



Tumor mineralization-based cancer diagnosis and therapy

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Abstract – Biomineralization is a phenomenon that involves the deposition of inorganic ions onto organic substrates, resulting in the formation of hard tissue materials. Tumor mineralization, on the other hand, encompasses two key aspects: tumor calcification and tumor iron mineralization. The occurrence of spontaneous tumor calcification and regional lymph node calcification in colorectal cancer, lung cancer, and glioblastoma has been established as a favorable prognostic factor in clinical settings. Building upon this understanding, we propose the concept and advance the development of a compound that artificially induces bionic mineralization around the surface of cancer cells. This process has demonstrated exceptional efficacy in inhibiting the growth and metastasis of cervical, breast, and lung tumors. Moreover, it has exhibited outstanding performance in the early-stage diagnosis of cancer. Consequently, we anticipate that this concept holds significant potential for cancer-targeted mineralization therapy and diagnosis, offering a novel avenue for the development of anticancer drugs.

Key words: Tumor mineralization, Tumor calcification, Biomineralization, Cancer diagnosis, Cancer therapy.

Introduction

Cancer is a devastating disease that poses a significant challenge to human longevity [1]. In the fight against cancer, chemotherapy has been a popular treatment option since the 1940s [2]. While surgeries can remove some solid tumors, chemotherapy is necessary to eliminate small and invisible cancer cells. However, most chemotherapy drugs not only disrupt the physiological processes of cancer cells but also have toxic effects on normal tissues, particularly the immune system. This can lead to complications such as cardiac arrest, neutropenia, and neuropathy [3]. Furthermore, chemotherapy has the potential to induce mutations in healthy tissues, which may result in long-term secondary effects including leukemia [4, 5]. Despite its widespread use, chemotherapy has shown limited contributions to overall patient survival [6].

In addition to chemotherapy, other commonly used treatments for cancer include targeted molecular therapies and immunotherapies. Unfortunately, these methods have shown

limited efficacy in treating carcinomas, such as lung cancers, primarily due to drug resistance and low response rates [7–10]. When it comes to cancer diagnosis, ultrasound and computerized tomography (CT) are standard screening tools. However, CT imaging faces challenges in detecting and differentiating tumor nodules in the early stages or when they are small, especially in distinguishing them from benign nodules [11].

Given the poor treatment efficacy and limited diagnosis ability of current methods, there is an urgent need for more effective, accurate, and minimally side-effect therapeutic and diagnostic approaches. Biomineralization is a process that involves the deposition of inorganic ions on organic substrates to form hard tissue materials [12, 13]. It plays a crucial role in the natural formation of bones and teeth [14]. Tumor mineralization encompasses two main aspects: tumor calcification and tumor iron mineralization.

In the clinic, tumor calcification has been identified as a benign prognostic factor in colorectal and lung cancer, although the underlying mechanisms are not fully understood [15, 16]. Some studies have suggested that tumor calcification may inhibit tumor cell proliferation, thus potentially having a thera-

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peutic effect on tumors [17]. Additionally, tumor calcification has shown promise in digital mammographic screening [18, 19]. However, inducing tumor calcification through exogenous calcium ions can lead to a high concentration of calcium in the body, potentially causing a hypercalcemia crisis [20]. This elevates the risk of complications such as cardiac arrest, renal failure [21], and even death. Therefore, it is important to explore methods of inducing tumor calcification using endogenous blood calcium.

In nature, certain bacterial cells have the ability to regulate iron mineralization by collecting iron ions and converting them into ferric hydroxide precipitation ($\text{Fe}(\text{OH})_3$) through adjustments in pH and redox conditions surrounding or inside the cell [22]. Although iron mineralization is not a natural physiological process in mammalian cells, it serves as an inspiration to induce tumor iron mineralization by precipitating $\text{Fe}(\text{OH})_3$. This approach creates a microenvironment within tumors that is characterized by a relatively high concentration of iron ions. Consequently, iron overload stress is induced in the mitochondria of tumor cells, which facilitates the activation of the tumor ferroptosis pathway. Simultaneously, iron mineralization provides a more powerful contrast agent compared to calcification, enabling faster response times and higher resolution and sensitivity for tumor diagnosis using clinical screening tools like ultrasound and computerized tomography. Thus, inducing tumor iron mineralization holds great potential for inhibiting tumors and improving clinical tumor diagnosis.

This paper provides a retrospective analysis of the physiological and pathological processes of calcification. It particularly focuses on the optimization of calcification induction using macromolecules such as polysaccharide-based conjugates [23] and polypeptides [24]. These tumor-targeted macromolecules promote tumor calcification through interaction with the intrinsic physiological ion concentrations of calcium. This process enhances the inhibitory effects on tumor growth and metastasis while increasing sensitivity in cancer patient screening and diagnosis. Additionally, this paper examines the use of Prussian blue/calcium peroxide nanocomposites [25], which induce iron mineralization in tumor cells and trigger oxidative stress to activate cellular apoptosis and ferroptosis pathways. As a result, the growth and metastasis of tumor cells are inhibited. This approach also greatly improves early-stage diagnosis of lung carcinomas and distinguishes them from benign nodules through medical imaging. These strategies for inducing tumor mineralization offer fresh insights into future therapy and screening diagnoses of cancer and hold immense potential for clinical applications.

