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Abstract

Tongue squamous cell carcinoma (TSCC) ranks among the most aggressive malignancies of the head and neck, marked by a highly acidic and immunosuppressive tumor microenvironment (TME) in which lactate accumulation persistently drives tumor-associated macrophages (TAMs) toward M2 polarization, promoting local recurrence. Given the superficial anatomical location and immunosuppressive TME of TSCC, developing local therapeutic strategies that combine spatiotemporal controllability with the capacity to reverse TME immunosuppression is of considerable significance. In this work, we construct near-infrared (NIR)-triggered phase-change core-shell

microspheres, CaO-ICG-Cap@LA, consisting of an indocyanine green (ICG) and CaO particle core encapsulated within a lauric acid (LA) phase-change shell in which capsaicin (Cap) is dissolved. Upon 808 nm NIR irradiation, ICG-mediated photothermal heating melts the LA shell (melting point $\sim 43^\circ\text{C}$), triggering the rapid release of capsaicin and CaO. The exposed CaO undergoes an exothermic hydration reaction ($\text{CaO} + \text{H}_2\text{O} \rightarrow \text{Ca(OH)}_2 + \Delta H$), the released heat augments local photothermal ablation; the resulting Ca(OH)_2 neutralizes TME lactate, disrupting lactate-driven M2 polarization and promoting TAM repolarization toward the M1 phenotype; and the liberated Ca^{2+} ions undergo massive influx through capsaicin-activated TRPV1 channels, inducing calcium overload-mediated cell death. In a CAL-27 xenograft model, CaO-ICG-Cap@LA (+ NIR) treatment produced marked tumor suppression, with extensive intratumoral calcium deposition and apoptotic signals, a shift in TAM polarization from M2 toward M1, and no evident pathological damage to major organs. By integrating TRPV1-mediated calcium overload, photothermal ablation enhancement, and lactate neutralization into a single platform, these phase-change microspheres offer a chemotherapy-independent combination strategy for TSCC treatment.

Keywords: phase-change microspheres, calcium oxide, TRPV1, calcium overload, lactate neutralization, macrophage polarization, photothermal therapy, tongue squamous cell carcinoma

1. Introduction

Accounting for a large share of oral malignancies, tongue squamous cell carcinoma (TSCC) stands among the most aggressive and lethal malignancies of the head and neck.¹⁻³ The rich vascular supply and lymphatic drainage of the tongue facilitate early regional lymph node metastasis;²⁻⁵ meanwhile, the TME of TSCC sustains an acidic and immunosuppressive milieu in which elevated lactate continuously polarizes TAMs toward the M2 phenotype.⁶⁻⁸ Although operative tumor excision together with

radiation treatment and chemotherapy represents the present clinical first-line approach, residual microscopic lesions and immune escape render local recurrence a major cause of treatment failure.⁹⁻¹² Therefore, given the superficial anatomical location and immunosuppressive TME of TSCC, developing local therapeutic strategies that integrate spatiotemporal controllability with the capacity to reverse TME immunosuppression is of considerable significance.

Near-infrared photothermal therapy (NIR-PTT) has garnered substantial attention owing to its minimally invasive nature and low systemic toxicity.¹³⁻¹⁶ However, photothermal ablation alone is limited by heterogeneous heat distribution within the tumor, with sublethal thermal stress capable of inducing thermotolerance in tumor cells, and is insufficient to eradicate residual lesions at the tumor margin.¹⁷⁻²¹ To overcome these limitations, PTT has frequently been combined with chemotherapeutic agents to enhance killing efficiency.²²⁻²⁴ Li et al. employed a cancer cell membrane-biomimetic strategy to construct a nanocarrier co-loaded with doxorubicin and indocyanine green, achieving synergistic photothermal-chemotherapy in an orthotopic oral cancer model.²⁵ Wu et al. encapsulated a cisplatin–indocyanine green coordination compound within RGD-modified exosomes, enabling NIR-triggered site-specific cisplatin release and demonstrating synergistic chemo-photothermal antitumor efficacy in an oral cancer model.²⁶ However, the introduction of chemotherapeutic agents in such combination strategies is accompanied by systemic toxic side effects and is constrained by multidrug resistance (MDR) of tumor cells.²⁷⁻³⁰ Hence, an pressing demand exists for combination therapeutic strategies that are independent of chemotherapeutic drugs.

In recent years, metal ion interference therapy has emerged as an antitumor strategy by disrupting intracellular ion homeostasis to induce tumor cell death.³¹⁻³⁴ Among these approaches, the regulation of calcium ion (Ca^{2+}) homeostasis plays a critical role in tumor cell proliferation and survival.³⁵⁻³⁷

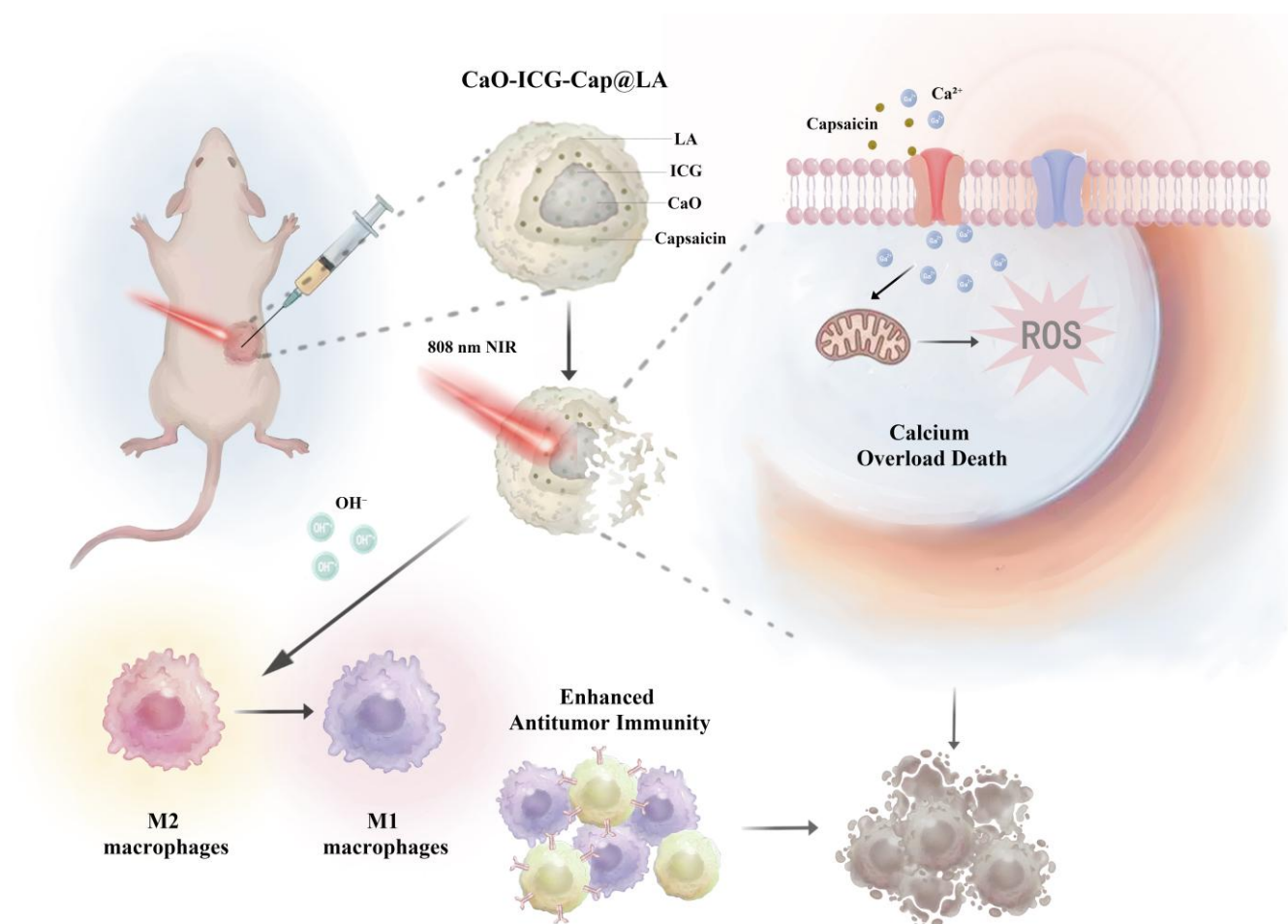
Transient receptor potential vanilloid subtype 1 (TRPV1), a non-selective cation channel, serves a

