>2</sub>O<sub>2</sub> into toxic hydroxyl radicals (HO<sup>•</sup>).<sup>[80-84]</sup> Therefore, a CaO<sub>2</sub> nanosystem coated by MnFe<sub>2</sub>O<sub>4</sub> NPs (CMFO) as a protective umbrella was constructed to treat

cancer by the synergistic effect of  $\text{Ca}^{2+}$  overload and CDT mediated by Mn/Fe ions (Figure 6e).<sup>[79]</sup> CMFO was stable in normal tissues and could lead to a specific self-cascade catalytic reaction in tumor sites. Being cancer cells are more sensitive to  $\text{Ca}^{2+}$  in the presence of  $\text{H}_2\text{O}_2$ , the overloaded  $\text{Ca}^{2+}$  could induce apoptosis of tumor cells rather than in normal cells. By inhibiting the efflux and promoting the influx mediated by generated  $\text{HO}^\bullet$ , higher  $\text{Ca}^{2+}$  overload occurred, causing an efficient mitochondrial and cellular apoptosis.  $\text{MnFe}_2\text{O}_4$  and  $\text{H}_2\text{O}_2$  could also attenuate tumor hypoxia and deplete intracellular glutathione, amplifying oxidative stress and enhancing ROS-related activities. Based on these processes, the complete elimination of the tumor was achieved after CMFO treatment (Figure 6f), and CMFO showed low systemic toxicity and high biocompatibility (Figure 6g and h). Therefore, these “intelligent” and biodegradable CMFO NPs provide a new strategy for tumor-specific ROS-related treatment and spatially selective self-cascade synergistic therapy. Similarly, a cell membrane coated  $\text{CaO}_2@\text{FePt-DOX}@PDA$  (PDA, polydopamine) hybrid was developed for the combined application of chemotherapy, chemodynamic therapy,  $\text{Ca}^{2+}$  overload, amplified oxidative stress, and photothermal therapy. The combined FePt could convert  $\text{H}_2\text{O}_2$  produced by  $\text{CaO}_2$  into  $\text{HO}^\bullet$ , leading to enhanced apoptosis.<sup>[85]</sup> In another work,  $\text{CaO}_2$  nanomaterials modified by  $\text{Fe}^{3+}/\text{TA}$  (tannic acid) polyphenol network were prepared to treat cancer by  $\text{Ca}^{2+}$  overload and CDT.<sup>[86,87]</sup> Notably,  $\text{HO}^\bullet$  generated via the Fenton reaction desensitized the intracellular calcium ion proteins, promoting the occurrence of calcium overload and accelerating the death of cancer cells.

Because  $\text{CaO}_2$  can also serve as a source of  $\text{O}_2$ ,  $\text{CaO}_2$ -based composites were constructed by loading chlorin e6 (Ce6) to achieve a PDT. Shen et al. designed  $\text{CaO}_2@\text{ZIF-Fe/Ce6}@PEG$  ( $\text{CaZFCP}$ ) to generate  $\text{H}_2\text{O}_2/\text{O}_2$  self-supply and simultaneously  $\text{Ca}^{2+}$  overload in tumor cells for enhanced CDT/PDT.<sup>[88]</sup> To increase tissue penetration of light, up-conversion nanoparticles (UCNPs) were used as a template for the fabrication of  $\text{UCNPs-Ce6}@RuR$  (ruthenium red) $@m\text{SiO}_2$  NPs (UCRS) coated with HA and  $\epsilon$ -polylysine (UCRSPH). The combined use of UCRSPH and stearic acid/HA modified  $\text{CaO}_2$  showed great potential for highly specific, efficient combined therapy against tumor cells by integrating TME improvement, self-reinforcing PDT, and  $\text{Ca}^{2+}$  overload <sup>[89]</sup>.