Tumor biomineralization

Biomineralization is a natural process in which living organisms produce minerals, resulting in the strengthening or hardening of mineralized materials. Examples of biomineralization include the formation of silicates in algae and diatoms, carbonates in invertebrates, and calcium phosphates and carbonates in the hard tissues of vertebrates. Calcification, a type of biomineralization, is a crucial biological process in mammals, playing a significant role in the formation of bones and

teeth [14]. Research indicates that the inorganic component of mammalian hard tissues primarily consists of calcium phosphate, with the apatite structure being the predominant form [26]. Calcification is not only important in normal physiological processes but is also involved in various pathological conditions such as atherosclerosis [27], lithiasis, and heterotopic ossification [28]. Moreover, calcification has been observed in certain types of tumors following radiotherapy or chemotherapy, suggesting its potential clinical significance [29]. Interestingly, studies have shown that calcification in tumors and regional lymph nodes may serve as a favorable prognostic factor in colorectal and lung cancer [30]. In 2008, researchers led by Ruikang Tang made a groundbreaking discovery in single-cell organism calcification, demonstrating its ability to alter cell behavior. This finding sparked interest among other scientists to explore the application of biomineralization in functionalizing cells, particularly in inhibiting tumor growth [31] (Figures 1a and 1b).

Cancer cell-specific calcification induced by polypeptides

In 2021, Jicheng Wu and his colleagues proposed a novel strategy for tumor therapy and diagnosis. They developed a polypeptide that mimics protein-like macromolecules and can induce calcification of tumor cells using endogenous blood calcium ions [24]. This approach offers a safe and selective means of targeting the tumor cells. The polypeptide, called calcification-inducing polypeptide (CiP), was designed with a calcium-binding motif, $(\text{E})_{24}$, at the C-terminus and a targeting motif, TDSILRSYDWTY, at the N-terminus, specifically binding to the plasma membranes of lung cancer cells [33].

The researchers successfully synthesized CiP and conducted experiments using protein immunoprecipitation and liquid chromatography-tandem mass spectrometry (LC-MS/MS) technology. This revealed that CiP can target the extracellular domain of erythropoietin-producing hepatoma receptor A2, which is significantly upregulated in various human cancer types compared to healthy tissues. This finding suggests that CiP has the potential to be used for both early diagnosis and targeted therapy in patients with lung cancer.

To further investigate the efficacy of CiP-induced tumor calcification, the researchers conducted experiments using A549 cancer cells and demonstrated its successful application in the early diagnosis of lung carcinoma. Video 1 showed the three-dimensional CT image of lung calcification in mice. Additionally, they showed that tumor calcification inhibits cancer growth and metastasis in mouse models. These findings highlight the potential of targeted calcification for clinical applications in tumor therapy and diagnosis, with no detectable systemic side effects (Figures 2a–2d, 3a–3e).

Cancer cell-specific calcification induced by polysaccharide

In 2021, Dr. Ben Wang and his team made significant advancements in the field. Ning Tang et al. developed a novel

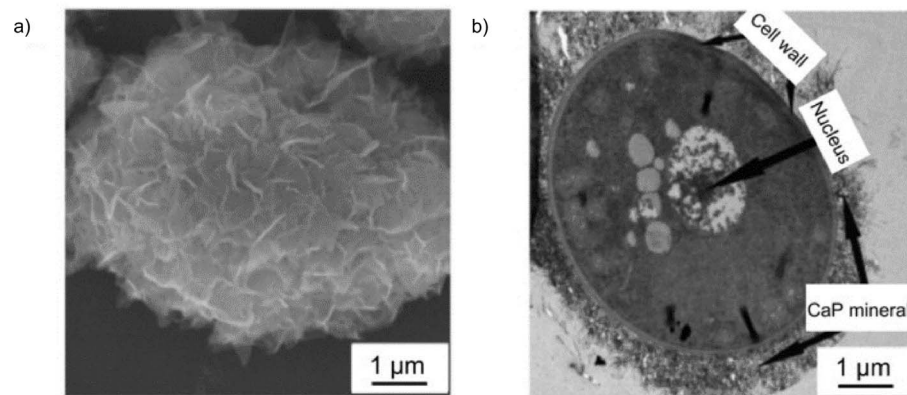
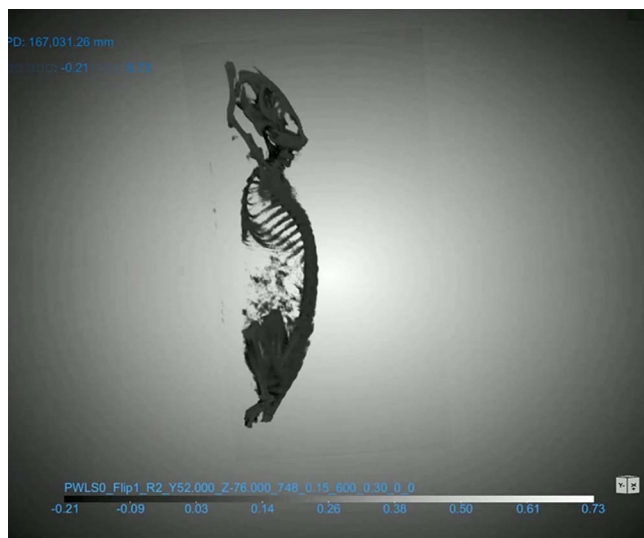