central function in cellular Ca^{2+} signaling.³⁸⁻⁴⁰ Studies have confirmed that TSCC cell lines such as CAL-27 exhibit high TRPV1 expression and are highly sensitive to capsaicin-induced channel activation.⁴¹ Capsaicin selectively activates TRPV1 on CAL-27 cells, eliciting a pronounced extracellular Ca^{2+} influx. The resulting disruption of intracellular Ca^{2+} homeostasis and mitochondrial dysfunction culminate in calcium overload-mediated cell death.⁴² Exploiting this sensitivity of TSCC cells to capsaicin offers a potential avenue for chemotherapy-independent tumor-selective killing.

However, tumor cell killing alone is insufficient to prevent recurrence, as the persistently immunosuppressive state of the TME constitutes a critical barrier to the clearance of residual tumor cells and their subsequent regrowth.⁴³⁻⁴⁶ Tumor cells undergo metabolic reprogramming via the Warburg effect, accumulating substantial quantities of lactate within the microenvironment.^{7,8,47,48} Lactate activates the HIF-1 α signaling pathway, driving TAM polarization toward the M2 phenotype and thereby facilitating tumor immune escape.^{49,50} Neutralizing TME lactate therefore represents a direct means of blocking M2 polarization. Calcium oxide (CaO) undergoes an exothermic reaction with water ($\text{CaO} + \text{H}_2\text{O} \rightarrow \text{Ca(OH)}_2 + \Delta\text{H}$); the resulting Ca(OH)_2 neutralizes lactate in situ, while the heat released augments local photothermal ablation. If the free Ca^{2+} synchronously released during this reaction is integrated with TRPV1-mediated calcium influx in TSCC cells, synergistic therapy combining enhanced hyperthermia efficacy, calcium overload, and lactate neutralization may be realized.

Based on the above analysis, this work developed NIR-triggered phase-change core-shell microspheres, CaO-ICG-Cap@LA. These microspheres comprise an indocyanine green (ICG) and CaO particle core encapsulated within a lauric acid (LA) shell in which capsaicin (Cap) is co-loaded. Upon 808 nm NIR irradiation, ICG-mediated photothermal heating melts the LA shell (melting point $\sim 43^\circ\text{C}$), triggering the rapid release of capsaicin and exposure of the CaO core. The exposed CaO undergoes an

exothermic hydration reaction ($\text{CaO} + \text{H}_2\text{O} \rightarrow \text{Ca(OH)}_2 + \Delta\text{H}$): the released heat augments local photothermal ablation; the resulting Ca(OH)_2 neutralizes TME lactate, disrupting lactate-driven M2 polarization and promoting TAM repolarization toward the M1 phenotype; and the liberated Ca^{2+} , together with capsaicin, activates TRPV1 channels, inducing calcium overload-mediated cell death. By integrating calcium overload, photothermal ablation, and lactate neutralization into a single platform, this work provides a chemotherapy-independent combination strategy for TSCC treatment.



Scheme 1. Schematic illustration of NIR-triggered CaO-ICG-Cap@LA phase-change microspheres for synergistic calcium overload, thermal ablation, and lactate neutralization therapy of TSCC.

2. Materials and Methods

2.1 Reagents and Materials

Nano calcium oxide (CaO NPs), indocyanine green (ICG), capsaicin (Cap), lauric acid (LA), polyvinyl alcohol (PVA), and cisplatin were purchased from Aladdin Biochemical Technology Co., Ltd. (Shanghai, China). Dialysis bags were obtained from Solarbio Science & Technology Co., Ltd. (Beijing, China). Penicillin–streptomycin solution, trypsin, DMEM, and FBS were procured from Servicebio (Wuhan, China).

The following assay kits and fluorescent probes were used for both in vitro and in vivo experiments: CCK-8 assay kit, Calcein-AM/PI live/dead double-staining kit, Fluo-4 AM intracellular calcium fluorescent probe, JC-1 mitochondrial membrane potential detection kit, mitochondrial superoxide indicator (MitoSOX Red), L-lactic acid (L-LA) assay kit, TUNEL apoptosis assay kit, Alizarin Red S, and hematoxylin and eosin (H&E) staining solution. All items were sourced from Servicebio (Wuhan, China).

Antibodies used for Western blotting and tissue multiplex immunofluorescence staining are listed below. Rabbit anti-iNOS, anti-Arg-1, anti-CD206, and anti-F4/80 antibodies were purchased from Immunoway. GAPDH served as the endogenous loading control, and its antibody was procured from Bioss (Beijing, China). All remaining reagents were of analytical grade and used as received.

2.2 Experimental Animals

All animal experiments were performed using female BALB/c nude mice (4–6 weeks, SPF grade, n = 25) housed at the Laboratory Animal Center of Lanzhou University. All procedures were reviewed and approved by the Laboratory Animal Ethics Committee of Lanzhou University (approval number: LZUKQ-2025-126) and conducted in accordance with the guidelines of the Laboratory Animal Science

Association.

2.3 Cell Culture Procedures

Human tongue squamous cell carcinoma CAL-27 cells and mouse monocytic macrophage RAW 264.7 cells were obtained from the Cell Resource Center of the Shanghai Institute of Life Sciences, Chinese Academy of Sciences. Cells were cultured at 37 °C in a humidified incubator with 5% CO₂ after thawing. Both cell lines were maintained in high-glucose DMEM (Hyclone) supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin/streptomycin (Solarbio). All experiments were performed using cells in the logarithmic growth phase.

2.4 Preparation of CaO-ICG-Cap@LA Phase-Change Microspheres

2.4.1 Preparation of CaO-ICG Core

CaO nanoparticles (10 mg) were dispersed in anhydrous ethanol (5 mL) and probe-sonicated in an ice bath for 2 min to obtain a homogeneous suspension. ICG (2 mg) was dissolved in anhydrous ethanol (1 mL) and added dropwise to the CaO suspension under continuous magnetic stirring. Stirring was continued for 2 h at room temperature in the dark, during which ICG adsorbed onto the CaO surface through electrostatic attraction. The resulting precipitate was collected by centrifugation (8000 rpm, 10 min), washed twice with anhydrous ethanol to remove free ICG, and dried under vacuum at room temperature overnight. The resulting dark green powder (CaO-ICG) was stored in a desiccator until further use.

2.4.2 Preparation of CaO-ICG-Cap@LA Core–Shell Microspheres

(1) Preparation of aqueous and oil phases. The aqueous phase consisted of 20 mL of 1% (w/v) PVA solution, preheated to 55 °C in a water bath. LA (60 mg) was placed in a glass vial and melted in a water bath at 55 °C.

(2) Formation of the solid–oil (S/O) dispersion. Capsaicin (5 mg) was added to the molten LA and dissolved by stirring. CaO-ICG powder (10 mg) was subsequently added and uniformly dispersed in the liquid LA by probe ultrasonication (50 W, 60 s), forming a solid–oil (S/O) dispersion.

