

Engineering Necroptosis-Induced Vesicular Nanocarriers for Homologous Targeted Melanoma Therapy

Zhenbin Dong, Yubo Niu, Jingxia Xu, Junfeng Wang, Wanting Yang, Guofeng Li, Xing Wang, and Wensheng Xie*



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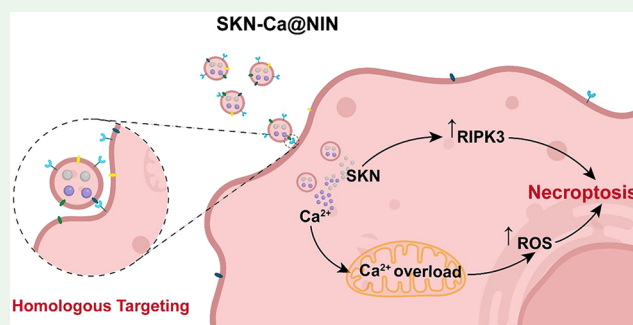
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ABSTRACT: Melanoma is a highly aggressive malignancy with limited therapeutic options, highlighting the need for efficient tumor-targeted drug delivery strategies based on nanomaterials. Herein, we report a necroptosis-induced biogenesis strategy for constructing tumor-derived biomimetic nanocarriers for homologous targeting in melanoma therapy. Shikonin-loaded amorphous calcium carbonate nanoparticles (SKN@Ca NPs) were synthesized and used as nanoinducers to trigger necroptosis in melanoma cells. During this process, cells released membrane-derived vesicles termed necroptotic bodies that simultaneously encapsulated internalized SKN@Ca NPs, resulting in the *in situ* formation of necroptosis-induced nanocarriers (SKN-Ca@NIN). The obtained SKN-Ca@NIN retained membrane proteins from parental tumor cells and therefore exhibited biomimetic vesicular structures and homologous targeting capability toward melanoma cells. *In vitro* studies demonstrated that SKN-Ca@NIN showed favorable biosafety toward normal cells while effectively inhibiting melanoma cell proliferation. Mechanistically, intracellular degradation of the calcium carbonate core triggered Ca^{2+} release, while shikonin promoted reactive oxygen species (ROS) accumulation, leading to severe ionic and oxidative stress that disrupted tumor cell homeostasis. This work provides an *in situ* necroptosis-induced nanocarrier platform for efficient tumor accumulation and therapy via a homologous targeting strategy.

KEYWORDS: necroptosis, biomimetic nanocarriers, homologous targeting, shikonin, melanoma



1. INTRODUCTION

Melanoma is one of the most aggressive forms of skin cancer and is characterized by rapid metastasis and poor prognosis.^{1,2} Despite recent advances in targeted therapy and immunotherapy, conventional chemotherapy remains an important treatment modality. However, the nonspecific distribution of chemotherapeutic agents often leads to severe systemic toxicity and limited therapeutic efficacy.^{3,4} Therefore, the development of tumor-targeted drug delivery systems has become an important strategy for improving melanoma treatment.⁵

Nanomedicine has shown great potential to enhance drug delivery efficiency and reduce systemic toxicity.^{6,7} Among various approaches, biomimetic nanocarriers based on cell membranes have attracted significant attention due to their unique biological functions, including immune evasion, prolonged circulation, and tumor targeting.^{8,9} In particular, cancer cell membrane-coated nanoparticles possess homologous targeting capability because they retain membrane adhesion molecules and tumor-associated antigens derived from parental cells. These features enable the nanoparticles to preferentially recognize and accumulate in tumors of the same cellular origin.¹⁰ Mechanistically, the *in situ* membrane strategy

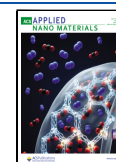
confers two critical functionalities to the nanocarriers: (i) stealth properties—primarily mediated by self-recognition markers such as CD47 that reduce phagocytic clearance and prolong systemic circulation; (ii) homologous targeting ability—driven by native membrane adhesion molecules and tumor-associated antigens, which facilitate preferential binding and internalization by the original tumor cell type.¹¹ However, most current biomimetic nanocarriers are fabricated using physical methods such as membrane extrusion, ultrasonication, or freeze–thaw cycles.^{12–14} These procedures can disrupt membrane structures and denature functional proteins, thereby impairing the nanocarriers' biological activity and targeting performance. Therefore, developing a mild, biologically driven strategy for constructing biomimetic nanocarriers while preserving membrane integrity remains an important challenge.

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Necroptosis is a regulated form of programmed necrotic cell death characterized by plasma membrane rupture and the release of intracellular vesicles.¹⁵ During this process, cells generate membrane-bound vesicular structures known as necroptotic bodies, which contain cellular components and preserve membrane proteins from the parental cells.^{11,16} These vesicles provide a natural biological platform that may serve as potential carriers for drug delivery. Shikonin, a natural naphthoquinone, exhibits potent antitumor activity primarily through the generation of reactive oxygen species (ROS) and activation of the RIPK1/RIPK3/MLKL necroptosis pathway.^{17,18} However, its clinical translation is severely hindered by poor water solubility, low bioavailability, and off-target toxicity when administered as a free drug. Moreover, its redox behavior is concentration-dependent and often insufficient to trigger robust oxidative stress in tumor cells without a suitable nanoscale delivery system. Amorphous calcium carbonate (ACC) is a pH-responsive inorganic material that remains stable under physiological conditions (pH 7.4) but undergoes rapid degradation in acidic microenvironments, such as endosomes/lysosomes (pH 4.5–5.5) or the tumor extracellular milieu.¹⁹ Upon degradation, ACC releases Ca^{2+} ions, leading to intracellular calcium overload, which disrupts mitochondrial membrane potential and exacerbates ROS-mediated damage. Notably, the nanoscale size of ACC particles dramatically accelerates its degradation kinetics and enables more uniform Ca^{2+} release compared to bulk or microscale counterparts.

Inspired by the biological process of necroptosis, we propose a necroptosis-induced biogenesis strategy to construct tumor-derived nanocarriers for melanoma therapy. In this study, shikonin-loaded amorphous calcium carbonate nanoparticles (SKN@Ca NPs) were synthesized and used as nanoinducers to trigger necroptosis in melanoma cells.²⁰ During the necroptotic process, cells released necroptotic bodies that simultaneously encapsulated the internalized SKN@Ca NPs, leading to the *in situ* formation of necroptosis-induced nanocarriers (SKN-Ca@NIN). Importantly, because these nanocarriers are generated directly from necroptotic melanoma cells without physical extrusion or sonication, they naturally preserve the integrity of parental membrane proteins—including both stealth-related markers (e.g., CD47) and homologous targeting-associated adhesion molecules. As a result, SKN-Ca@NIN inherits the dual functionality of immune evasion and homologous targeting, which distinguishes it from artificially fabricated biomimetic carriers. The resulting nanoplateform combines necroptosis-induced *in situ* generation with homologous targeting and Ca^{2+} /ROS-mediated cytotoxicity, providing a novel strategy for precision melanoma therapy.