Figure 1. Calcified cells. a) Yeast cell with a calcium phosphate (CaP) mineral coat after the layer-by-layer (LbL) treatment. b) Ultrathin section image of the encapsulated cell.



Video 1. The three-dimensional CT image of lung calcification in mice. <https://vcm.edpsciences.org/10.1051/vcm/2024001#V1>.

polysaccharide-based conjugate that combines folate and polysialic acid (PolySia). They also addressed the issue of abnormally high-concentration calcium solutions in vivo, which can lead to severe conditions such as cardiac arrest, necrotizing pancreatitis, renal failure, and even death. The researchers successfully achieved tumor calcification using physiological levels of blood calcium and phosphate.

The overexpression of the folate receptor in certain types of tumors, including ovarian, lung, and breast carcinomas, is well-documented [34, 35]. Taking advantage of this, the team utilized folate to target tumor cells. Furthermore, PolySia provides carboxylate groups that facilitate the enrichment of calcium from the blood, inducing spontaneous and selective cancer cell calcification. To confirm the specificity of folate-polySia, the researchers employed human cervical epithelial (Ect1/E6E7) cells as a model for folate receptors (FR)-deficient normal cells and human cervical cancer (HeLa) cell lines as an FR-rich cancer cell model. They also conducted experiments by

attaching 5-amino fluorescein to folate-polySia to demonstrate its selective binding to specific tumor cells. Results showed fluorescence signals both on the surface and inside the cytoplasm of HeLa cells, while no signal was observed on Ect1/E6E7 cells.

Through their comprehensive efforts and further experiments comparing doxorubicin (a traditional chemotherapy drug) and folate-polySia, the team confirmed that folate-polySia effectively inhibits tumor growth by affecting the aerobic glycolysis of cancer cells. Importantly, this approach exhibited an ideal anticancer effect with minimal side effects on the host, as compared to traditional chemotherapy drugs. In conclusion, Ning Tang's research provides valuable insights into macro-molecular drug development and offers a novel strategy for extra-cellular containment and elimination of cancer cells through polysaccharide-induced calcification [23] (Figures 4a–4g).

Cancer cell-specific inhibition induced by iron mineralization

In 2021, Jicheng Wu's studies on tumor calcification revealed that the method's effectiveness in inhibiting tumors was limited due to its slow speed. This also resulted in a reduced ability to prevent tumor metastasis. Additionally, the slow speed of calcification hindered early-stage tumor diagnosis by not providing enough points for medical imaging within a short timeframe. To address these limitations, Kaixin Zhang and coworkers developed a new method inspired by the natural process of iron mineralization, where certain bacterial cells gather iron ions and transform them into insoluble forms similar to calcification [22]. Their approach centered around a nano transformational concept of tumor iron mineralization, utilizing Prussian blue ($\text{Fe}_4[\text{Fe}(\text{CN})_6]_3$, PB)– CaO_2 nanocomposites as a precursor (Figure 5). This material, approved by the U.S. Food and Drug Administration as an antidote for thallium intoxication, showed promise as a potential exogenous iron pool. The researchers effectively combined hollow mesoporous Prussian blue (HPB) with CaO_2 , which demonstrated