(3) High-shear emulsification and cold-quench solidification. Under constant temperature at 55 °C, the PVA aqueous phase was stirred with a high-shear homogenizer at 8000 rpm, and the S/O dispersion was added dropwise to form an O/W emulsion. The emulsion was immediately transferred to an ice–water bath (0–4 °C) and gently stirred with a magnetic stirrer for 10 min to complete the cold-quench solidification process.

(4) Purification and freeze-drying. After solidification, the microspheres were collected by centrifugation at 3000 rpm for 5 min, washed twice with ice-cold ultrapure water, freeze-dried, sealed, and stored at 4 °C protected from light.

2.5 Photothermal Performance Evaluation of Phase-Change Microspheres

CaO-ICG-Cap@LA microspheres were dispersed in deionized water at a concentration of 1 mg/mL. Based on the actual ICG loading calculated from the microsphere synthesis feed ratio, an equivalent amount of free ICG solution was prepared as a control, and PBS was used as the blank. Each sample (1 mL) was placed in a quartz cuvette and irradiated with an 808 nm NIR laser (2.0 W/cm²) for 10 min. Real-time temperature changes were recorded using an infrared thermal camera, and temperature–time curves were plotted.

2.6 Light-Controlled Release of Capsaicin and Ca²⁺

Dialysis tubing (MWCO 3.5 kDa) was boiled in water for 10 min prior to use. CaO-ICG-Cap@LA microspheres were suspended in deionized water at 1 mg/mL, and 1 mL of the suspension was loaded into a dialysis bag and sealed at both ends. The bag was submerged in 50 mL of PBS (pH 7.4) and

maintained at 37 °C under constant magnetic stirring at 300 rpm. Continuous 808 nm NIR laser irradiation (2.0 W/cm²) was delivered to the microsphere suspension enclosed in the dialysis bag, while the control group was maintained without laser exposure. At each designated time point (0, 20, 40, 60, 80, 100, and 120 min), a 1 mL aliquot of the release medium was withdrawn and immediately replaced with an equal volume of pre-warmed PBS to maintain a constant total volume. Capsaicin levels in each sample were quantified by absorbance at 280 nm on a UV–Vis spectrophotometer, with concentrations interpolated from a standard calibration curve. For Ca²⁺ quantification, another 1 mL aliquot was acidified with 5 µL of concentrated hydrochloric acid to dissolve calcium salt precipitates, and the Ca²⁺ concentration was measured by inductively coupled plasma mass spectrometry (ICP-MS).

2.7 Detection of Intracellular Free Ca²⁺

The cell-permeable fluorescent probe Fluo-4 AM was used for qualitative observation and semi-quantitative analysis of intracellular free Ca²⁺. To permit cell attachment, CAL-27 cells in the logarithmic growth phase were seeded into 6-well plates and incubated for 24 h at 37 °C under 5% CO₂. The medium was then removed and replaced with treatment solutions according to the following groups: Control; CDDP; ICG+NIR; CaO-ICG-Cap@LA (– NIR); CaO-ICG-Cap@LA (+ NIR). Following a 4 h co-incubation, the ICG+NIR and CaO-ICG-Cap@LA (+ NIR) groups were exposed to vertical irradiation from an 808 nm NIR laser at 2.0 W/cm² for 5 min. The treatment medium was then aspirated, and the cells were rinsed with PBS. Freshly prepared Fluo-4 AM working solution was added to each well, and the plate was incubated at 37 °C in the dark for 30 min. After washing with PBS, cells were imaged by confocal laser scanning microscopy (CLSM), and semi-quantitative analysis was performed using ImageJ software.

2.8 Detection of Mitochondrial Membrane Potential and Mitochondrial Superoxide

To permit cell attachment, CAL-27 cells in the logarithmic growth phase were seeded into 6-well plates and incubated for 24 h at 37 °C under 5% CO₂. The medium was then removed and replaced with treatment solutions according to the following groups: Control; CDDP; ICG+NIR; CaO-ICG-Cap@LA (– NIR); CaO-ICG-Cap@LA (+ NIR). After 4 h of co-incubation, the ICG+NIR and CaO-ICG-Cap@LA (+ NIR) groups were vertically irradiated with an 808 nm NIR laser at 2.0 W/cm² for 5 min, after which the cells were returned to the incubator and cultured for an additional 12 h.

Mitochondrial membrane potential detection: The treatment solutions were aspirated, and the cells were washed with PBS. Cells were then loaded with freshly prepared JC-1 working solution and incubated in the dark at 37 °C for 20 min. After washing with PBS, mitochondrial membrane potential changes were visualized by CLSM.

Mitochondrial superoxide detection: Cells were stained with a freshly prepared MitoSOX Red working solution under the same grouping and treatment conditions and incubated at 37 °C for 30 min in the dark. After washing with PBS, the cells were imaged by CLSM.

2.9 Measurement of Extracellular Lactate Concentration and Detection of Macrophage Polarization Markers

A Transwell co-culture system was employed to establish an in vitro model of tumor–immune interaction between CAL-27 cells and RAW 264.7 macrophages. Logarithmic-phase CAL-27 cells (5×10^5 cells/well) were seeded into the upper Transwell chamber, and RAW 264.7 macrophages (5×10^5 cells/well) were seeded into the lower chamber. After 24 h of co-culture, the spent medium was removed and replaced with treatment solutions according to the following groups: Control; CDDP; ICG+NIR; CaO-ICG-Cap@LA (+ NIR). After 4 h of co-incubation, the ICG+NIR and CaO-ICG-

Cap@LA (+ NIR) groups were vertically irradiated with an 808 nm NIR laser at 2.0 W/cm² for 5 min, after which the co-culture system was returned to the incubator and cultured for an additional 12 h.

Following incubation, the upper Transwell chamber containing CAL-27 cells was removed, and the supernatant was collected. The extracellular L-lactic acid (L-LA) concentration was measured using an L-LA assay kit according to the manufacturer's instructions, with absorbance read at 570 nm on a multifunctional microplate reader. RAW 264.7 macrophages were harvested from the lower chamber, and total protein was extracted using RIPA lysis buffer containing protease and phosphatase inhibitors. After BCA quantification, lysates were normalized to equal protein concentrations, combined with loading buffer, and denatured by boiling. Equal amounts of protein were resolved by SDS-PAGE (220 V, approximately 30 min) and electroblotted onto PVDF membranes pre-activated in methanol (10 min) using a wet transfer system operated at 400 mA in an ice bath. The transfer time was set according to the molecular weight of each target protein: iNOS (~130 kDa), 135 min; Arg-1 (~40 kDa) and GAPDH (~37 kDa), 40 min. After transfer, the PVDF membranes were blocked with protein-free rapid blocking solution at room temperature for 20 min, then incubated with diluted primary antibodies (rabbit anti-iNOS, rabbit anti-Arg-1, and mouse anti-GAPDH) at 4 °C with gentle shaking overnight, followed by rewarming at room temperature for 1 h on the following day. The membranes were washed three times with TBST, incubated with HRP-conjugated secondary antibodies (1:5000) at room temperature for 2 h, and washed three additional times with TBST. Signals were developed with ECL substrate and captured by imaging. Protein band intensities were quantified using ImageJ software, and relative expression levels were determined by normalizing to GAPDH.