2. EXPERIMENTAL SECTION

2.1. Materials

Anhydrous calcium chloride, ammonium bicarbonate, and anhydrous ethanol was purchased from Macklin Biochemical Co., Ltd. Shikonin (SKN, HPLC $\geq 98\%$) was purchased from Tianjin Zancheng Technology Co., Ltd. RPMI 1640 basal medium, RPMI 1640 complete medium, phosphate-buffered saline (PBS), trypsin (with/without 0.25% EDTA), fetal bovine serum (FBS), and 4% paraformaldehyde (PFA) fixative were purchased from Gibco (Thermo Fisher Scientific, USA). Reagents for biological evaluations, including the bicinchoninic acid (BCA) protein assay kit, Triton X-100 cell lysis buffer, 4',6-diamidino-2-phenylindole (DAPI), Coumarin 6, Rhodamine phalloidin, Annexin V-FITC Apoptosis Detection

Kit, Calcein-AM, Cell cycle detection kit, Fluo-4 AM, 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA), and Cell counting kit-8 (CCK-8) were purchased from Solarbio Science & Technology Co., Ltd. (Beijing, China).

2.2. Synthesis of SKN@Ca NPs

Fully degradable shikonin-loaded amorphous calcium carbonate nanoparticles (SKN@Ca NPs) were synthesized *via* a biomineralization strategy. Briefly, CaCl_2 (150 mg) and shikonin (7.5 mg, HPLC $\geq 98\%$) were dissolved in 100 mL of anhydrous ethanol under magnetic stirring at room temperature. The solution was sealed with perforated Parafilm and placed in a vacuum desiccator containing 10 g NH_4HCO_3 . Mineralization was conducted at 37 °C for 48 h. The precipitate was collected by centrifugation (8,000 rpm, 10 min, 4 °C), resuspended in 10 mL anhydrous ethanol, and stored at 4 °C. For concentration determination, 0.5 mL of the suspension was lyophilized (−55 °C, 24 h), and the dried mass was weighed. The SKN content in SKN@Ca was quantified by UV absorption spectrometry. The SKN concentration was 15.6 $\mu\text{g}/\text{mL}$ for SKN@Ca.

2.3. Preparation and Isolation of SKN-Ca@NIN and SKN@NIN

Autologous SKN-Ca@NIN was prepared *via* a necroptosis-induced *in situ* biogenesis strategy under sterile conditions. B16F10 cells were cultured in T75 flasks to 100% confluence, washed twice with PBS (pH 7.4), and incubated with 20 mL RPMI 1640 complete medium containing 300 $\mu\text{g}/\text{mL}$ SKN@Ca NPs at 37 °C with 5% CO_2 for 24 h. The culture supernatant was collected and subjected to sequential centrifugation at 4 °C and 2500 rpm for 5 min to remove cell debris, 8000 rpm for 10 min to eliminate free SKN@Ca NPs, and 10,000 rpm for 20 min to pellet SKN-Ca@NIN. The final pellet was resuspended in 1 mL sterile RPMI 1640 basal medium and stored at 4 °C. The intact tumor cell membrane structure of SKN-Ca@NIN allowed total membrane protein content to be used as a surrogate for its relative concentration, which was quantified using a bicinchoninic acid (BCA) protein assay kit. SKN-Ca@NIN samples were resuspended in serum-free RPMI 1640 medium to avoid interference from fetal bovine serum, then diluted 2-, 4-, and 8-fold. A volume of 20 μL of each diluted sample was added in triplicate. Subsequently, 200 μL of BCA working solution was added to each well, followed by incubation at 37 °C for 30 min in the dark. Absorbance at 562 nm was measured using a microplate reader, and protein concentrations were calculated based on the BSA standard curve. SKN@NIN was prepared using the same procedure, except that B16F10 cells were treated with free SKN at 4 $\mu\text{g}/\text{mL}$ (without CaCO_3) instead of SKN@Ca NPs. The SKN content in SKN-Ca@NIN was quantified by Ultraviolet–Visible spectrophotometer (UV–vis Spectrophotometer, UV-6100, Mapada Instruments Co., Ltd., Shanghai, China). The SKN concentration was 2.35 $\mu\text{g}/\text{mL}$ for SKN-Ca@NIN, the SKN loading content in SKN-Ca@NIN is 0.247% (w/w).

2.4. Characterization

The morphology and microstructure of SKN@Ca NPs and SKN-Ca@NIN were characterized by transmission electron microscopy (TEM, Hitachi HT7700, Japan) operated at an acceleration voltage of 100 kV. For TEM observation, the nanoparticle suspension was diluted to an appropriate concentration, dispersed by ultrasonication (KQ3200DE, Kunshan Ultrasonic Instruments Co., China) to avoid aggregation, and then a small drop of the suspension was placed onto a carbon-coated copper grid and air-dried before imaging. Particle size was measured by ImageJ software (Rasband, USA) based on TEM images. The crystal structure of SKN@Ca NPs was analyzed by X-ray powder diffraction (XRD, Bruker D2 PHASER, Germany) over a 2θ range of 10°–80°. Fluorescence imaging was performed using a laser scanning confocal microscope (LSCM, Leica TCS SP8), and flow cytometry analysis was conducted on a CytoFLEX flow cytometer (Beckman Coulter, USA).