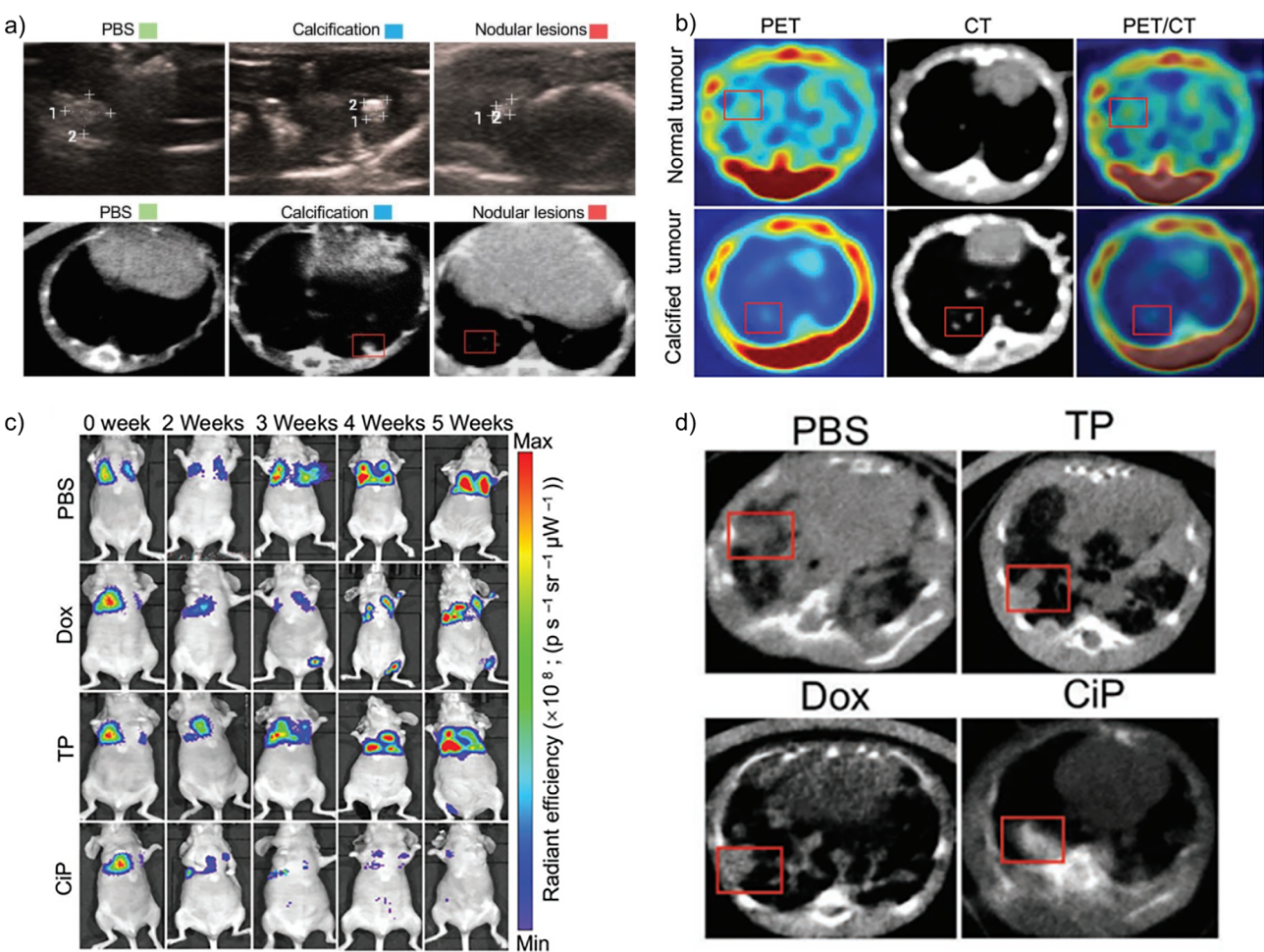


Figure 2. CT image of polypeptide-induced calcification. a) Ultrasound imaging, CT images, and quantification of calcification signals in lung tumors ($n = 8$), calcified lung tumors ($n = 8$), and lung nodules ($n = 10$) in the lungs of mice. The red box indicates a positive lesion. b) PET/CT scanning of the whole body of the mice and quantification of calcification signals in the lung tissues. The red box indicates the positive area. c) Bioluminescence images of mice subjected to the different treatments. d) CT imaging and quantification of calcification signals in mice. The red box indicates the positive area.

the ability to induce intracellular pH elevation and consistent oxidative stress in tumor cells through reactive oxygen species (ROS). Furthermore, internalized HPB-CaO₂ triggered a rapid elevation of OH⁻ ions, promoting iron mineralization (Fe(OH)₃) through the combination of Fe (II) or Fe (III). The overgenerated Fe (II) or Fe (III) also catalyzed H₂O₂ to generate more ROS, activating, the ferroptosis pathways and enhancing the antitumor efficiency.

To investigate the potential for early tumor diagnosis, the group conducted specific *in vivo* and *in vitro* experiments. These experiments confirmed that iron-based mineralized particles accumulated in tumor cells could serve as contrast agents, providing higher resolution and sensitivity for tumor diagnosis using CT, ultrasound, or magnetic resonance imaging. *In vitro* experiments further validated that HC-induced mineralization enhanced magnetic resonance imaging. Moving to the next stage, the *in vivo* antitumor ability of HC was evaluated in tumor-bearing mice with A549 lung cancer cells. The results demonstrated a higher tumor cell death rate and a remarkable

ability to suppress tumor metastasis through HC treatment. Additionally, μ CT analysis on major organs confirmed the safety of this material [25] (Figures 6a–6e).