2.10 CCK-8 Cell Viability Assay

CAL-27 cells in the logarithmic growth phase were seeded into 96-well plates at a density of 5×10^3 cells/well and cultured overnight. The medium was then replaced with fresh medium containing CaO-ICG-Cap@LA microspheres at concentrations of 0, 25, 50, 100, and 200 µg/mL, and the cells were

incubated for 24 h. Thereafter, the medium was aspirated, and fresh medium supplemented with CCK-8 reagent was added to each well, followed by incubation at 37 °C for an additional 2 h. Absorbance was measured at 450 nm on a microplate reader. Cell viability was calculated using the following equation:

$$\text{Cell viability (\%)} = (\text{OD}_{\text{experimental}} - \text{OD}_{\text{blank}}) / (\text{OD}_{\text{control}} - \text{OD}_{\text{blank}}) \times 100\%$$

2.11 Live/Dead Cell Double Staining

Logarithmic-phase CAL-27 cells were seeded into 6-well plates and cultured for 24 h to allow attachment. The spent medium was then removed and replaced with treatment solutions according to the following groups: Control; CDDP; ICG+NIR; CaO-ICG-Cap@LA (– NIR); CaO-ICG-Cap@LA (+ NIR). After 4 h of co-incubation, the ICG+NIR and CaO-ICG-Cap@LA (+ NIR) groups were vertically irradiated with an 808 nm NIR laser at 2.0 W/cm² for 5 min, followed by an additional 12 h of culture. The medium was aspirated, and the cells were rinsed with PBS. Each well received a working staining solution of Calcein-AM and propidium iodide (PI), and the plate was incubated at 37 °C in the dark for 30 min. After washing with PBS, the cells were imaged by CLSM.

2.12 Establishment of Tumor Xenograft Model and In Vivo Treatment

Logarithmic-phase CAL-27 cells were trypsinized, centrifuged, washed, and resuspended in sterile PBS to a concentration of 5×10^7 cells/mL. A 100 μ L aliquot of the cell suspension ($\sim 5 \times 10^6$ cells) was drawn into a 1 mL sterile syringe and subcutaneously inoculated into the right flank of female BALB/c nude mice (4–6 weeks). Every two days, the longest (L) and shortest (W) diameters of each tumor were measured, and the tumor volume (V) was calculated using the formula $V = L \times W^2 / 2$. Once tumor volumes reached 50–100 mm³, the tumor-bearing mice were randomly allocated to five groups (n = 5):

Control group: intratumoral injection of PBS. CDDP group: intratumoral injection of cisplatin solution. ICG+NIR group: intratumoral injection of free ICG solution; at 30 min post-injection, the tumor site was irradiated with an 808 nm NIR laser at 2.0 W/cm² for 10 min. CaO-ICG-Cap@LA (– NIR) group: intratumoral injection of CaO-ICG-Cap@LA microsphere suspension without laser irradiation. CaO-ICG-Cap@LA (+ NIR) group: intratumoral injection of CaO-ICG-Cap@LA microsphere suspension; at 30 min post-injection, the tumor site was irradiated with an 808 nm NIR laser at 2.0 W/cm² for 10 min.

The above treatments were repeated once every three days for a total of five administrations.

2.13 In Vivo Antitumor Efficacy and Systemic Biosafety Evaluation

Following the group assignments and treatments described in Section 2.12, mouse body weight and tumor volume were recorded every two days. Upon completion of the treatment period, the mice were sacrificed, and the heart, liver, spleen, lungs, kidneys, and tumor tissues were collected, fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned at a thickness of 4 μm.

H&E staining: Tissue sections were dewaxed, dehydrated through a graded ethanol series, stained with hematoxylin for 5 min and eosin for 2 min, dehydrated, cleared, and mounted. Histopathological changes were observed under an optical microscope.

Alizarin Red S staining: Following dewaxing, tumor sections were incubated with Alizarin Red S solution (pH 4.2) for 5 min, counterstained with hematoxylin, mounted, and examined under an optical microscope to assess calcium salt deposition.

TUNEL staining: Staining was performed according to the manufacturer's instructions of the TUNEL apoptosis detection kit, and apoptotic cells were observed under a fluorescence microscope.

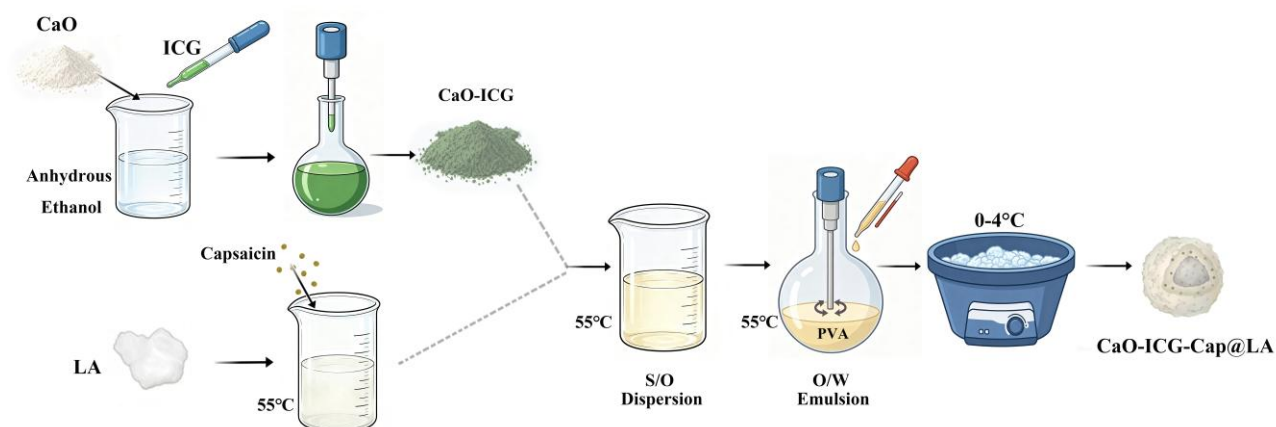
Multiplex immunofluorescence staining: Tumor sections were dewaxed, subjected to antigen retrieval, permeabilization, and blocking, and then incubated with primary antibodies against F4/80 (pan-macrophage marker), CD206 (M2 marker), and iNOS (M1 marker) at 4 °C overnight. After washing with PBS, the sections were incubated with the corresponding species-specific fluorophore-conjugated secondary antibodies at room temperature for 1 h in the dark, followed by DAPI counterstaining. Images were acquired by confocal laser scanning microscopy (CLSM), and the polarization phenotype of intratumoral macrophages was analyzed.

2.14 Statistical Analysis

All data are expressed as mean $\pm$ standard deviation (SD). Between-group differences were assessed using Student's t-test for two-group comparisons and one-way analysis of variance (ANOVA) with Tukey's post hoc test for comparisons involving three or more groups. Statistical significance was defined as $p < 0.05$ ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$; ns, not significant). All experiments were independently replicated at least three times.

3. Findings and Interpretation

3.1 Preparation and Characterization of CaO-ICG-Cap@LA Microspheres



Scheme 2. Schematic illustration of the preparation of CaO-ICG-Cap@LA phase-change microspheres.

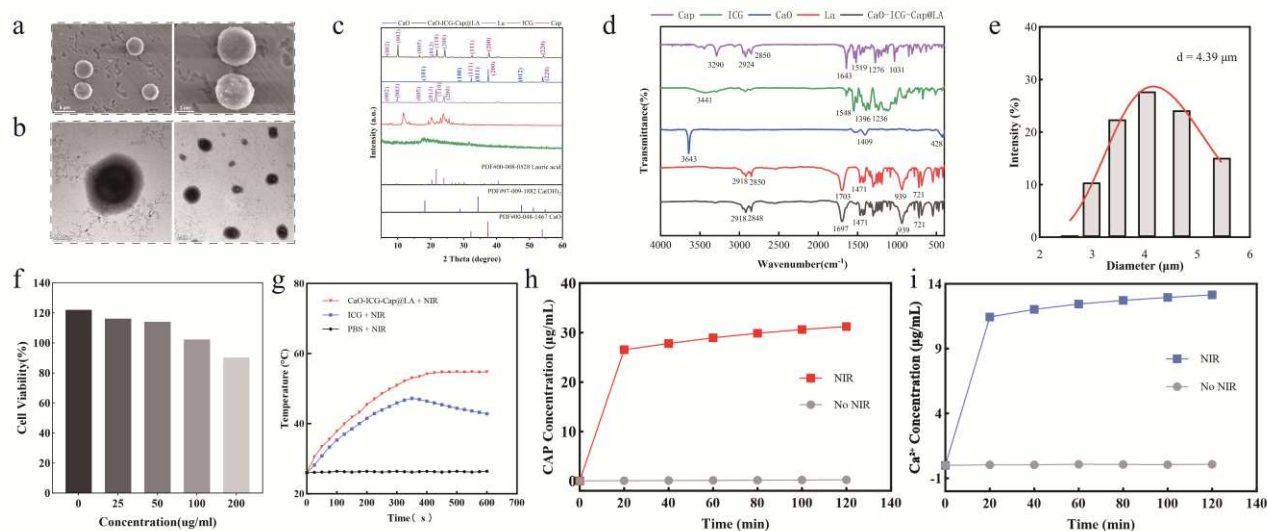


Figure 1. Morphology, structure, crystalline phase, size distribution, photothermal performance, release profiles, and cytotoxicity of CaO-ICG-Cap@LA microspheres. (a) SEM image. (b) TEM image. (c) XRD patterns. (d) FTIR spectra. (e) Hydrodynamic size distribution by DLS. (f) Cell