2.5. Western Blot

B16F10 cells were seeded in 6-well plates (5×10^5 cells/well) and cultured overnight. After adherence, cells were treated with SKN@Ca

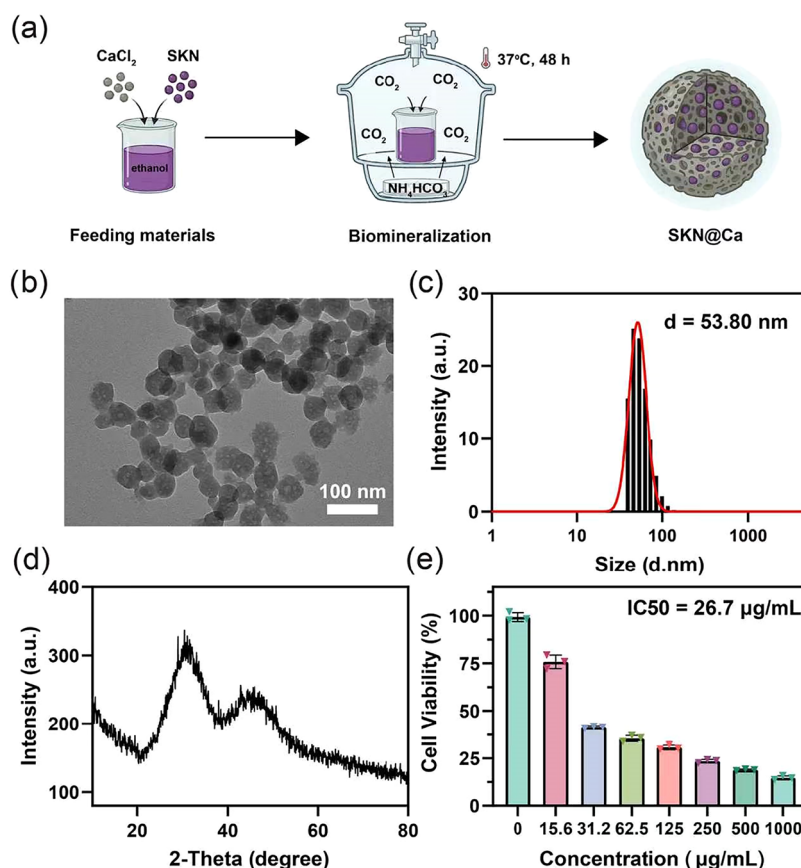


Figure 1. Preparation and characterization of SKN@Ca NPs. (a) Schematic diagram of the synthetic procedure *via* biomineralization; (b) TEM images of SKN@Ca NPs; (c) Size distribution of SKN@Ca NPs measured by DLS. (d) XRD pattern of SKN@Ca NPs; (e) Cell viability of B16F10 cells after treatment with various concentrations of SKN@Ca NPs.

NPs (100 μg/mL) for 24 h; the control group received equal-volume PBS. After incubation, cells were washed with precooled PBS, lysed with RIPA buffer (with 1% PMSF) on ice, centrifuged (12,000 rpm, 4 °C, 15 min), and the supernatant (total protein) was collected. Protein concentration was determined by the BCA assay. Equal protein (30 μg/lane) was denatured, loaded onto 10% separating gel/5% stacking gel, and electrophoresed (80 V for stacking gel, 120 V for separating gel). Proteins were transferred to PVDF membrane (300 mA, 90 min), blocked with 5% nonfat milk in TBST for 2 h at room temperature. The membrane was incubated with primary antibodies against RIPK3 and GAPDH (per manufacturer's dilution) at 4 °C overnight, washed with TBST, then incubated with HRP-conjugated secondary antibody (1:5000 in TBST) for 1.5 h in the dark. After TBST washing, protein bands were visualized by ECL and imaged.

2.6. *In Vitro* Cytotoxicity Measurement

The *in vitro* antitumor activity of SKN-Ca@NIN was evaluated using a CCK-8 assay. B16F10 cells in the logarithmic phase were seeded into 96-well plates at 5×10^3 cells per well in 200 μL RPMI 1640 complete medium and incubated at 37 °C with 5% CO₂ for 24 h. After adherence, cells were treated with 100 μL of complete medium containing SKN-Ca@NIN at 0, 30, 60, 120, 230, 480, and 710 μg/mL, with six replicates per group and a blank control without cells. Following 24 h incubation at 37 °C with 5% CO₂, the medium was removed and replaced with 100 μL of CCK-8 working solution prepared in serum-free RPMI 1640 (10:1, v/v). After incubation at 37 °C for 2 h in the dark, 80 μL of supernatant was transferred to a new 96-well plate, and absorbance at 450 nm was measured using a microplate reader. The half-maximal inhibitory concentration (IC₅₀) was determined by nonlinear regression using GraphPad Prism.

2.7. *In Vitro* Homologous Targeting Evaluation

In vitro homologous targeting and tumor selectivity of SKN-Ca@NIN were evaluated using B16F10 melanoma cells as homologous cells and 4T1 breast cancer cells as heterologous controls. Experimental conditions, including seeding density (5×10^3 cells per well), drug concentration gradients, incubation time (24 h at 37 °C with 5% CO₂), and CCK-8 assay procedure, were consistent with Section 2.5. Cell viability was assessed with six replicates per group, and cytotoxicity curves of B16F10 and 4T1 cells were compared to evaluate selective uptake and targeted antitumor efficacy of SKN-Ca@NIN.

2.8. *In Vitro* Biocompatibility

The *in vitro* cytocompatibility of SKN-Ca@NIN was evaluated using the L929 mouse fibroblast cell line. L929 cells were seeded into 96-well plates at 5×10^3 cells per well and incubated at 37 °C with 5% CO₂ for 24 h, then treated with SKN-Ca@NIN at 0, 30, 60, 120, 230, 480, and 710 μg/mL with six replicates per group. After 24 h, cell viability was determined by the CCK-8 assay, normalized to the untreated control, and compared with that of B16F10 cells to evaluate the safety margin and therapeutic window.

2.9. Live Cell Imaging *via* Calcein-AM Staining

B16F10 cells were seeded into 35 mm confocal dishes at 1×10^6 cells/mL in 1 mL RPMI 1640 complete medium and incubated at 37 °C with 5% CO₂ for 12 h, then treated with SKN-Ca@NIN at 0, 30, 60, 120, 230, 480, and 710 μg/mL for 24 h. Cells were washed 3 times with prewarmed PBS, incubated with 0.5 mL Calcein-AM working solution (0.1–5 μM) at 37 °C for 30 min in the dark, washed twice with PBS, and further incubated with 1 mL serum-free medium for 30 min. After two additional washes, fluorescence images were acquired