Summary and outlook

The current progress in research has revealed the significant potential of strategies that induce mineralization in tumor cells for cancer treatment and diagnosis. The mineralization process, which involves the deposition of minerals around tumor cells, has shown to be effective in exerting cytotoxicity against tumor cells while remaining non-toxic to normal cells and the organism. Furthermore, this process allows for the early detection and diagnosis of cancer through medical imaging techniques.

Currently, there are two potential methods for inducing tumor mineralization in clinical applications: tumor calcification and iron mineralization. Tumor calcification involves the use of

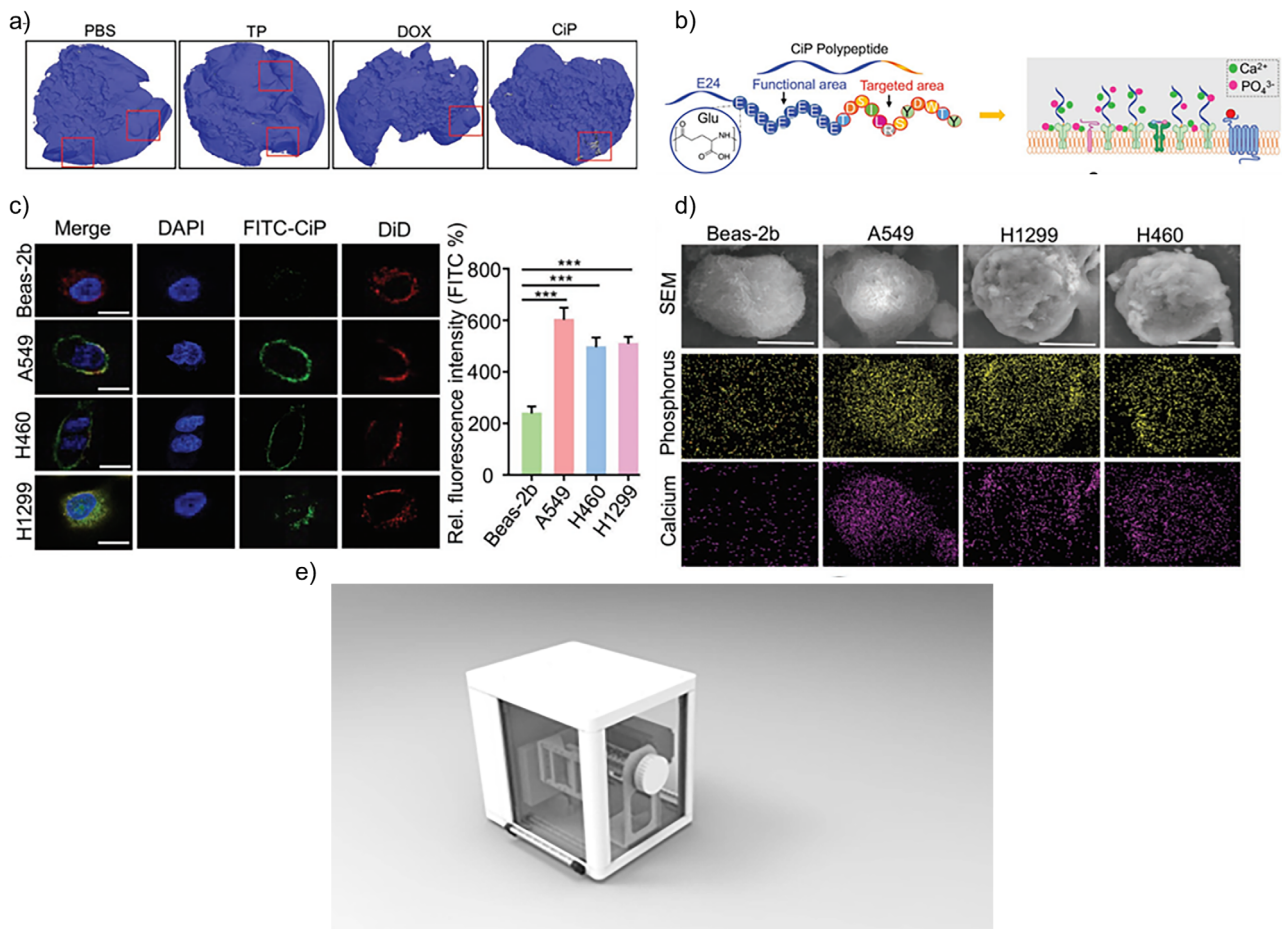


Figure 3. Image of CiP-induced tumor calcification. a) 3D-reconstructed images and quantitative analysis of lungs from tumor-bearing mice obtained by ex vivo micro-CT scanning; the red box indicates tumor location and calcification signal. b) Schematic of CiP targeting specific cell membranes and inducing calcification. c) Selective adhesion of FITC labeled CiP (200 $\mu\text{g mL}^{-1}$) on different lung cancer cells. d) The presence of a calcified shell around the cancer cells was confirmed by SEM and element mapping of EDX. e) Small animal CT equipment for taking CT.

organic macromolecules with two structural domains. One domain is designed to target tumor cells, while the other domain chelates calcium ions, thereby promoting the accumulation of calcium minerals around the tumor cells. On the other hand, tumor iron mineralization utilizes nanomaterials that are internalized by tumor cells. These nanomaterials alter the pH and cause the precipitation of iron ions from an exogenous iron pool, leading to the mineralization of iron in the tumor cells and activating pathways associated with cellular apoptosis and ferroptosis.