viability by CCK-8 assay. (g) Photothermal temperature curves under 808 nm NIR irradiation. (h) Cumulative capsaicin release. (i) Cumulative Ca^{2+} release.

Core-shell microspheres CaO-ICG-Cap@LA , with a lauric acid (LA) phase-change shell encapsulating an anhydrous CaO and indocyanine green (ICG) core, were prepared according to the method described in Section 2. The synthesis process is illustrated in **scheme 2**. Scanning electron microscopy (SEM) revealed regularly spherical microspheres with a dense surface (**Figure 1a**). Transmission electron microscopy (TEM) confirmed a distinct core-shell structure with a shell thickness of approximately 0.5–1.0 μm (**Figure 1b**). The LA shell of this thickness provides effective physical isolation of the core, serving as the structural basis for preventing premature CaO hydration during storage and in vivo delivery.

In the FTIR spectrum of CaO-ICG-Cap@LA microspheres (**Figure 1d**), the characteristic absorption peaks of capsaicin (Cap), ICG, and CaO were absent, and the overall spectral profile and principal absorption peak positions closely matched those of pure LA, indicating the encapsulation of Cap, ICG, and CaO within the LA shell. XRD analysis (**Figure 1c**) further characterized the crystalline phase composition of each component: ICG exhibited an amorphous diffuse diffraction curve with no sharp diffraction peaks; the diffraction peaks of pure LA matched standard card PDF#00-008-0528, corresponding to the (002), (003), (005), (013), (110), and (200) crystal planes; the characteristic diffraction peaks of CaO corresponded to PDF#00-048-1467 for the (111), (200), and (220) planes, with minor Ca(OH)_2 impurity peaks (PDF#97-009-1882) detected, attributable to slight surface hydration of CaO upon exposure to air. The XRD pattern of CaO-ICG-Cap@LA microspheres retained the characteristic diffraction peaks of both CaO and crystalline LA, with markedly reduced Ca(OH)_2 impurity peak intensity compared with pure CaO, indicating that the CaO crystalline phase was preserved intact during the synthesis process and that LA maintained a well-ordered crystalline structure on the microsphere surface. Amorphous ICG and Cap, lacking characteristic diffraction

signals and present at relatively low loading levels, were masked by the baseline of the crystalline phases.

Dynamic light scattering (DLS) analysis(**Figure 1e**) revealed that the microspheres exhibited a lognormal size distribution in aqueous phase ($R^2 = 0.9754$), with a geometric mean hydrodynamic diameter of $4.39 \mu\text{m}$ ($\text{GeoSD} = 1.26$). This micron-scale particle size favors local depot retention following peritumoral injection, thereby avoiding the rapid vascular clearance typical of nanoparticles.^{51,52} The zeta potential of CaO-ICG-Cap@LA microspheres was $-28.6 \pm 3.2 \text{ mV}$, indicating favorable electrostatic stability in aqueous phase. This negative surface charge mainly originates from deprotonation of carboxyl groups ($-\text{COO}^-$) on the LA shell surface and physical adsorption of the emulsifier PVA, with the high absolute surface potential providing sufficient inter-particle electrostatic repulsion that favors dispersion under physiological conditions.

3.2 Photothermal Performance and Drug Release of the Microspheres

An infrared thermal camera captured the thermal profiles of multiple samples under uninterrupted exposure to 808 nm NIR laser irradiation at a power density of 2.0 W/cm^2 (**Figure 1g**). Both the ICG solution and the CaO-ICG-Cap@LA microsphere suspension exhibited rapid temperature increases during the initial irradiation phase. The ICG solution reached a peak temperature and subsequently declined owing to photobleaching and thermal degradation. In contrast, the CaO-ICG-Cap@LA microsphere suspension surpassed the LA phase-change melting point ($\sim 43^\circ\text{C}$), continued to heat to $50.4 \pm 1.2^\circ\text{C}$, and maintained this plateau temperature throughout the entire irradiation period. This thermal stability is attributable to two factors: the LA shell physically protected the encapsulated ICG from photobleaching; and the CaO exposed upon shell melting underwent an exothermic hydration reaction ($\text{CaO} + \text{H}_2\text{O} \rightarrow \text{Ca(OH)}_2 + \Delta H$), contributing additional chemical thermal energy.

A dynamic dialysis method was used to characterize the NIR-triggered release from the microspheres, and the concentrations of capsaicin and Ca^{2+} released into the medium were quantified by UV–Vis spectrophotometry and inductively coupled plasma mass spectrometry (ICP-MS), respectively (**Figure 1h, i**). Without NIR irradiation, the cumulative release of both capsaicin and Ca^{2+} remained close to zero over the 120 min monitoring period, indicating that the solid LA shell effectively prevented leakage of the encapsulated contents. Upon exposure to 808 nm NIR irradiation, the microspheres exhibited rapid release of capsaicin and Ca^{2+} within the first 20 min, after which the release rate gradually decreased and approached a plateau. The cumulative release concentrations of capsaicin and Ca^{2+} at 120 min reached approximately 31 $\mu\text{g/mL}$ and 13 $\mu\text{g/mL}$, respectively. This release behavior is attributed to ICG-mediated photothermal heating, which raised the LA shell temperature above its melting point, triggering shell melting and the subsequent rapid release of the encapsulated contents.