Similar to  $\text{CaCO}_3$ ,  $\text{CaO}_2$  can be combined with drugs able to further inhibit  $\text{Ca}^{2+}$  efflux and enhance  $\text{Ca}^{2+}$  overload. Transferrin (Tf) could be destroyed by  $\text{H}_2\text{O}_2$  and could release  $\text{Fe}^{3+}$ , converting  $\text{H}_2\text{O}_2$  into  $\text{HO}^\bullet$  through the Fenton reaction.<sup>[90,91]</sup> Therefore, CUR and Tf co-loaded  $\text{CaO}_2$  ( $\text{CaO}_2/\text{Tf}/\text{CUR}$ ) NPs were formulated.<sup>[92]</sup> The cooperation of CUR and exogenous  $\text{Ca}^{2+}$  increased the intracellular  $\text{Ca}^{2+}$  level, reaching an efficient  $\text{Ca}^{2+}$  overload. Besides,  $\text{Fe}^{3+}$

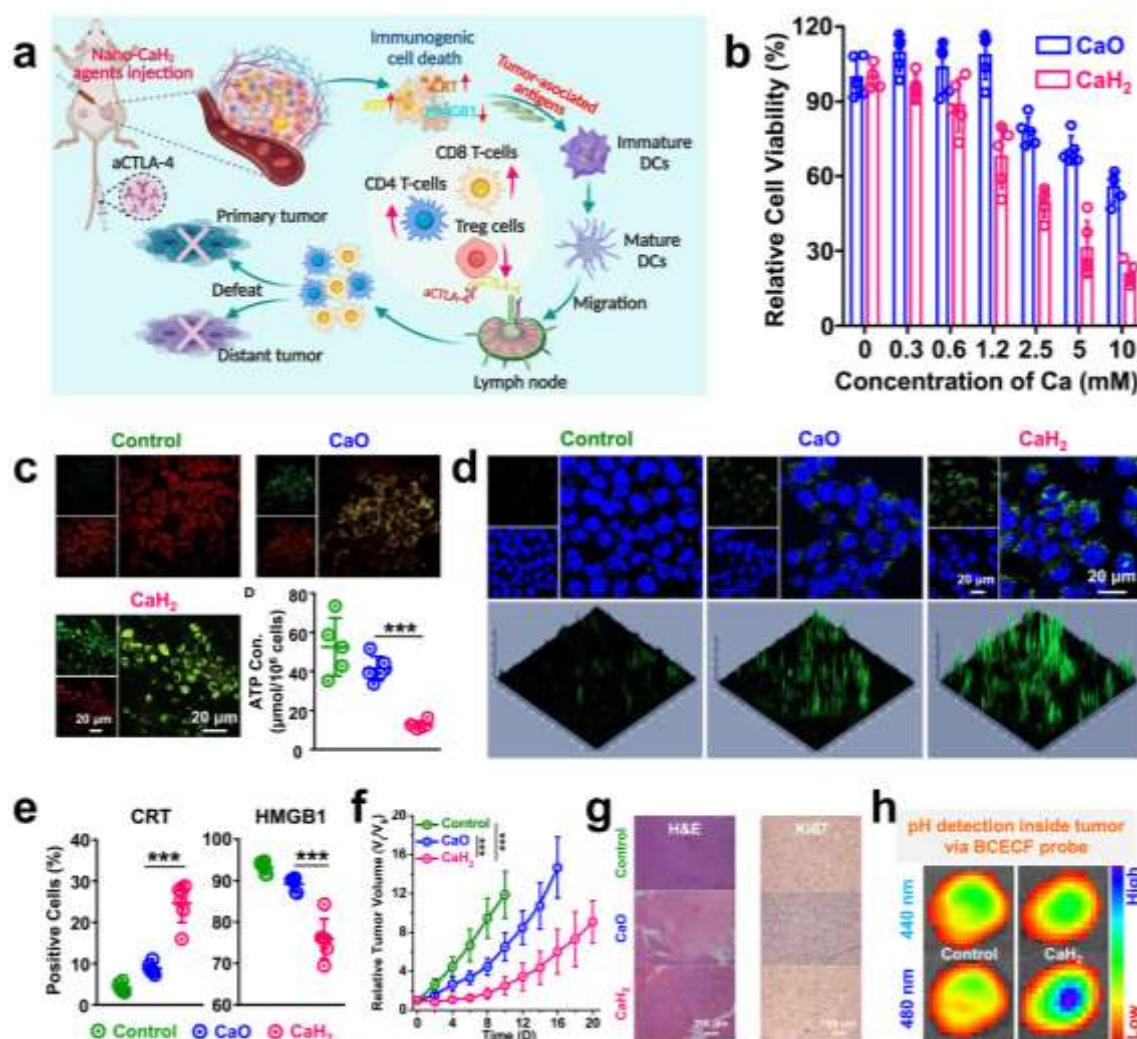
transformed  $\text{H}_2\text{O}_2$  into  $\text{HO}^\bullet$  and finally induced cancer cells apoptosis. Compared to the  $\text{CaO}_2$  group without the drugs, a higher cellular uptake was observed with  $\text{CaO}_2$  modified with Tf, confirming that Tf-conjugated NPs had a higher selectivity to cancer cells, via an endocytosis process mediated by the Tf receptor.<sup>[93]</sup> Cells treated with  $\text{CaO}_2/\text{Tf}/\text{CUR}$  NPs had the highest  $\text{Ca}^{2+}$  concentration among all groups, leading to efficient mitochondrial damage and apoptosis. *In vivo* results confirmed that  $\text{CaO}_2/\text{Tf}/\text{CUR}$  NPs possessed an excellent antitumor effect, with a lot of apoptotic cells identified in tumor tissues. Further, Transient Receptor Vanilloid 1 (TRPV1) thermal-sensitive cation channel can promote apoptosis by regulating  $\text{Ca}^{2+}$  influx after thermal activation (threshold  $\sim 43^\circ\text{C}$ ).<sup>[94-96]</sup> NIR-responsive DPPC-DSPE-PEG2000- $\text{NH}_2@$ PDPP@ $\text{CaO}_2@$ DOX NPs were designed.<sup>[97]</sup> Under NIR irradiation, the PTE promoted  $\text{Ca}^{2+}$  influx. Combined with  $\text{Ca}^{2+}$  release from  $\text{CaO}_2$  by the acid response, efficient  $\text{Ca}^{2+}$  overload was achieved. Finally, breast cancer tumor growth was inhibited obviously by the combination of PTT, chemotherapy, and  $\text{Ca}^{2+}$  overload.