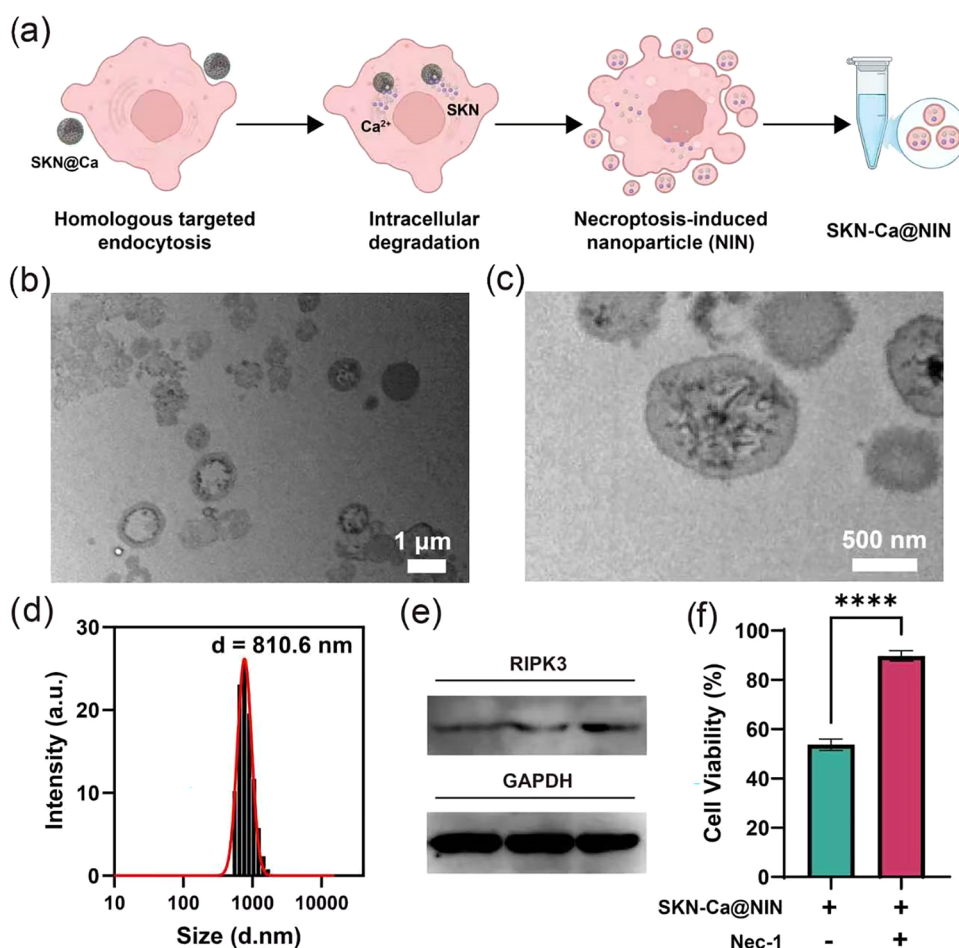


Figure 2. Preparation and characterization of SKN-Ca@NIN. (a) Schematic diagram of the synthetic procedures to generate SKN-Ca@NIN; (b, c) TEM images of SKN-Ca@NIN; (d) Size distribution of SKN-Ca@NIN measured by DLS. (e) Western blot validation of necroptosis *via* RIPK3 expression. (f) Cell viability of B16F10 cells treated with SKN-Ca@NIN nanoparticles combined with/without Nec-1.

using a confocal microscope (LSM 880, Zeiss) under identical settings.

2.10. Cellular Uptake Evaluation

Cellular uptake was evaluated in B16F10 and 4T1 cells using confocal microscopy. SKN-Ca@NIN was labeled with Coumarin 6 by stirring at room temperature in the dark for 6 h, followed by dialysis in anhydrous ethanol using a 3500 Da membrane. The labeled nanoparticles were diluted to 81.7 $\mu\text{g/mL}$ and 163.4 $\mu\text{g/mL}$. Cells were seeded into 35 mm dishes at 2×10^5 cells/mL in 1 mL medium and incubated for 12 h, then treated with 1 mL labeled SKN-Ca@NIN for 8 h. Cells were washed 3 times with PBS, fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.5% Triton X-100 for 10 min, stained with Rhodamine-Phalloidin for 30 min and DAPI for 10 min, washed 3 times, and imaged by CLSM (LSM 880, Zeiss). Excitation/emission wavelengths were 364/454 nm for DAPI, 443/530 nm for Coumarin 6, and 540/570 nm for Rhodamine.

2.11. Cell Cycle Analysis by Flow Cytometry

B16F10 cells were seeded into 6-well plates at 1×10^6 cells per well and incubated for 12 h, then treated for 12 h with Control, SKN@Ca NPs, SKN@NIN, and SKN-Ca@NIN at a final SKN concentration of 1 $\mu\text{g/mL}$, corresponding to 2.1 mg/mL, 0.32 mg/mL, and 0.48 mg/mL, respectively. Cells were collected, washed twice with precooled PBS, fixed in 70% ethanol at 4 °C for 1 h, washed twice, stained with PI/RNase A for 30 min in the dark, and analyzed using a CytoFLEX flow cytometer at 488 nm. Data were processed with FlowJo software.

2.12. Intracellular Calcium (Ca^{2+}) Overload Validation

B16F10 cells were seeded into 35 mm dishes at 1×10^6 cells/mL in 1 mL medium and incubated for 12 h, then treated with SKN-Ca@NIN at 81.7 $\mu\text{g/mL}$ and 163.4 $\mu\text{g/mL}$ for 8 h. Cells were washed 3 times with PBS, incubated with 0.5 mL Fluo-4 AM (0.1–5 μM) at 37 °C for 30 min in the dark, washed 3 times, and imaged in 1 mL serum-free medium using CLSM (TCS SP8, Leica) under identical settings.

2.13. Reactive Oxygen Species (ROS) Generation Assay

B16F10 cells were seeded into 35 mm dishes at 1×10^6 cells/mL in 1 mL medium and incubated for 12 h, then treated with SKN-Ca@NIN at 81.7 $\mu\text{g/mL}$ and 163.4 $\mu\text{g/mL}$ for 8 h. Cells were washed 3 times with PBS, incubated with 0.5 mL DCFH-DA (0.1–5 μM) at 37 °C for 30 min in the dark, washed 3 times, and imaged using CLSM (LSM 880, Zeiss), with fluorescence intensity reflecting intracellular ROS levels.

2.14. Apoptosis and Necrosis Assay

B16F10 cells were seeded into 6-well plates at 1×10^6 cells per well and incubated for 12 h, then treated for 24 h with Control, SKN@Ca NPs, SKN@NIN, and SKN-Ca@NIN at a final SKN concentration of 1 $\mu\text{g/mL}$. Cells were collected using EDTA-free trypsin, washed twice with PBS, and resuspended in 100 μL binding buffer at $1\text{--}5 \times 10^6$ cells/mL. Annexin V-FITC (5 μL) was added and incubated for 5 min in the dark, followed by 5 μL PI and 400 μL PBS. Samples were analyzed within 1 h using a CytoFLEX flow cytometer. Excitation/emission wavelengths were 488/530 nm for Annexin V-FITC and 488/615 nm for PI.