3.3 TRPV1 Channel Activation and Induction of Intracellular Calcium Overload

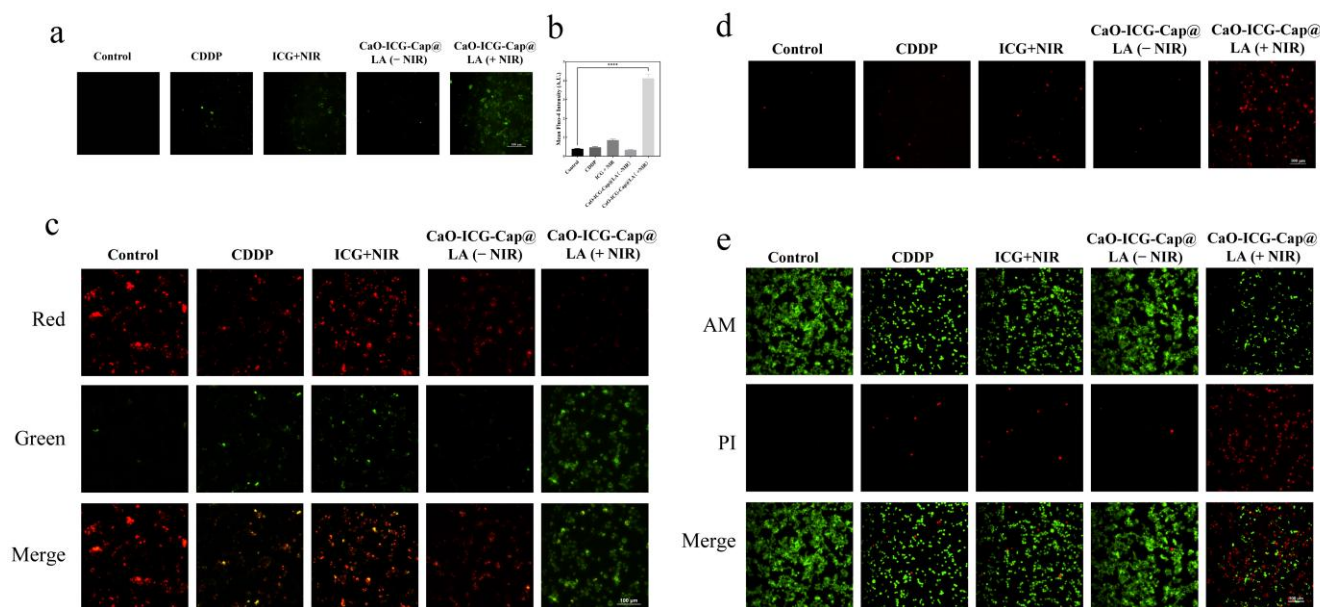


Figure 2. TRPV1-mediated calcium overload, mitochondrial dysfunction, and cytotoxicity in CAL-27 cells. (a) Fluo-4 AM fluorescence images of intracellular Ca^{2+} . The scale bar was 200 nm. (b) Semi-quantitative analysis of mean fluorescence intensity (MFI) of Fluo-4. (Data are presented as mean $\pm$ SD.

p < 0.05, **p < 0.01, *p < 0.001, ****p < 0.0001; ns, not significant.*) (c) JC-1 staining for mitochondrial membrane potential. (d) MitoSOX Red staining for mitochondrial superoxide. (e) Calcein-AM/PI live/dead staining.

Capsaicin specifically binds to and activates the TRPV1 channel, triggering extracellular Ca^{2+} influx. To verify whether CaO-ICG-Cap@LA microspheres can initiate this process upon NIR triggering, Fluo-4 AM was used as a Ca^{2+} fluorescent probe for intracellular Ca^{2+} imaging and semi-quantitative analysis in CAL-27 cells (**Figure 2a,b**). Green fluorescence was not observed in the control or CDDP group, consistent with intracellular Ca^{2+} concentrations remaining at basal levels under resting conditions and following chemotherapeutic drug treatment. The CaO-ICG-Cap@LA (– NIR) group similarly showed no detectable fluorescence signal, demonstrating that the LA shell effectively prevented CaO and capsaicin leakage in the absence of NIR triggering. The ICG+NIR group displayed weak, punctate fluorescence, suggesting that photothermal effects alone were insufficient to perturb intracellular Ca^{2+} homeostasis. In contrast, the CaO-ICG-Cap@LA (+ NIR) group exhibited intense whole-cell green fluorescence, and semi-quantitative analysis using ImageJ showed that the mean fluorescence intensity (MFI) of this group was significantly higher than that of all control groups ($p < 0.001$). These results indicate that upon NIR-triggered LA shell melting, the synchronously released capsaicin activated TRPV1 channels on the CAL-27 cell membrane, and the Ca^{2+} liberated from CaO hydration underwent massive influx through the opened TRPV1 channels, leading to intracellular calcium overload.

3.4 Detection of Mitochondrial Membrane Potential and Mitochondrial Superoxide

Excessive intracellular Ca^{2+} influx can trigger the opening of the mitochondrial permeability transition pore (mPTP), leading to mitochondrial membrane potential depolarization and a subsequent increase in mitochondrial superoxide levels.⁵³ Mitochondrial membrane potential changes were evaluated using the

JC-1 fluorescent probe across different treatment groups(**Figure 2c**). The aggregation state of JC-1 is highly dependent on mitochondrial membrane potential: in healthy mitochondria, JC-1 forms J-aggregates that emit bright red fluorescence, whereas upon mitochondrial damage and membrane potential decline, JC-1 exists as monomers emitting green fluorescence. The control, cisplatin, ICG + NIR, and CaO-ICG-Cap@LA (– NIR) groups all displayed predominantly red fluorescence, indicating that the mitochondrial membrane potential remained normal. In the CaO-ICG-Cap@LA (+ NIR) group, the red fluorescence diminished while the green fluorescence intensified, indicating depolarization of the mitochondrial membrane potential. The MitoSOX Red probe was used to detect mitochondrial superoxide levels (**Figure 2d**). The control and CaO-ICG-Cap@LA (– NIR) groups showed only low background red fluorescence. The ICG+NIR group exhibited a modest increase in fluorescence intensity. The CaO-ICG-Cap@LA (+ NIR) group displayed intense red fluorescence throughout the cells, markedly distinct from all control groups. These results demonstrate that NIR-triggered release of capsaicin and Ca^{2+} from CaO-ICG-Cap@LA microspheres induced TRPV1-mediated calcium overload, which in turn caused mitochondrial membrane potential collapse and substantial superoxide accumulation.

3.5 Extracellular Lactate Clearance and Alterations in Macrophage Polarization Phenotype

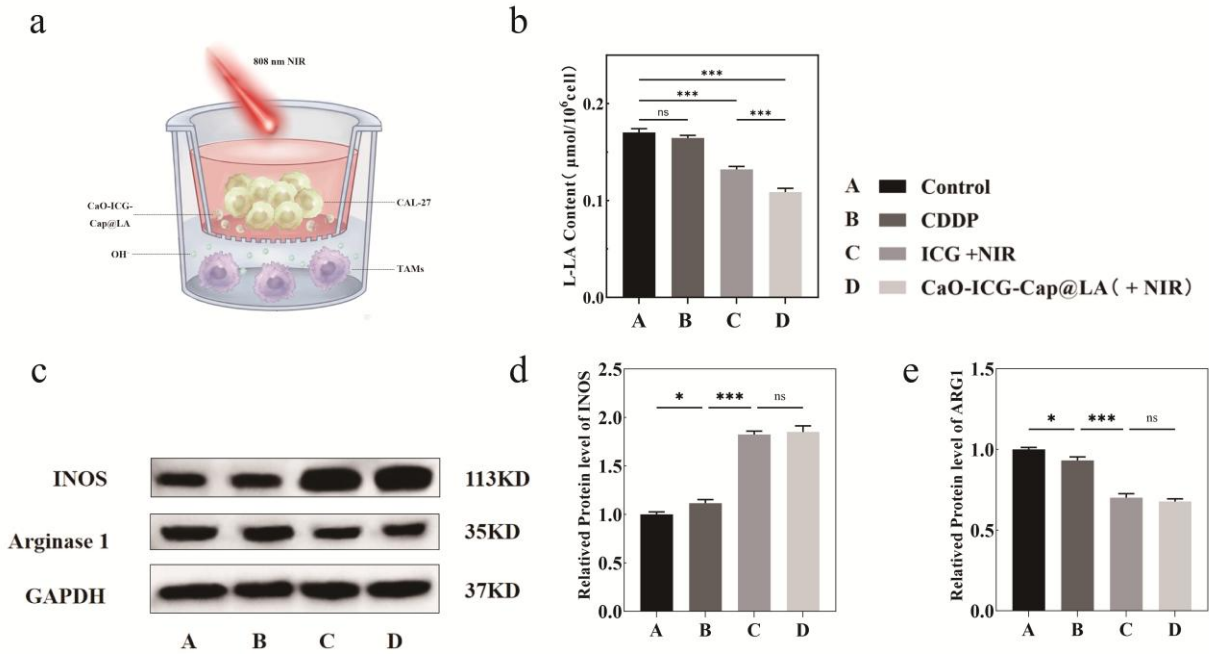


Figure 3. Microsphere-mediated lactate neutralization and macrophage repolarization in a Transwell co-culture model. (a) Schematic of the Transwell system (CAL-27 cells in upper chamber, RAW 264.7 macrophages in lower chamber). (b) Extracellular L-LA concentrations in each treatment group. (c) Representative Western blot bands of iNOS, Arg-1 and GAPDH. (d, e) Relative protein expression of iNOS (d) and Arg-1 (e) normalized to GAPDH. Data are presented as mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; ns, not significant.