In addition, it has been confirmed that NO can promote the release of  $\text{Ca}^{2+}$  from ER by activating the opening of ryanodine receptors (RyRs).<sup>[98-101]</sup> Therefore, PDA-modified and arginine-loaded mesoporous  $\text{CaO}_2$  NPs were designed.<sup>[102]</sup>  $\text{H}_2\text{O}_2$  generated from  $\text{CaO}_2$  can oxidize arginine to release locally NO. Subsequently, NO stimulates  $\text{Ca}^{2+}$  release. This  $\text{Ca}^{2+}$  together with the contribution of calcium from  $\text{CaO}_2$  NP decomposition could synergistically and efficiently cause  $\text{Ca}^{2+}$  overload. By the combination of PTT, immune regulation,  $\text{Ca}^{2+}$  overload, and gas therapy, these NPs showed great potential to inhibit 4T1 tumor growth and formation of lung metastases.

## 2.4 Other Types of $\text{Ca}^{2+}$ -based Nanomaterials

Recently, novel  $\text{Ca}^{2+}$ -based nanomaterials are emerging to enhance  $\text{Ca}^{2+}$  overload and promote cancer cell apoptosis. Hydrogen is an endogenous gas with high diffusibility and it has exhibited excellent antitumor performance as evidenced over the past decades.<sup>[103]</sup> Calcium hydride ( $\text{CaH}_2$ ) NPs were designed for cancer treatment by combining the advantages of  $\text{Ca}^{2+}$  overload, TME modulation, and immune and hydrogen therapy (**Figure 7a**).<sup>[104]</sup>  $\text{CaH}_2$  NPs can release abundant  $\text{H}_2$ , and change the pH into neutral or even alkaline from acid by the generated hydroxyl radicals. Compared with  $\text{CaO}$ ,  $\text{CaH}_2$  exhibited more serious toxicity and efficient apoptosis, which might be attributed to the combination of hydrogen and more effective  $\text{Ca}^{2+}$  overload (Figure 7b). Furthermore,  $\text{CaH}_2$  caused more damages to mitochondria, and evidently decreased the generation of ATP compared with  $\text{CaO}$  (Figure 7c). As expected, the intracellular  $\text{Ca}^{2+}$  level increased after  $\text{CaH}_2$  treatment rather than using  $\text{CaO}$  (Figure 7d). Interestingly,

CaH<sub>2</sub> NPs can also cause efficient ICD, which could activate T cells by antigen presentation (Figure 7e).