In the case of tumor calcification, organic macromolecules assembled from peptides, polysaccharides, and other compounds possess the necessary structural domains. The targeting domain is designed to interact with specific receptors expressed on the surface of tumor cells, such as FR and erythropoietin-producing hepatoma receptor A2. The calcium chelating domain contains highly negatively charged residues, such as carboxylate and phosphate groups. This design allows the macromolecules to selectively interact with the tumor cell membrane without undergoing endocytosis, reducing the likelihood

of drug resistance compared to conventional chemotherapy approaches. Additionally, these macromolecules utilize physiological levels of intracellular calcium ions to promote tumor calcification, eliminating the need for introducing supraphysiological concentrations of calcium ions as required in previous studies. This design strategy helps avoid the risk of hypercalcemia crisis, which can have severe consequences such as cardiac arrest, renal failure, and even death, thereby impeding clinical applications.

In the case of tumor iron mineralization, nanocomposites are required to provide an exogenous iron pool and have the ability to alter the environmental pH. The designed nanocomposite material, HPB- CaO_2 (HC), consists of hollow mesoporous Prussian blue (HPB) and CaO_2 . HPB provides active sites for iron ions and efficient drug loading, while CaO_2 , when loaded onto HPB, increases the concentration of OH^- ions through its degradation process. This increase in OH^- ions leads to the precipitation of iron ions released from HPB and the simultaneous release of H_2O_2 , which activates the ferroptosis pathway. Tumor cells, due to their higher metabolic levels