A Transwell co-culture system (CAL-27 cells in the upper chamber, RAW 264.7 macrophages in the lower chamber) was employed to evaluate the effects of CaO-ICG-Cap@LA microspheres on TME lactate levels and macrophage polarization (Figure 3a). As shown in Figure 3b, the extracellular L-lactic acid (L-LA) concentrations in the control and CDDP groups showed no significant difference and both remained at high levels. The L-LA concentration in the ICG+NIR group was lower than that of the control group, attributable to the reduction in lactate-producing tumor cells resulting from

photothermal ablation. The CaO-ICG-Cap@LA (+ NIR) group exhibited the lowest L-LA concentration, differing significantly from all control groups. This outcome is ascribed to two factors: the reduction in tumor cell number through combined photothermal effects and TRPV1-mediated calcium overload, and the direct neutralization of extracellular lactate by Ca(OH)_2 generated from CaO hydration via the acid–base reaction $\text{Ca(OH)}_2 + 2\text{HLac} \rightarrow \text{Ca(Lac)}_2 + 2\text{H}_2\text{O}$.

Western blot analysis of RAW 264.7 macrophages in the lower chamber (**Figure 3c**) showed that, relative to the control group, the CaO-ICG-Cap@LA (+ NIR) group exhibited reduced Arg-1 (M2 marker) and elevated iNOS (M1 marker) protein expression. The ICG+NIR group also exhibited a partial reversal, although of a smaller magnitude than that observed in the CaO-ICG-Cap@LA (+ NIR) group. The control, CDDP, and CaO-ICG-Cap@LA (– NIR) groups were all characterized by high Arg-1 expression and low iNOS expression. These results indicate that CaO-ICG-Cap@LA microspheres, upon NIR triggering, neutralize lactate through Ca(OH)_2 .

3.6 In Vitro Cytotoxicity

The CCK-8 assay was employed to assess the in vitro cytotoxic effects of CaO-ICG-Cap@LA microspheres on CAL-27 cells (**Figure 1f**). In the absence of NIR irradiation, cells co-incubated with microspheres at all tested concentrations ($\leq 200 \mu\text{g/mL}$) for 24 h maintained a viability above 85%, meeting the ISO 10993-5 standard for non-cytotoxicity, and indicating that the LA shell effectively prevented leakage of ICG, CaO, and capsaicin prior to phototriggering. Cell status across treatment groups was examined by Calcein-AM/PI live/dead double staining (**Figure 2e**). The control and CaO-ICG-Cap@LA (– NIR) groups displayed predominantly uniform green fluorescence with virtually no red dead-cell signal. The CDDP group exhibited patchy red fluorescence, consistent with the partial tolerance of CAL-27 cells to chemotherapeutic agents. The ICG+NIR group showed localized red fluorescence regions, although the killing efficiency was limited by the single-mechanism nature of photothermal ablation. The CaO-ICG-Cap@LA (+ NIR) group was dominated by red fluorescence

across the field of view, accompanied by extensive cellular debris, indicating widespread loss of cell membrane integrity. This outcome is attributed to the synergistic killing effect produced by the synchronously released capsaicin and Ca^{2+} through TRPV1-mediated calcium overload and photothermal ablation following NIR-triggered shell melting.

3.7 In Vivo Antitumor Efficacy

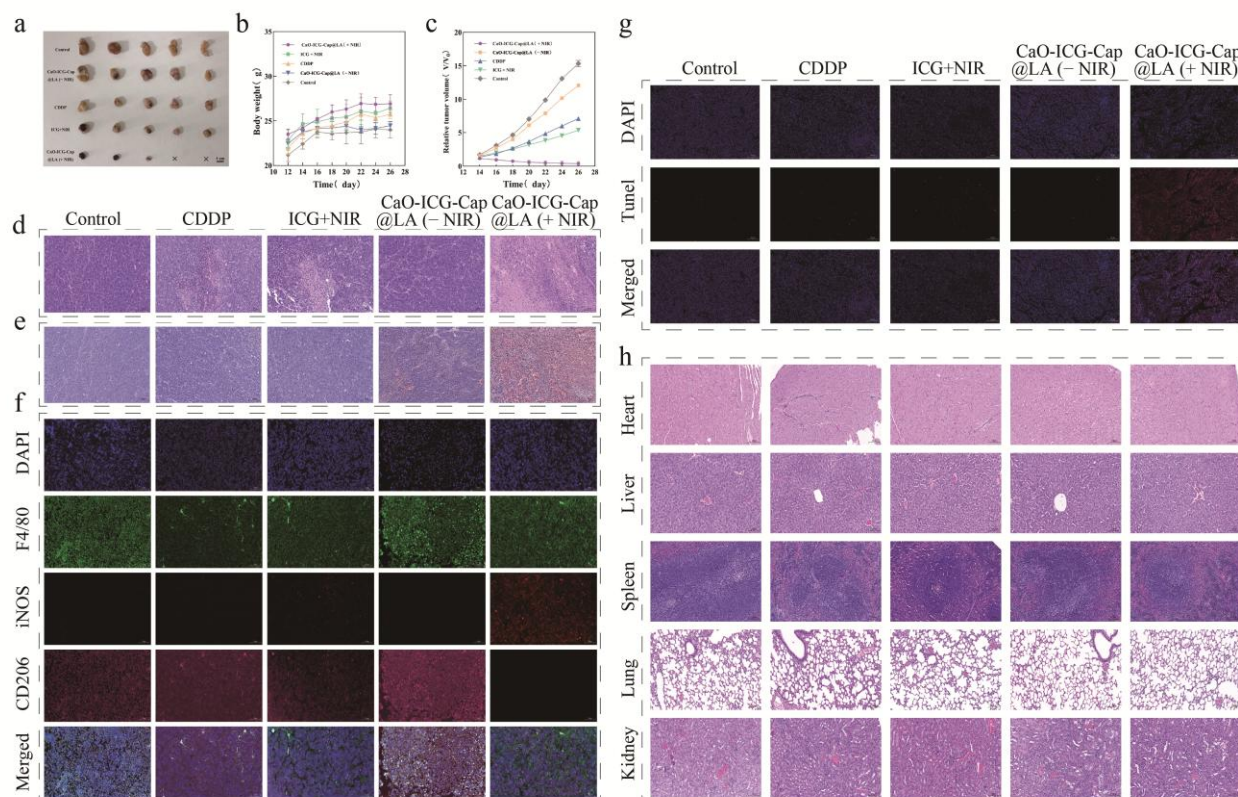


Figure 4. In vivo antitumor efficacy and biosafety evaluation of CaO-ICG-Cap@LA microspheres in a CAL-27 subcutaneous xenograft model. (a) photographs of excised tumors from each group. (b) Body weight changes during treatment. (c) Relative tumor volume growth curves. (d) H&E staining of tumor sections. (e) Alizarin Red S staining for intratumoral calcium deposition. (f) Multiplex immunofluorescence staining of F4/80, CD206, and iNOS for macrophage polarization. (g) TUNEL staining of tumor sections. (h) H&E staining of major organs (heart, liver, spleen, lung, and kidney).