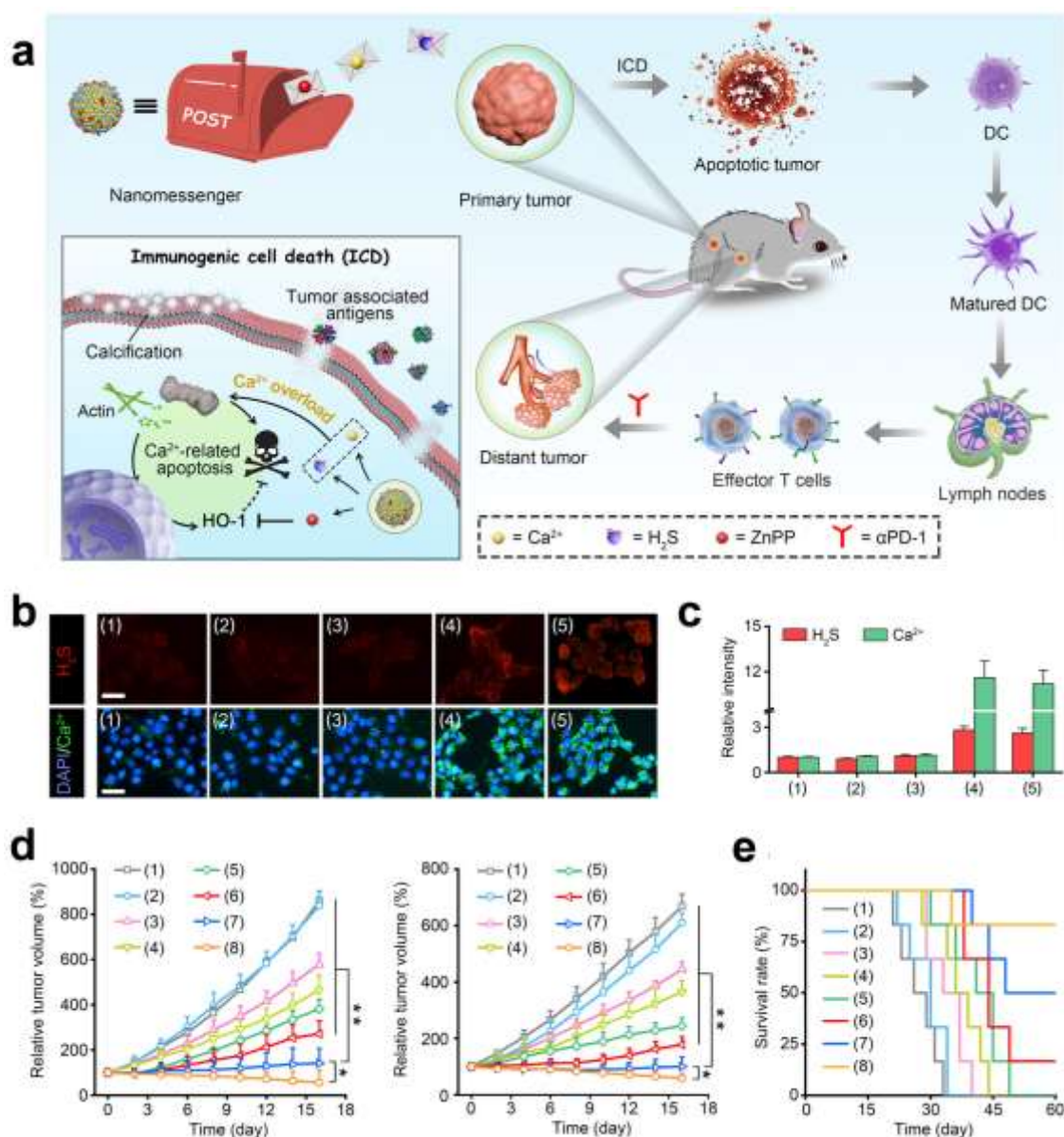


**Figure 7.** a) Schematic illustration of CaH<sub>2</sub> NPs mediating the synergistic effect of Ca<sup>2+</sup> overload, TME regulation, and hydrogen-immune therapy; b) Cell viability. (c) Mitochondrial damage and ATP generation inhibition; d) Ca<sup>2+</sup> content; e) ICD characterization; f) Tumor volume changes after CaH<sub>2</sub> and CaO treatment; g) H&E and Ki67 staining; h) pH value changes inside tumors. Reproduced with permission from Ref. [104]. Copyright 2022, Elsevier Ltd.