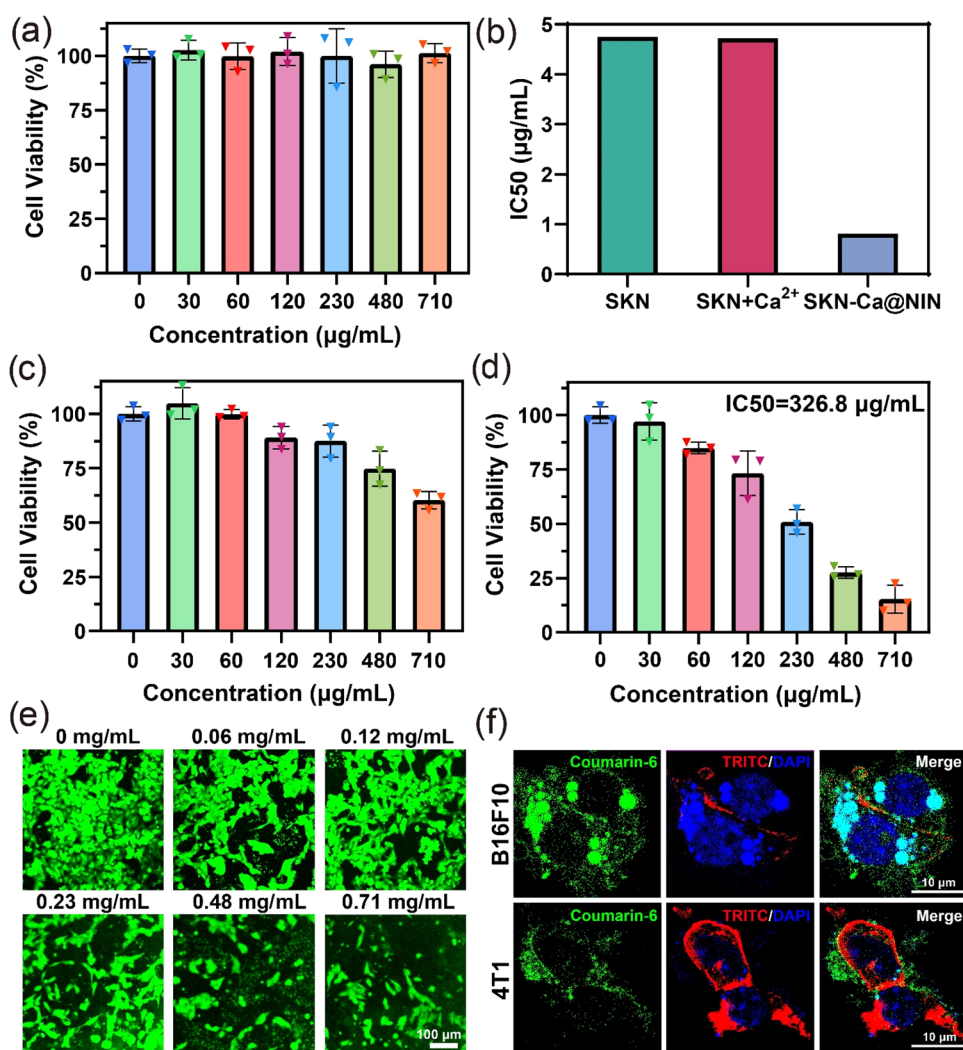


Figure 3. *In vitro* biocompatibility and cytotoxicity of SKN-Ca@NIN. (a) Cell viability of L929 cells after incubation with SKN-Ca@NIN; (b) IC_{50} values of free SKN, SKN+Ca²⁺, and SKN-Ca@NIN (the content of SKN in all groups were the same) against B16F10 cells; (c) Cell viability of 4T1 cells after incubation with SKN-Ca@NIN; (d) cell viability of B16F10 cells after incubation with SKN-Ca@NIN for 24 h and staining with Calcein-AM probe; (e) CLSM images of B16F10 cells after incubation with different concentrations of SKN-Ca@NIN for 24 h; (f) CLSM image of B16F10 and 4T1 cell after incubation with Coumarin-6 labeled SKN-Ca@NIN for 8 h.

2.15. Necroptosis Inhibitor Coincubation Assay

B16F10 melanoma cells were seeded in 96-well plates at a density of 5×10^3 cells per well and cultured overnight. Then, cells were treated with 100 μ L of medium containing 326.8 μ g/mL SKN-Ca@NIN alone or 326.8 μ g/mL SKN-Ca@NIN plus 100 μ g/mL necroptosis inhibitor (Nec-1, Cat. No. IN0910) for 24 h. Cell viability was assessed using the CCK-8 assay according to the manufacturer's protocol. Absorbance was measured at 450 nm using a microplate reader.

2.16. Statistical Analysis

Results are presented as mean $\pm$ standard deviation (SD). Statistical significance was determined using Student's *t* test or one-way ANOVA followed by Tukey's post hoc test. Significance levels were set as follows: **** $p < 0.0001$; *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; ns = not significant.

3. RESULTS AND DISCUSSION

3.1. Preparation and Characterization of SKN@Ca NPs

In this study, Shikonin-loaded amorphous calcium carbonate nanoparticles (SKN@Ca NPs) were synthesized *via* a biomimetalization method using CaCl₂ and NH₄HCO₃

precursors (Figure 1a).²¹ TEM images showed that the obtained nanoparticles exhibited a relatively uniform spherical morphology with nanoscale dimensions (Figure 1b). The DLS results showed that the hydrodynamic diameter was about 53.80 nm (Figure 1c), indicating a suitable size of the nanoparticles. XRD analysis displayed a broad diffraction pattern characteristic of amorphous calcium carbonate, confirming the amorphous structure of the nanoparticles²² (Figure 1d). Unlike crystalline calcium carbonate, ACC has a porous internal structure that enables efficient drug loading and exhibits efficient pH-responsive degradation ability. In the acidic microenvironment of endosomes and lysosomes, ACC undergoes rapid dissolution, facilitating the sustained release of both shikonin and Ca²⁺ ions within tumor cells. This dual-release profile is expected to synergistically amplify ionic and oxidative stress.