3.7.1 Tumor Growth Inhibition

The in vivo antitumor efficacy of CaO-ICG-Cap@LA microspheres was evaluated in a CAL-27 subcutaneous xenograft model in nude mice. Tumor-bearing mice were randomly allocated to five groups (n = 5): control, cisplatin, ICG + NIR, CaO-ICG-Cap@LA (– NIR), and CaO-ICG-Cap@LA (+ NIR). Tumors were dissected at the end of the treatment period (**Figure 4a**). The control and CaO-ICG-Cap@LA (– NIR) groups exhibited rapid tumor volume growth with no significant difference between them, indicating that the LA shell effectively prevented payload leakage in the absence of NIR triggering and that the microspheres remained inert in vivo. The cisplatin and ICG+NIR groups showed partial tumor growth inhibition, although the tumor volumes remained relatively large, consistent with the known limitations of single-modality chemotherapy and photothermal therapy. The CaO-ICG-Cap@LA (+ NIR) group exhibited smaller tumor volumes compared with all control groups, with some tumors displaying scab formation and shrinkage. H&E staining (**Figure 4d**) revealed extensive nuclear pyknosis, karyorrhexis, and coagulative necrosis in the tumor tissues of the CaO-ICG-Cap@LA (+ NIR) group, whereas the other groups retained relatively high tumor cell density and intact tissue morphology.

3.7.2 Intratumoral Calcium Deposition

Calcium deposition in tumor tissues was detected by Alizarin Red S staining combined with hematoxylin counterstaining (**Figure 4e**). The control, cisplatin, and ICG+NIR groups all stained negative, with no evident calcium deposition observed. The CaO-ICG-Cap@LA (– NIR) group showed only occasional small punctate calcium foci in the extracellular matrix. In contrast, the CaO-ICG-Cap@LA (+ NIR) group exhibited extensive brick-red calcium deposition aggregates in necrotic regions and within cells. These results indicate that upon NIR-triggered shell melting, the CaO core was exposed and underwent a hydration reaction in vivo, releasing Ca^{2+} and resulting in massive intratumoral calcium deposition.

3.7.3 Detection of Intratumoral Macrophage Polarization Phenotype

Multiplex immunofluorescence staining (antibodies targeting F4/80 (pan-macrophage), CD206 (M2), and iNOS (M1)) was performed to evaluate the polarization status of intratumoral TAMs (**Figure 4f**). The control, cisplatin, and CaO-ICG-Cap@LA (– NIR) groups all displayed F4/80 and CD206 co-localization signals in the tumor stroma, with relatively low iNOS signals, indicating that the microenvironment without metabolic intervention was dominated by an M2-type immunosuppressive state. The ICG+NIR group showed attenuated CD206 signals and partially elevated iNOS signals compared with the control group, exhibiting a partial reversal trend, which was attributable to the reduction in lactate-producing tumor cells caused by photothermal ablation. The CaO-ICG-Cap@LA (+ NIR) group demonstrated diminished CD206 signals and enhanced iNOS signals. These results indicate that the Ca(OH)_2 generated from CaO core hydration neutralized TME lactate, alleviating the lactate-driven M2 polarization and promoting TAM repolarization toward the M1 phenotype. Given that the nude mouse model lacks adaptive immunity, the Ca(OH)_2 -mediated TME alkalization and M1 polarization observed herein may be further amplified through dendritic cell maturation and CD8^+ T cell priming in immunocompetent hosts;⁵⁴ this hypothesis remains to be validated in immunocompetent models.

3.7.4 TUNEL Staining for Detection of Tumor Tissue Apoptosis

TUNEL staining analysis was performed on tumor sections from each group aimed at assessing the therapeutic efficacy of CaO-ICG-Cap@LA microspheres (**Figure 4g**). The control and CaO-ICG-Cap@LA (– NIR) groups showed almost no TUNEL-positive fluorescence signal. The cisplatin and ICG+NIR groups displayed only scattered punctate apoptotic signals resulting from single-modality chemotherapy or thermal damage. The CaO-ICG-Cap@LA (+ NIR) group exhibited markedly enhanced TUNEL-positive fluorescence, with apoptotic cells distributed over a large and diffuse area. This outcome is primarily attributable to the synergistic action of three mechanisms following NIR-

triggered shell melting: capsaicin released from the microspheres activated TRPV1 channels, inducing calcium overload; the CaO hydration reaction provided chemical thermal energy that augmented photothermal effects; and the resulting Ca(OH)_2 neutralized TME lactate and relieved immunosuppression, collectively inducing tumor cell apoptosis.

3.8 In Vivo Biosafety Evaluation

H&E staining of the major organs (heart, liver, spleen, lungs, and kidneys) from each group (**Figure 4h**) revealed normal cell morphology and tissue architecture in all groups, with no evident inflammatory infiltration or pathological damage, indicating that CaO-ICG-Cap@LA microspheres exhibit favorable in vivo biosafety at the experimental dose.

4. Conclusion

In this study, we developed NIR-triggered phase-change core-shell microspheres, CaO-ICG-Cap@LA, for local ablation and microenvironment-modulating therapy of tongue squamous cell carcinoma (TSCC). The microspheres exhibited favorable colloidal stability in aqueous phase, and their 4–5 μm particle size conferred a local depot effect following peritumoral injection, circumventing the rapid systemic clearance typically associated with conventional nano-formulations. In vitro and in vivo experiments demonstrated that upon 808 nm NIR irradiation, the photothermal conversion of the encapsulated ICG drove solid-to-liquid phase transition of the LA shell, achieving on-demand release of the payloads. The therapeutic mechanism of this platform is based on three synergistic aspects:

1. TRPV1-mediated calcium overload: The synchronously released capsaicin activates TRPV1 channels on the TSCC cell membrane, driving massive influx of Ca^{2+} liberated from CaO hydration, disrupting intracellular ion homeostasis, and inducing mitochondrial dysfunction and

calcium overload-mediated cell death, thereby circumventing the drug resistance issues associated with conventional chemotherapeutic agents.

2. Synergistic photothermal–chemical thermal ablation: The exposed CaO undergoes an exothermic reaction with water ($\text{CaO} + \text{H}_2\text{O} \rightarrow \text{Ca(OH)}_2 + \Delta\text{H}$), with the released heat augmenting local photothermal ablation.
3. Lactate neutralization-driven TAM polarization reversal: The Ca(OH)_2 generated from CaO hydration neutralizes lactate accumulated in the TME, disrupting the lactate-mediated M2 polarization signaling and driving TAM repolarization from the M2 toward the M1 phenotype.

In a CAL-27 xenograft model, the CaO-ICG-Cap@LA (+ NIR) group exhibited marked tumor suppression. H&E staining revealed no evident pathological damage to major organs, indicating that the microspheres possess favorable in vivo biosafety at the experimental dose. In summary, this work integrates ion interference therapy with TME metabolic modulation, providing a chemotherapy-independent combination strategy for TSCC treatment.

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Author contributions

Conceptualization: D.H., C.C. Supervision: D.H. Methodology: C.C. (microspheres construction); C.C., J.Z., J.G. (cellular experiments, in vivo antitumor experiments); Formal Analysis: C.C., J.G., X.W. (fluorescent and electronic imaging, photothermal performance, drug release quantification, Western blot quantification). Visualization: J.G., X.W. (schematic drawing). Writing – Original Draft: C.C., J.Z. Writing – Review & Editing: C.C., D.H.

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Availability of data and materials

No datasets were generated or analysed during the current study.

Ethics approval and consent to participate

This article does not contain any studies with human participants or animals performed by any of the authors. All animal experimental protocols were performed strictly following the guidelines established by the Association of Laboratory Animal Sciences and the Center for Laboratory Animal Sciences at Lanzhou University (approval number: LZUKQ-2025-126).

Consent for publication

All authors have read and approved the final manuscript and consent to its publication in Cancer Nanotechnology.

Conflicts of interest

The authors declare no conflicts of interest.

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