After being treated with CaH<sub>2</sub> *in vivo*, tumor growth was inhibited by the combination of Ca<sup>2+</sup> overload, H<sub>2</sub> effect, microenvironment pH change, and activated immune responses (Figure 7f). A large population of apoptotic cancer cells appeared (Figure 7g) and the pH value inside the tumor increased (Figure 7h) after CaH<sub>2</sub> treatment. In addition, with the assistance of an immune checkpoint blockade (using an anti-T-lymphocyte-associated antigen 4 antibody, aCTLA-4), CaH<sub>2</sub> NPs showed higher efficacy in both primary and distant tumors. It was obvious that, in both tumors, the percentage of total T cell and cytotoxic T lymphocyte (CTL)

infiltration remarkably increased in the combination treatment group (CaH<sub>2</sub> NPs + aCTLA-4) compared with the CaH<sub>2</sub> NPs alone and the control groups. The combination treatment could greatly decrease the percentage of T<sub>reg</sub> cells in the bilateral tumors, and also promote the polarization of macrophages from M2 to M1 type, favorable to achieving antitumor immunity. Therefore, while CaH<sub>2</sub> NPs could modulate the TME and induce cancer cell apoptosis to trigger CTL-mediated antitumor immunity, the CTLA-4 blockade could further enhance the systemic antitumor immune responses, bringing a better therapeutic effect.

H<sub>2</sub>S is a molecular marker that regulates the activity of calcium channels.<sup>[105]</sup> It is well accepted that H<sub>2</sub>S can down-regulate the permeability of Ca<sup>2+</sup> channels, inhibiting Ca<sup>2+</sup> efflux. Based on this evidence, CaS can serve as another ideal candidate to mediate H<sub>2</sub>S-enhanced Ca<sup>2+</sup> overload for cancer therapy. Zhao et al. designed a CaS-based nanosystem stabilized with PAA and zinc protoporphyrin (ZnPP@PAA-CaS) (**Figure 8a**).<sup>[106]</sup> ZnPP@PAA-CaS could release more Ca<sup>2+</sup> and H<sub>2</sub>S in the acidic microenvironment, revealing a notable clear acid-responsive decomposition ability (Figure 8b and c). As a consequence, Ca<sup>2+</sup> overload was triggered while H<sub>2</sub>S further enhanced intracellular Ca<sup>2+</sup> level, seriously damaging the mitochondria, regulating cytoskeleton disassembly for cell division/migration inhibition, and causing cell apoptosis. Besides, the protective role of HO-1, a cytoprotective enzyme protecting the cancer cells from death induced by oxidative stress, could be repressed by ZnPP. Meanwhile, ZnPP@PAA-CaS NPs could cause ICD to inactivate T cells by DCs. The *in vivo* experiments demonstrated that ZnPP@PAA-CaS NPs could impede the growth of both primary tumors and distant metastases, and increase the survival rate of mice, especially with the assistance of αPD-1 (PD-1 antibody) (Figure 8d and e). Meanwhile, the main organs of mice treated with ZnPP@PAA-CaS NPs remained normal and all mice kept a regular body weight during treatments, indicating the high biosafety and biocompatibility of these NPs. In summary, the combination of ZnPP@PAA-CaS NPs mediated Ca<sup>2+</sup> overload, H<sub>2</sub>S therapy, protective pathway inhibition, and immunotherapy possesses a high efficiency for cancer therapy.



