Enhanced Photothermal Therapy of Breast Cancer via Co-Loading With Autophagy Inhibitor

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Abstract

Photothermal therapy (PTT) usually causes hyperthermia and damages healthy tissues. Developing a PTT platform using mild irradiation, while enhance the therapeutical effects attracts increasing attention. Here, this study developed a theranostic poly(D,L-lactic-co-glycolic acid) (PLGA) nanoplatform loaded with a near-infrared (NIR) dye (new indocyanine Green IR820), autophagy inhibitor (chloroquine, CQ) and a fluorescence imaging agent (ZnCdSe/ZnS quantum dot, QD) by the double emulsion solvent evaporation technique ($W_1/O/W_2$).

The resulting hybrid PLGA nanoparticles with IR820/ZnCdSe/ZnS/CQ co-loading (termed PIFC NPs) approximated 240 nm in diameter and had excellent monodispersity, fluorescence and size stability, and biocompatibility. PIFC NPs displayed photothermal effects, and the released CQ remarkably decreased autolysosome degeneration by lysosomes in cancer cells, thereby enhancing the suppressive effect on autophagy as well as resistance to photothermia. Anticancer effects were enhanced both in cellular and animal experiments attributed to the combined effects exerted by PIFC NPs and mild NIR irradiation. Moreover, PIFC NPs significantly accumulated in tumors because of enhanced permeability and retention (EPR) effect, enabling high-spatial resolution, real-time fluorescence imaging of solid tumors. The present study developed a novel PTT platform showing potentially enhanced therapeutic efficacy.

1. Introduction

Multifunctional nanodrugs combining diagnostic and therapeutic functions currently constitute a novel approach in cancer treatment.^[1, 2] Ideally, theranostic platforms should both exert efficient therapeutic effects and low side effects. Therapeutic agents and imaging compounds are essential for the fabrication of nanotheranostics for cancer therapy.^[3, 4] On one hand, photothermal therapy (PTT), an important light-dependent therapeutic approach, is considered as a novel, efficient and noninvasive method for cancer treatment, with reduced tumor recurrence and elevated selectivity. In PTT, light energy is absorbed to yield heat energy by photothermal molecules to kill cancer cells.^[5, 6] Albumin nanoparticles carrying PTT compounds exerted excellent anticancer effects. IR820, a photosensitizer, after NIR light irradiation, efficiently converts light to heat, acting as PTT agent and inhibiting malignant cells.^[7, 8] Photothermal therapy shows broad application prospects, but the problems arising in the actual treatment process need to be addressed urgently. At present, photothermal therapy is mostly applied in superficial tumors. The treatment temperature needs to reach 50 °C or higher to effectively inhibit tumor cells.^[9, 10] Meanwhile, excessive temperatures can cause severe burns to the skin. The burned skin further induces a series of self-defense reactions in the body, such as the release of inflammatory factors, increasing the risk of tumor metastasis and recurrence.^[11] Therefore, photothermal treatment of tumors at lower temperatures is more in line with practical clinical application. However, photothermal treatment at lower temperatures cannot effectively inhibit tumor growth, and its tumor treatment effect is not significant.^[12, 13] According to previous reports, tumor cells actually have varying degrees of anti-photothermal effects, leading to ineffective tumor ablation. Such resistance mainly originates from an "autophagy" mechanism in the cell.^[14, 15] Autophagy represents an important cellular pathway that breaks down impaired organelles, aged proteins and other constituents, reusing them as cellular nutrients for self-renewal.^[16, 17] Additionally, autophagy has a vital function in resisting cell stress as well as various treatment methods.^[18, 19] Indeed, autophagy can repair and reverse cell damage caused by heating, resulting in incomplete cell necrosis.^[20–22] In order to overcome this treatment resistance, severer conditions (e.g., high temperature and extended irradiation time) are applied in PTT, which may cause accidental damage to nearby non-cancerous tissues.^[23, 24] Therefore, developing an advanced PTT platform that can effectively inhibit autophagy is very attractive, and could help achieve more targeted and specific inhibition of tumors.

On the other hand, in comparison with alternative imaging techniques, e.g., computed tomography (CT) and magnetic resonance imaging (MRI),^[25, 26] fluorescence imaging has been assessed for bioimaging and medical application thanks