In addition, cytotoxicity evaluation demonstrated that SKN@Ca NPs induced concentration-dependent toxicity on B16F10 melanoma cells, with an IC_{50} value of 26.7 μ g/mL (Figure 1e). The relatively low IC_{50} suggests that the nanoparticle formulation enhances the bioavailability of

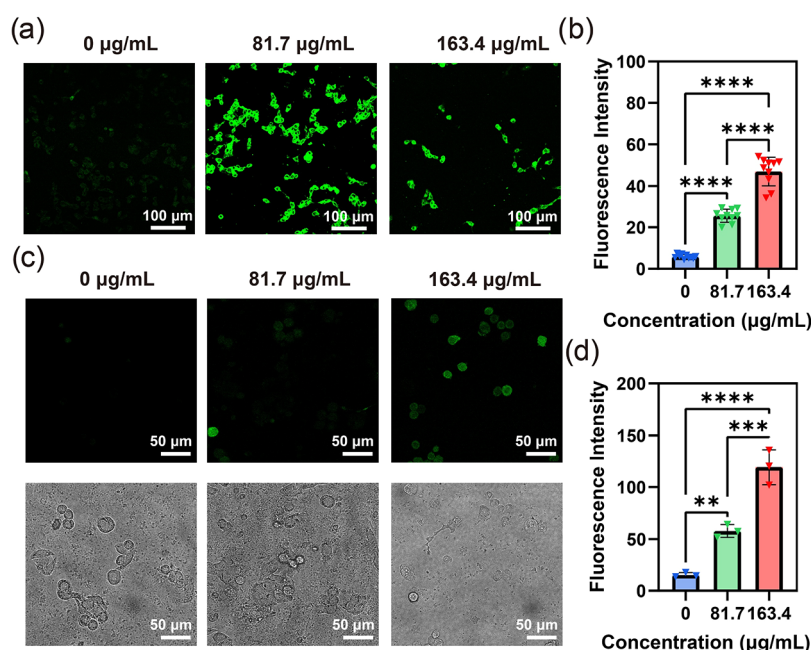


Figure 4. Intracellular Ca²⁺ and ROS overload induced by SKN-Ca@NIN. (a, b) CLSM images and corresponding fluorescence intensity of B16F10 cells after incubation with SKN-Ca@NIN and staining with Fluo-4 AM probe; (c, d) CLSM images and corresponding fluorescence intensity of B16F10 cells after incubation with SKN-Ca@NIN and staining with probe DCFH-DA.

shikonin while enabling efficient intracellular delivery of calcium ions. Collectively, these results establish SKN@Ca NPs as a suitable nanoinducer for triggering cellular stress responses, forming the basis for subsequent necroptosis induction and biomimetic nanocarrier generation.

3.2. Necroptosis-Induced Formation of SKN-Ca@NIN

Based on the successful preparation and preliminary activity of SKN@Ca, we next explored its ability to trigger necroptosis and generate tumor-derived biomimetic nanocarriers *in situ*. To construct biomimetic nanocarriers through a biological process, SKN@Ca NPs were incubated with B16F10 melanoma cells to induce necroptosis. During the necroptotic process, tumor cells release membrane-derived vesicular structures known as necroptotic bodies. Importantly, the internalized SKN@Ca NPs were degraded, released Ca²⁺, and SKN were encapsulated within these vesicles, leading to the formation of necroptosis-induced nanocarriers (SKN-Ca@NIN). The encapsulation efficiency of SKN in SKN-Ca@NIN was approximately 15.8%. TEM images revealed that SKN-Ca@NIN exhibited a typical vesicular morphology with membrane-like structures (Figure 2b,c). The hydrodynamic diameter of the SKN-Ca@NIN was determined to be 810.6 nm by DLS, which is consistent with the formation of cell-derived vesicles (Figure 2d).¹⁶

To further confirm that the formation of SKN-Ca@NIN was driven by necroptosis rather than other forms of cell death, we analyzed the expression of RIPK3, a key effector protein in the necroptosis signaling pathway. As shown in Figure 2e, B16F10 cells treated with SKN@Ca NPs exhibited significantly upregulated RIPK3 expression, indicating the activation of necroptosis. This result is consistent with previous reports that SKN can induce necroptosis in cancer cells by activating the RIPK1/RIPK3/MLKL signaling axis. The induction of necroptosis is critical for the *in situ* formation of necroptotic bodies that encapsulate internalized nanoparticles, as these vesicles are released specifically during the necroptotic process.

Furthermore, the necroptosis inhibitor coincubation measurement showed that Nec-1 could significantly inhibit the cytotoxicity of SKN-Ca@NIN (Figure 2f), confirming the induced necroptosis signaling pathway. Collectively, these findings confirm that SKN@Ca NPs serve as effective nanoinducers of necroptosis, facilitating the biogenesis of tumor-derived biomimetic nanocarriers while preserving the membrane protein integrity essential for subsequent homologous targeting.

3.3. In Vitro Cytotoxicity and Homologous Targeting Ability

With the successful biogenesis of SKN-Ca@NIN confirmed, we next evaluated its biosafety, homologous targeting performance, and tumor inhibition capacity *in vitro*. Cytotoxicity assays using L929 fibroblast cells demonstrated that SKN-Ca@NIN exhibited relatively low toxicity across the tested concentration range, with cell viability remaining above 80% even at the highest concentration (Figure 3a). This favorable biocompatibility result could be explained by the biomimetic vesicular structure, which mitigates nonspecific cytotoxicity toward normal cells *via* the protective effect of the tumor-derived membrane coating. The half maximal inhibitory concentration (IC₅₀) of free SKN to B16F10 was about 4.75 μg/mL (Figure 3b). Although an additional supplement with Ca²⁺ ions could not enhance the cytotoxicity of SKN, as-synthesized SKN-Ca@NIN significantly strengthened the antitumor efficacy of SKN, with a low equal IC₅₀ value of about 0.81 μg/mL, which indicates the advantage of necroptosis-induced nanocarriers.

In contrast, SKN-Ca@NIN displayed potent cytotoxic effects against tumor cells. Both B16F10 melanoma cells and 4T1 breast cancer cells exhibited concentration-dependent inhibitory effects in cytotoxicity assays (Figure 3c,d). Notably, the inhibitory effect was markedly more pronounced in homologous B16F10 cells compared with heterologous 4T1 cells; the IC₅₀ for B16F10 cells was determined to be 326.8