**Figure 8.** a) Schematic illustration of ZnPP@PAA-CaS mediated signaling cascade for antitumor immunotherapy; b,c) Intracellular H<sub>2</sub>S and Ca<sup>2+</sup> content change; d) Volume changes of primary and distant tumors; e) Survival curves of mice after the different treatments. In b and c, (1) blank; (2) PAA-Ca; (3) ZnPP; (4) PAA-CaS; (5) ZnPP@PAA-CaS. In d and e, (1) blank; (2) PAA-Ca; (3) ZnPP; (4) αPD-1; (5) PAA-CaS; (6) ZnPP@PAA-CaS; (7) PAA-CaS + αPD-1; (8) ZnPP@PAA-CaS + αPD-1. Reproduced with permission from ref. 83. Copyright 2021, American Chemical Society.

### 3. Perspectives

In this review, different types of Ca<sup>2+</sup>-based nanomaterials for cancer therapy have been summarized, and the strategies to enhance Ca<sup>2+</sup> overload discussed. Besides CaCO<sub>3</sub>, CaO<sub>2</sub>, and CaP, which have been widely studied, some new Ca<sup>2+</sup>-based nanomaterials are also emerging,

such as  $\text{CaH}_2$  and  $\text{CaS}$ . All of them result in a promising platform for  $\text{Ca}^{2+}$  overload-based cancer therapy. Nevertheless,  $\text{Ca}^{2+}$  overload-mediated cancer therapy remains in its infancy, and more endeavors are urgently needed to address some limitations (such as low cellular uptake of nanomaterials, slow  $\text{Ca}^{2+}$  release, and the inhibition of  $\text{Ca}^{2+}$  overload by homeostasis regulation) to further improve the performance and obtain the satisfactory efficiency. Meanwhile, the clinical translation of  $\text{Ca}^{2+}$ -based nanomaterials faces three main challenges. First, the enrichment efficiency of  $\text{Ca}^{2+}$ -based nanomaterials in tumor sites is still low after intravenous injection. Second, the efficiency of monotherapy by  $\text{Ca}^{2+}$  overload still needs to be improved. Third, the long-term biosafety of  $\text{Ca}^{2+}$ -based nanomaterials needs to be confirmed, although the short-term biosafety is satisfactory. It is worth noting that when the concentration of intracellular increases to 1.5-2 folds, apoptosis is triggered and the tumors start to be inhibited to a certain extent.<sup>[38,41,43,59,64,68]</sup> Interestingly, once the concentration of the intracellular  $\text{Ca}^{2+}$  exceeds 10 folds, significant apoptosis happens and the tumor can be inhibited efficiently.<sup>[76,79,104,106]</sup>

Generally, the rate of cellular uptake of nanomaterials deserves further optimization, necessary not only for  $\text{Ca}^{2+}$  overload-mediated cancer therapy but for most nanomedicine therapies such as PTT, PDT, and CDT. Indeed, the prerequisite of obtaining a satisfactory curative effect is delivering enough synthesized NPs into cancer cells while avoiding uptake by normal cells. The surface modification of nanomaterials has shown great potential to enhance nanomaterials' internalization and obtain targeting capability. HA, FA, erythrocyte membrane, and homologous cancer cell membrane are currently common choices. Meanwhile, enhancing the uptake capacity (via phagocytosis or pinocytosis) is an alternative strategy.<sup>[107-110]</sup> For example, Hou et al. designed a hollow mesoporous nanoplatfrom made of Prussian blue NPs encapsulating idrossiclorochina and coated with mannose for cancer therapy, which can facilitate cellular internalization via sugar receptor-mediated endocytosis.<sup>[111]</sup> while Han et al. used cationic liposomes constituted of oleic acid, a collagen mimetic cleavable lipopeptide, and glutamate-rich segment to enhance endocytosis through surface charge reversal in response to matrix metalloproteinase 9.<sup>[112]</sup> Furthermore, the shape of nanomaterials allows to also control of the uptake rate and targeting capability. Cong et al. demonstrated that mesoporous silica nanorods can be taken up by cancer cells more efficiently than nanospheres, while the metabolism and endocytosis of nanorods were suppressed in healthy cells.<sup>[113]</sup> Moreover, the surface modification and the morphological regulation also endow  $\text{Ca}^{2+}$ -based nanomaterials with the ability to better target cancer cells rather than normal cells, which is beneficial to improve biosafety and promote further clinical applications. However, there are no detailed