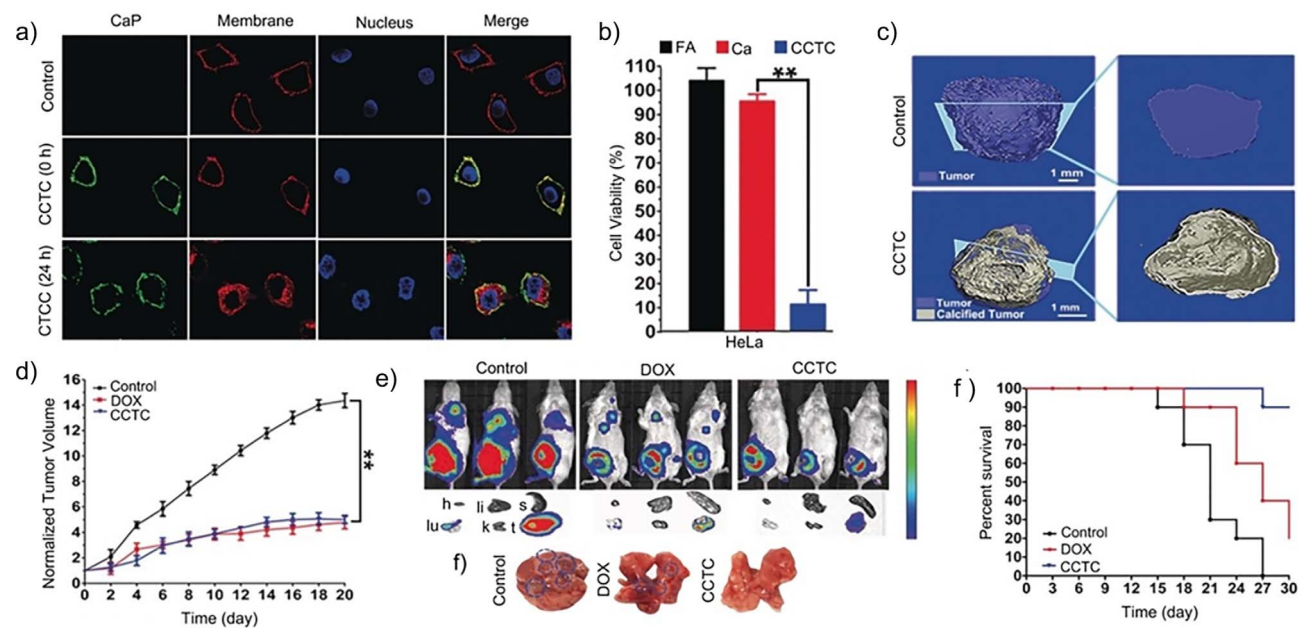


Figure 4. Image and data of polysaccharide-induced tumor calcification. a) Fluorescent detection of calcified HeLa cells by confocal laser scanning microscopy (CLSM) shows the form of calcium phosphate (CaP) after cancer cell targeting calcification (CCTC) treatment, calcium mineral was stained by Calcein (green), the cell membrane was stained by PKH26 (red), and the nucleus was stained by Hoechst33342 (blue). b) Quantitative analysis of cell viability with different treatments (***P* < 0.01). c) Micro-computed tomography (micro-CT) detection of the tumors shows in vivo CCTC treatment effects on tumors. The left column shows a three-dimensional reconstruction, and the right was a typical tomography section marked on the left. d) Normalized tumor growth curves in the control, DOX, and CCTC treatment groups (volume vs. time). e) Tumor metastasis detection by in vivo imaging (mice) and ex vivo imaging (organs); organs included the heart. f) Optical observation of pulmonary metastasis (circles). g) Survival rates of the tumor mice after DOX and CCTC treatment (*n* = 10). ***P* < 0.01.

and lower levels of catalase, endocytose more HC and generate more H₂O₂ compared to normal cells. This characteristic results in the selective killing of tumor cells while sparing normal cells. However, current research indicates that HC degrades too quickly under physiological conditions, necessitating further investigation into methods to slow down its degradation.

It is essential to identify the optimal molecular structures and concentrations of drugs that can induce tumor cell calcification without causing side effects in the organism. Additionally, the design of organic molecules or nanocomposite materials should consider different administration routes, such as oral or aerosolized inhalation, which have become increasingly popular in clinical practice due to their convenience. Moreover, besides calcification and iron mineralization, other mineral components, such as silica or iron oxide, may also serve as potential constituents for the mineral shell of tumor cells. These considerations require interdisciplinary research spanning the fields of materials science, medicine, and biology.

Based on the research above, HPB-CaO₂ (HC) and organic macromolecules assembled from peptides, polysaccharides, and other compounds can specifically target tumor cells and induce tumor mineralization. Moreover, these methods have powerful contrast effects, fast response time, high resolution, and sensitivity. In this way, we can diagnose tumors and distinguish them from benign nodules through medical imaging. So specific tumor mineralization induction holds immense potential for

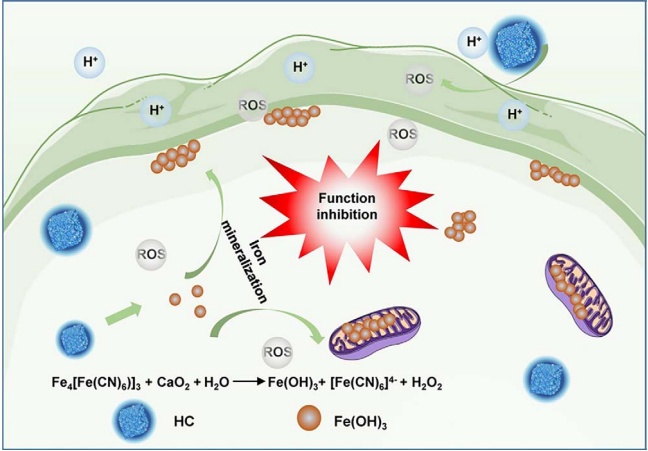


Figure 5. Scheme of HC-induced iron mineralization in tumor cells.

clinical early tumor diagnosis. At the same time, HPB-CaO₂ (HC) can trigger oxidative stress to activate cellular apoptosis and ferroptosis pathways. As a result, the growth and metastasis of tumor cells are inhibited. Beyond all doubt, HC contributes to the development of anti-tumor drugs.

Furthermore, several intriguing questions warrant further exploration. HC degrades too quickly under physiological conditions. Its degrading pathway needs further exploration.