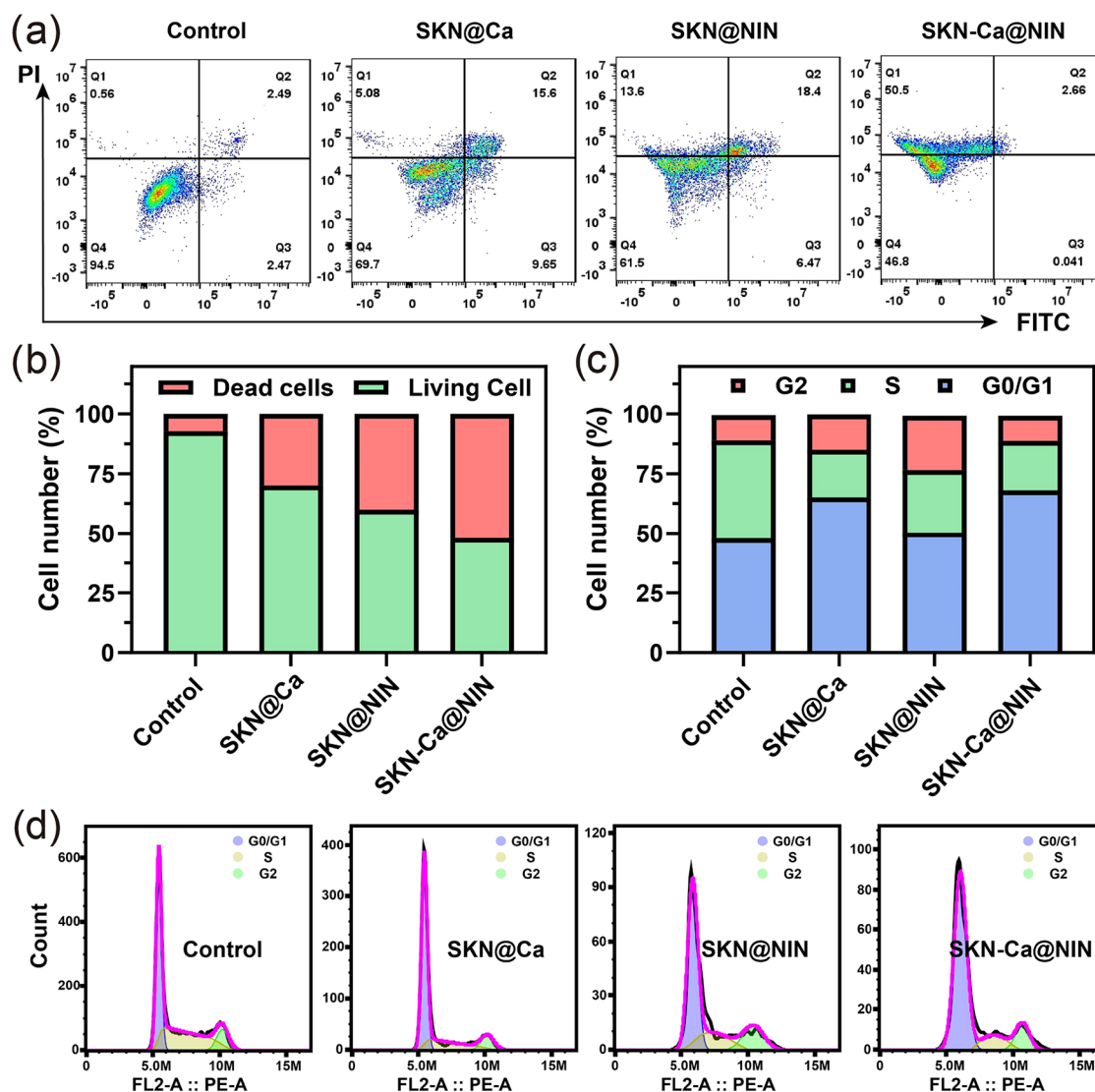


Figure 5. Antitumor mechanism of SKN-Ca@NIN. (a, b) Flow cytometric analysis of apoptosis/necrosis for SKN-Ca@NIN-treated B16F10 cells; (c, d) Cell cycle assay of B16F10 cells after treatment with various nanocarriers.

$\mu\text{g/mL}$, whereas the IC_{50} for 4T1 cells could not be reliably calculated within the tested concentration range, indicating substantially lower sensitivity. This differential cytotoxicity suggests that SKN-Ca@NIN possesses selective antitumor activity toward cells of the same origin. Moreover, the live cell staining results exhibited the same trend (Figure 3e): with the increase of SKN-Ca@NIN concentration, the green fluorescent signal gradually weakened, which was consistent with the aforementioned CCK-8 assay results. To further confirm the homologous targeting ability of SKN-Ca@NIN, cellular uptake behavior was examined by confocal laser scanning microscopy (CLSM). Following incubation with Coumarin-6-labeled SKN-Ca@NIN, significantly stronger intracellular fluorescence signals were observed in B16F10 cells compared with 4T1 cells (Figure 3f). The B16F10 cells exhibit more potent green fluorescence than the 4T1 cells, indicating the higher uptake efficiency of SKN-Ca@NIN. This preferential internalization can be attributed to the retention of functional membrane proteins—such as adhesion molecules and tumor-associated antigens—derived from the parental B16F10 cells on the surface of SKN-Ca@NIN. These conserved surface features

enable homologous recognition and facilitate targeted delivery, thereby enhancing the therapeutic efficacy against melanoma cells while minimizing off-target effects.

3.4. SKN-Ca@NIN Induces Intracellular Ca^{2+} and ROS Overload

To investigate the mechanism underlying the cytotoxicity of SKN-Ca@NIN, we examined intracellular Ca^{2+} and ROS levels, both of which are critical mediators of cellular stress and cell death. Fluo-4 AM staining revealed a pronounced increase in intracellular Ca^{2+} fluorescence signals in SKN-Ca@NIN-treated B16F10 cells compared with untreated controls (Figure 4a,b). This elevation is primarily attributable to the degradation of the amorphous calcium carbonate core within acidic intracellular compartments, such as endosomes and lysosomes, which triggers the sustained release of Ca^{2+} ions. The resulting Ca^{2+} overload is known to disrupt mitochondrial membrane potential, activate Ca^{2+} -dependent proteases, and exacerbate oxidative stress.²³

Concurrently, ROS generation was assessed using the DCFH-DA probe. CLSM images demonstrated a marked increase in green fluorescence intensity in SKN-Ca@NIN-

treated cells (Figure 4c,d), indicating substantial ROS accumulation. This effect is likely driven by shikonin, a known inducer of ROS through mitochondrial dysfunction and NADPH oxidase activation.²⁴ This reciprocal amplification disrupts intracellular homeostasis, culminating in necroptosis—a programmed cell death pathway driven by calcium overload and ROS—that causes irreversible cell damage and death. Collectively, these findings indicate that SKN-Ca@NIN exerts its antitumor effects through a dual mechanism involving ionic and oxidative stress, which together overwhelm the adaptive capacity of tumor cells and ultimately execute necroptosis.