studies about the effect of the shape of  $\text{Ca}^{2+}$ -based nanomaterials on triggering intracellular  $\text{Ca}^{2+}$  overload yet, which needs more effort and attention to uncovering this relation. Meanwhile, the enhanced permeability and retention effect and transcytosis bring  $\text{Ca}^{2+}$ -based nanomaterials to the tumor sites, reducing the potential side effects on normal tissues.<sup>[114,115]</sup> Furthermore, how to inhibit the exocytosis of  $\text{Ca}^{2+}$ -based nanomaterials after internalization by cancer cells and related studies to get an effective therapeutic strategy are still lacking. Promoting  $\text{Ca}^{2+}$  release after the uptake of nanomaterials is also pivotal for cancer therapy exploiting  $\text{Ca}^{2+}$  overload. Varied  $\text{Ca}^{2+}$ -based nanomaterials of different natures and compositions show different abilities to trigger  $\text{Ca}^{2+}$  overload. Due to the high stability and the slow release of  $\text{Ca}^{2+}$ , CaP nanomaterials alone usually cause a weak  $\text{Ca}^{2+}$  overload.  $\text{CaCO}_3$  has a rapidly acid response to release more  $\text{Ca}^{2+}$ . With the increase in solubility,  $\text{CaO}_2$ ,  $\text{CaS}$ , and  $\text{CaH}_2$  particles will easily release high doses of  $\text{Ca}^{2+}$ . However, to maintain the stability of  $\text{Ca}^{2+}$ -based nanomaterials before entering the cells, appropriate surface modifications are required. Meanwhile, varied methods have been developed to accelerate the dissolution of  $\text{Ca}^{2+}$ -based nanomaterials and  $\text{Ca}^{2+}$  release. As discussed above, CuS-mediated PTT could accelerate  $\text{Ca}^{2+}$  release from tricalcium phosphate nanoparticles. Efficient decomposition of  $\text{Ca}^{2+}$ -based nanomaterials in cancer cells can be considered an efficient way to increase the level of  $\text{Ca}^{2+}$ . Therefore, increasing the content of  $\text{H}^+$  will promote the degradation of  $\text{Ca}^{2+}$ -based nanomaterials due to the acid response, triggering a rapid release of  $\text{Ca}^{2+}$ . Glucose oxidase catalyzes the transformation of glucose into gluconic acid and hydrogen peroxide, enhancing the intracellular acidity of cancer cells.<sup>[116,117]</sup> A liquid metal-glucose oxidase- $\text{CaCO}_3$  composite was prepared, endowed with the capacity to accelerate the release of  $\text{Ca}^{2+}$  by gluconic acid.<sup>[59]</sup> It is well known that self-regulation of  $\text{Ca}^{2+}$  homeostasis is the main barrier to efficient  $\text{Ca}^{2+}$  overload.<sup>[118-121]</sup> This process can be broken by inducing  $\text{Ca}^{2+}$  release from ER, promoting  $\text{Ca}^{2+}$  influx, and inhibiting  $\text{Ca}^{2+}$  efflux. ER is the intracellular  $\text{Ca}^{2+}$  storage organelle and can regulate intracellular  $\text{Ca}^{2+}$  concentration. Some molecules, such as ionafarnib, can activate caspase family proteins which can trigger ER stress to release  $\text{Ca}^{2+}$ .<sup>[122]</sup> Meanwhile,  $\text{Ca}^{2+}$  channel inhibitors (e.g., CUR or T-type calcium channel blockers) have been used to hamper the efflux of  $\text{Ca}^{2+}$ .<sup>[68,123]</sup> Furthermore, NO also could stimulate  $\text{Ca}^{2+}$  release from ER by activating the opening cellular channels, leading to intracellular  $\text{Ca}^{2+}$  overload.<sup>[102]</sup> Some methods to promote  $\text{Ca}^{2+}$  influx have also been exploited. KAE and CAP are efficient regulators for  $\text{Ca}^{2+}$  influx promotion.<sup>[64,66]</sup> Alternatively, ultrasound as an exogenously physical stimulus has been used to induce  $\text{Ca}^{2+}$  influx.<sup>[68]</sup> Interestingly, hydrogen peroxide and hydroxyl radicals have displayed the dual abilities to promote influx and inhibit efflux of  $\text{Ca}^{2+}$ ,<sup>[75,76,79]</sup> while  $\text{H}_2\text{S}$  was recently