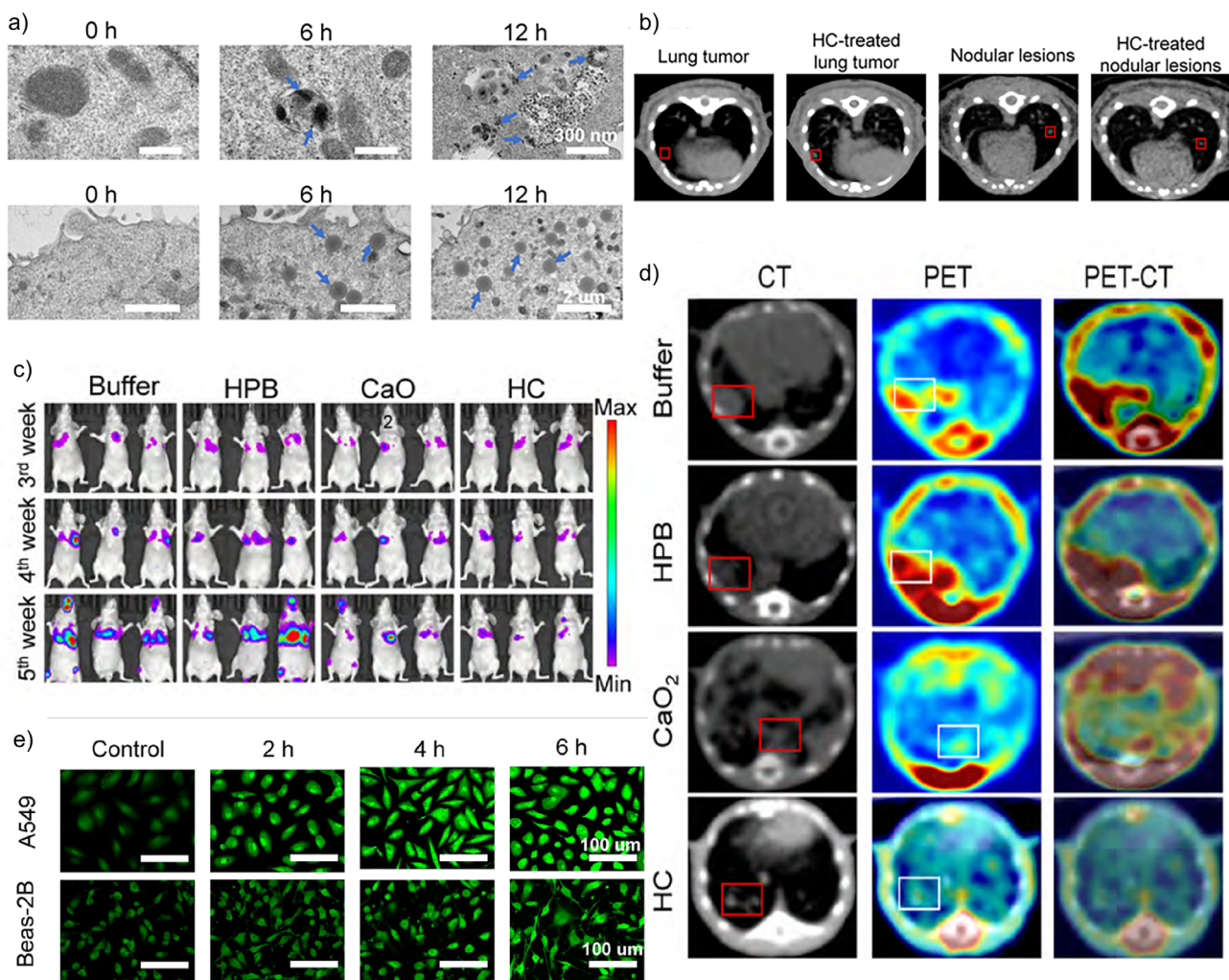


Figure 6. Images related to iron mineralization. a) TEM observation of iron mineralization on mitochondria and effects of HC on the intracellular distribution of lipids in A549 cells at different time points (0, 6, and 12 h). b) CT images (on a voxel size of $0.035 \times 0.035 \times 0.2 \text{ mm}^3$) of lung tumors, mineralized lung tumors, lung nodules, and HC-treated lung nodules; quantified CT value of HC-treated tumors or nodules/untreated tumors or nodules ($n = 3$). c) Luminescence photographs of tumor-burdened mice treated with different formulations over the 3-week treatment period. d) PET/CT analysis of the treatment efficiency of different formulations (Maximum resolution of CT = $40 \mu\text{m}$ and PET $< 1.4 \text{ mm}$). e) Confocal fluorescence microscopy images of A549 and Beas-2B cells stained by intracellular pH probe, 2',7'-bis(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester (BCECF-AM) after time-dependent HC treatments.

Some methods such as altering its chemical construction or adding some adjuvant may be practical to slow down its degradation. The biological changes triggered by tumor cell mineralization and the way it impact various signaling pathways should be further researched. This is important for clinical application and research of anti-tumor drugs. Furthermore, the response of the body's immune system to mineralization within tumor tissue. Some cannot be ignored. This matters for the prevention of adverse drug reactions. In addition, there are still some questions that remain to be solved. Can strategies for inducing tumor cell mineralization be employed to address drug-resistant tumor cells? Is there any situation where this strategy is not applicable? More studies and efforts are needed in this field to fully understand these questions before tumor mineralization-based cancer diagnosis and therapy can be realized in clinical settings.

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Conflict of interests

The authors declare that they have no conflict of interest.

Data availability statement

The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Author contribution statement

Ben Wang designed the study. Zhenyu Hu contributed to the literature search, wrote the abstract, and designed the video and figure. Jiahang Han wrote the paper and got the data for the figures. Muzhi Li wrote the introduction. Haoyu Wang wrote the summary and outlook. Hao Shou, Qingyan Zhang, Jicheng Wu, Ning Tang, and Ben Wang revised the manuscript. All authors read and approved the final manuscript.

Ethics approval

All animal experiment protocols were reviewed and approved by the Zhejiang University Animal Care and Use Committee (Approval Number: ZJU20200002) and complied with all relevant ethical regulations.

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