to elevated sensitivity, simple operation and cost effectiveness.^[27–30] Consequently, albumin nanoparticles carrying PTT compounds and fluorescent dyes were designed to effectively diagnose and treat cancer. Recently, due to synergistic effects exerted by both polymer nanospheres and inorganic nanomaterials, polymer-inorganic hybrid nanospheres attract increasing attention. Specifically, biocompatible compounds that can emit fluorescence may help monitor the nanospheres in cells and body, revealing tumor locations and reflecting treatment responses. In comparison with organic fluorescent dyes, inorganic fluorescent emitters, including quantum dots (QDs), have stronger fluorescence intensity, higher photostability, wider excitation wavelength range and smaller emission spectrum.^[31] Based on the above properties, extensive research has focused on the use of quantum dots in multicolor cell, tissue and animal imaging protocols.^[27, 32] However, low biocompatibility and stability under a variety of physiological conditions limit the biomedical/clinical application of QDs. Multiple approaches are under development for overcoming the above limitations. The most successful strategy has involved QD coating with biocompatible hydrosoluble polymers, which improves the colloidal stability and biocompatibility of QDs in aqueous solutions and biological environments.^[28, 33] However, such a thin polymer layer on QDs hardly meets the requirement of carrying sufficient active molecules. Using biocompatible polymer nanospheres to wrap QDs instead of thin polymer layers would provide ample space to load other activators (e.g., drugs and inorganic nanoparticles), which can spread their value for disease treatment and diagnosis, making them multifunctional nanomaterials. In addition, quantum dots in the polymer matrix may reduce the shell shedding of QDs, which usually leads to photodegradation. Meanwhile, most of the previously reported constructions of QD-related polymer nanocarriers require complex synthetic processes and could cause environmental toxicity because of the use of surfactants and/or organic solvents.^[34, 35]

Herein, a PLGA-based multifunctional treatment system, with both fluorescence imaging and autophagy inhibition, was examined for PTT effectiveness with mild irradiation. The system uses PLGA as the nanocarrier of the composite, with chloroquine (CQ) loaded in the PLGA core to form an internal water phase and ZnCdSe/ZnS QD and IR820 used as the oil phase (Fig. 1). Interestingly, efficient inhibition of autophagy was accomplished by interfering lysosomes and reducing autolysosome degradation by CQ released in malignant cells.^[36] Integrating the strong photothermal features and autophagy inhibition of malignant cells, the PIFC NPs system exerted anticancer effects in vivo and in vitro under mild near-infrared irradiation. Therefore, this study provides a novel potential platform for PTT-based tumor therapy with greater efficacy.

2. Materials And Methods

2.1 Chemical and Reagents.

Poly(lactic-co-glycolic) acid (PLGA) (50:50; MW, 10000), Poly(vinyl alcohol) (PVA), chloroquine diphosphate, LysoTracker Green and 4',6-diamidino-2-phenylindole (DAPI) were provided by Sigma (USA). New indocyanine Green (IR820) was provided by Macklin (China). Cell counting kit-8 (CCK-8) was from Melone (China). ZnCdSe/ZnS QD was provided by Wuhan Jiayuan Quantum Dots (China). All chemicals were directly utilized with no further purification.

2.2 Synthesis of PIFC NPs

PIFC NPs were synthesized by the double emulsion (water/oil/water: $W_1/O/W_2$) technique, with some modifications. In brief, PLGA (20 mg) and IR820 (1 mg) were added to dichloromethane (1 mL), followed by ZnCdSe/ZnS QD solution (0.1 mL, 3 mg/mL) and CQ (0.2 mL, 2.5 mg/mL). The above mixture was then emulsified in an ice water bath for 30 seconds with an ultrasound probe (Sonics & Materials, USA). The resulting mixture was further mixed with cold PVA solution (5 mL, 4% w/v). The resulting emulsion was re-emulsified in an ice water bath for 3 minutes using the above ultrasound probe, with subsequent addition of water (15 mL) and stirring until dichloromethane volatilization. Finally, PIFC NPs were obtained by centrifugation (12000 rpm, 20 minutes) and washed thrice with DI water.

2.3 Characterizations.

Transmission electron microscopy (TEM) was performed on a JEM 2001F (JEOL, Japan) for image acquisition. An energy dispersive spectrometer (EDS) was also utilized for material characterization. UV-Vis spectroscopy was carried out on a UVmini-1240 (Shimadzu, Japan). Dynamic light scattering (DLS)-based zeta potential and size distribution were determined with a nano-ZS90 Zetasizer (Malvern Panalytical, UK).

2.4 Loading of IR820 and CQ.

Subsequently, PIFC NPs were centrifuged at 12000 rpm for 20 minutes, and underwent three deionized water washes for removing unloaded CQ and IR820. The drug loading rates were examined using the resulting supernatant. Based on CQ and IR820 absorbance at approximately 344 nm and 680 nm, respectively, calibration curves for the concentrations of CQ and IR820 were generated.

In order to assess drug release under acidic pH, two dialysis bags (Biosharp USA, molecular weight cutoff approximating 3500 kDa) containing 1 mL of PIFC NPs were immersed in 8 mL of PBS (pH 7.4 or pH 5.0) under constant temperature and stirring. At various times, 0.2 mL dialysis solution was obtained for examination, maintaining the dialysate volume by addition of PBS (0.2 mL). Absorption was read at 344 nm on a UV-Vis spectrometer, and the amount of released CQ was determined in the collected sample based on the abovementioned calibration curve.