found to be able to down-regulate the permeability of the  $\text{Ca}^{2+}$  channel and inhibit  $\text{Ca}^{2+}$  efflux.<sup>[106]</sup> Another potential way to interfere with  $\text{Ca}^{2+}$  homeostasis is curtailing the conduction due to the excess of  $\text{Ca}^{2+}$ .<sup>[1,4,11,13]</sup> Varieties of signaling molecules are needed to maintain  $\text{Ca}^{2+}$  homeostasis. Therefore, antagonizing the conduction way of  $\text{Ca}^{2+}$  is helpful to induce continuous  $\text{Ca}^{2+}$  overload. Naturally, the combination of enhanced endocytosis,  $\text{Ca}^{2+}$  efflux inhibition,  $\text{Ca}^{2+}$  efflux promotion, triggering  $\text{Ca}^{2+}$  release from ER, and accelerated  $\text{Ca}^{2+}$  release from nanomaterials can further enhance the  $\text{Ca}^{2+}$  overload to obtain a better therapeutic effect. Moreover,  $\text{Ca}^{2+}$ -based nanomaterials prepared from soluble calcium salts (such as  $\text{CaCl}_2$ ,  $\text{CaBr}_2$ ,  $\text{Ca}(\text{NO}_3)_2$ , calcium aspartate, and calcium gluconate) for  $\text{Ca}^{2+}$  overload-mediated cancer therapy by suitable surface modification and encapsulation (referring to the preparation of NaCl NPs) will be an attractive choice to cause  $\text{Ca}^{2+}$  burst.<sup>[124,125]</sup>

One point that needs to be emphasized is that once cancer cell apoptosis happened, the intracellular  $\text{Ca}^{2+}$  would be released into the environment and then diffuses into the surrounding. It was reported that the extracellular concentration of  $\text{Ca}^{2+}$  is ~1300 times that of the intracellular calcium concentration (~100 nM).<sup>[126]</sup> Therefore, there is only a slight increase in the extracellular  $\text{Ca}^{2+}$  concentration after cancer cell apoptosis. The slight increase of extracellular  $\text{Ca}^{2+}$  concentration does not damage normal cells and tissues, but promotes tissue regeneration, especially in bone repair. Indeed, varied  $\text{Ca}^{2+}$ -based scaffolds and hydrogels have been developed for bone tissue engineering by releasing  $\text{Ca}^{2+}$  to stimulate osteogenic differentiation and mineralization.<sup>[127-132]</sup>

In summary, in the arena of fighting against cancer,  $\text{Ca}^{2+}$ -based nanomaterials and  $\text{Ca}^{2+}$  overload cancer therapies are demonstrating their distinctive power. The variety of enhancement strategies for  $\text{Ca}^{2+}$  overload can promote a better design of  $\text{Ca}^{2+}$ -based functional nanomaterials. It is expected that this review provides practical assistance for the utilization of  $\text{Ca}^{2+}$  overload and inspires the development of new tumor treatments.

## **Acknowledgements**

The authors greatly acknowledge the financial support from the National Natural Science Foundation of China (No. 82100974), Shandong Province Major Scientific and Technical Innovation Project (No. 2021SFGC0502), Shandong Province Key Research and Development Program (No. 2021ZDSYS18), Shandong Province Natural Science Foundation (ZR2021QH241 and ZR202102180927), Qilu Young Scholars Program of Shandong University. This work was supported by the Interdisciplinary Thematic Institute SysChem via the IdEx Unistra (ANR-10-IDEX-0002) within the program Investissement d'Avenir. We also

wish to acknowledge the Centre National de la Recherche Scientifique (CNRS) and the International Center for Frontier Research in Chemistry (icFRC). Figure 1 and ToC figure were partly from Servier Medical Art with some modification (<http://smart.servier.com/>), licensed under a Creative Commons Attribution 3.0 Generic License (<https://creativecommons.org/licenses/by/3.0/>).

### Conflict of Interest

The authors declare no conflict of interest.

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