3.5. Necroptotic Cell Death and Cell Cycle Analysis

Having established that SKN-Ca@NIN induces synergistic ionic and oxidative stress in melanoma cells (Figure 4), we next sought to verify the downstream consequences on cell fate and proliferative capacity. Flow cytometry was employed to evaluate the effects of SKN-Ca@NIN on cell death and cell cycle progression, providing further insight into its antitumor mechanism. Annexin V-FITC/PI staining revealed a significant increase in the proportion of dead cells following SKN-Ca@NIN treatment compared with the control group (Figure 5a,b), confirming effective induction of tumor cell death. Notably, the observed cell death profile exhibited characteristics consistent with necroptosis, aligning with the RIPK3 upregulation (Figure 2e) and supporting that SKN-Ca@NIN induces programmed necrosis rather than classical apoptosis.

In addition, cell cycle analysis was performed to assess the impact of SKN-Ca@NIN on proliferative capacity. As shown in Figure 5c,d, treatment with SKN-Ca@NIN resulted in a marked G0/G1 phase arrest, with the proportion of cells in G0/G1 increasing from approximately 47.5% in the control group to 67.4%. Concurrently, the proportions of cells in the S phase and G2 phase decreased significantly, indicating effective blockade of cell cycle progression. This G0/G1 arrest is likely associated with the sustained intracellular Ca^{2+} overload and ROS stress induced by SKN-Ca@NIN, as both factors are known to modulate the expression and activity of cyclin-dependent kinases and their regulatory subunits. Collectively, these results demonstrate that SKN-Ca@NIN exerts its antitumor effects through a dual mechanism: directly inducing tumor cell death *via* necroptosis while simultaneously inhibiting proliferation by imposing G0/G1 cell cycle arrest. This combined strategy disrupts both the viability and replicative potential of melanoma cells, highlighting the therapeutic potential of SKN-Ca@NIN as a multifunctional biomimetic nanoplatform.

In addition to the direct antitumor effects discussed above, the necroptosis-induced SKN-Ca@NIN holds significant potential for combined immune activation, given that necroptosis is a highly immunogenic form of cell death. The tumor-derived vesicles naturally encapsulate damage-associated molecular patterns (DAMPs) and tumor neo-antigens, which could promote dendritic cell maturation and T-cell priming, potentially converting the primary tumor into an *in situ* vaccine. Future studies could evaluate the ability to induce systemic antitumor immunity by the SKN-Ca@NIN, and the combined therapeutic efficacy together with immune checkpoint blockade. Moreover, this biogeneration strategy can be extended to other nanoparticle cargos and tumor types to produce personalized, autologous nanocarriers.

4. CONCLUSION

In summary, we developed a necroptosis-driven biogenesis strategy to construct tumor-derived biomimetic nanocarriers for melanoma therapy. Shikonin-loaded calcium carbonate nanoparticles (SKN@Ca NPs) were used as nanoinducers to trigger necroptosis in melanoma cells, leading to the release of necroptotic bodies that encapsulated the nanoparticles and formed SKN-Ca@NIN. The resulting nanoplatform retained tumor membrane proteins and exhibited homologous targeting capability toward melanoma cells. Mechanistically, the degradation of the calcium carbonate core triggered intracellular Ca^{2+} release, while shikonin promoted ROS accumulation, resulting in severe oxidative and ionic stress that disrupted tumor cell homeostasis. This work provides a unique strategy to construct an *in situ* necroptosis-induced nanocarrier platform for efficient tumor therapy and immune response *via* a homologous targeting strategy.

AUTHOR INFORMATION

Corresponding Author

Wensheng Xie — State Key Laboratory of Organic–Inorganic Composites, Beijing Laboratory of Biomedical Materials, Beijing University of Chemical Technology, Beijing 100029, P. R. China; orcid.org/0000-0001-7700-9626; Email: xws@mail.buct.edu.cn

Authors

Zhenbin Dong — State Key Laboratory of Organic–Inorganic Composites, Beijing Laboratory of Biomedical Materials, Beijing University of Chemical Technology, Beijing 100029, P. R. China

Yubo Niu — The Key Laboratory of Biomedical Information Engineering of the Ministry of Education, School of Life Science and Technology, Xi'an Jiaotong University, Bioinspired Engineering and Biomechanics Center (BEBC), Xi'an Jiaotong University, Xi'an 710049, P. R. China

Jingxia Xu — State Key Laboratory of Organic–Inorganic Composites, Beijing Laboratory of Biomedical Materials, Beijing University of Chemical Technology, Beijing 100029, P. R. China

Junfeng Wang — State Key Laboratory of Organic–Inorganic Composites, Beijing Laboratory of Biomedical Materials, Beijing University of Chemical Technology, Beijing 100029, P. R. China

Wanting Yang — State Key Laboratory of Organic–Inorganic Composites, Beijing Laboratory of Biomedical Materials, Beijing University of Chemical Technology, Beijing 100029, P. R. China

Guofeng Li — State Key Laboratory of Organic–Inorganic Composites, Beijing Laboratory of Biomedical Materials, Beijing University of Chemical Technology, Beijing 100029, P. R. China

Xing Wang — State Key Laboratory of Organic–Inorganic Composites, Beijing Laboratory of Biomedical Materials, Beijing University of Chemical Technology, Beijing 100029, P. R. China; orcid.org/0000-0002-9990-1479

Complete contact information is available at:
<https://pubs.acs.org/10.1021/acsanm.6c01766>

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS

SKN, shikonin; ACC, amorphous calcium carbonate; ROS, reactive oxygen species; CCK-8, cell counting Kit-8; TEM, transmission electron microscopy; XRD, X-ray diffraction; CLSM, confocal laser scanning microscopy; LSCM, laser scanning confocal microscopy; DAPI, 4',6-diamidino-2-phenylindole; PBS, phosphate buffered saline; BCA, bicinchoninic acid; FBS, fetal bovine serum; PFA, paraformaldehyde; EDTA, ethylenediaminetetraacetic acid; ECL, enhanced chemiluminescence; PVDF, poly(vinylidene fluoride); RIPK3, receptor-interacting protein kinase 3; IC₅₀, half-maximal inhibitory concentration; SD, standard deviation; RPMI 1640, Roswell Park Memorial Institute 1640; ANOVA, analysis of variance; DCFH-DA, 2',7'-dichlorodihydrofluorescein diacetate; PI, propidium iodide; TBST, Tris-buffered saline with Tween 20; SKN@Ca NPs, shikonin-loaded amorphous calcium carbonate nanoparticles; SKN-Ca@NIN, necroptosis-induced nanocarriers loaded with SKN and Ca²⁺; SKN@NIN, necroptosis-induced nanocarriers loaded with SKN

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