2.5 Photothermal Effects.

Totally, 1 mL PIFC NPs (0, 150, 300, 450, 600 and 750 $\mu\text{g/mL}$ in water) was submitted to irradiation by an 808-nm laser for 5 min (0.5, 1.0 and 1.5 W/cm^2 , respectively), using an infrared thermal imaging camera for temperature recording every 1 minute (E60, FLIR, USA).

In order to evaluate the photostability of PIFC NPs, 1 mL PIFC aqueous solution (PIFC NPs at 600 $\mu\text{g/mL}$ and IR820 at 80 $\mu\text{g/mL}$) and free IR820 (80 $\mu\text{g/mL}$) were irradiated with a 808-nm laser till temperature stabilization. After turning off the laser, solution cooling occurred, and temperature recording was carried out at an interval of 1 min. This on/off cycle was repeated four times.

2.6 Cellular Uptake Assays

To evaluate the cellular uptake behavior of PIFC NPs, breast cancer MDA-MB-231 cells underwent seeding in 6-well plates and 24 h incubation at 37°C under 5% CO_2 . Then, medium with PIFC NPs was used for further culture for 0.5, 1 and 2 h, respectively. After PBS washes, the nucleus and lysosomes underwent staining with DAPI and LysoTracker Green, respectively. Finally, a confocal laser scanning microscope (CLSM, Olympus, Japan) was utilized for data analysis.

2.7 In vitro Cytotoxicity Assessment.

Breast cancer NIH-3T3 and MDA-MB-231 cells underwent seeding in 96-well plates at 1×10^4 /well and culture at 37°C and 5% CO_2 for 12 hours. Then, different concentrations (0, 150, 300, 450, 600 and 750 $\mu\text{g/mL}$) of PIFC NPs were supplemented for a 24-hour incubation as above. Upon medium aspiration, 10 μL CCK-8 solution in 100 μL DMEM/L-15 (with 10% FBS) was added per well for another 2 hours. Finally, absorbance at 450 nm was obtained on a microplate reader (BioTek Epoch, Service Card).

2.8 In vitro Evaluation of Therapeutic Effects.

MDA-MB-231 cells underwent seeding in 96-well plates at 1×10^4 /well and culture at 37°C under 5% CO_2 for 12 hours. CQ (39.86 $\mu\text{g/mL}$), PIF NPs (600 $\mu\text{g/mL}$) and PIFC NPs (600 $\mu\text{g/mL}$ PIFC NPs, 39.86 $\mu\text{g/mL}$ CQ) were incubated with cells for 4 hours, followed by irradiation at 808 nm (1.0 W/cm^2 , 5 min). CCK-8 was utilized for cell viability assessment.

Autophagy suppression-sensitized PTT effects in cultured cells were further assessed. MDA-MB-231 cells underwent seeding in 96-well plates at 1×10^4 /well. Then, PIF NPs and PIFC NPs were supplemented to cells at various levels (0, 150, 300, 450, 600 and 750 $\mu\text{g/mL}$ for PIFC NPs; 0, 9.965, 19.93, 29.895, 39.86 and 49.825 $\mu\text{g/mL}$ for CQ) for a 4-h co-incubation. This was followed by 808-nm irradiation (1.0 W/cm^2 , 5 min) and cell viability assessment by CCK-8.

The effect of autophagy suppression-sensitized PTT was determined in cultured cells for various irradiation times. MDA-MB-231 cells underwent seeding in 96-well plates at 1×10^4 /well. PIF NPs (600 $\mu\text{g/mL}$) and PIFC NPs (600 $\mu\text{g/mL}$ PIFC NPs and 39.86 $\mu\text{g/mL}$ CQ) were added to cells for 4-h incubation and 808-nm irradiation (1.0 W/cm^2) for 0, 1, 2, 3, 4 and 5 min, respectively. Finally, cell viability assessment utilized CCK-8.

2.9 Western Blot.

MDA-MB-231 cells underwent seeding in 6-well plates at 4×10^5 cells per well, and were incubated with control medium, CQ, PIF NPs and PIFC NPs for 4 h. This was followed by 808-nm irradiation (1.0 W/cm^2 , 5 min). The harvested cells (trypsinization) were fully lysed with the RIPA buffer containing protease inhibitors (Beyotime, China). After adding SDS buffer, the sample was boiled for 8 min, and protein separation was carried out by 12% SDS-PAGE. This was followed by electro-transfer of the separated proteins to the NC membrane (Beyotime) at low temperature for 45 min and blocking with the blocking solution for 2 h. Then, the NC membrane underwent incubation with anti-LC3 (Proteintech, China), P62 (Proteintech, China) and anti-Actin (Abcam, UK) primary antibodies at appropriate dilutions overnight at 4°C . After incubation with HRP-linked Affinipure Goat Anti-Rabbit IgG (H + L) (Abcam) for 2 h at ambient, enhanced chemiluminescence was utilized for visualization.

2.10 LysoTracker Green Staining Assay.

Upon incubation with CQ (39.86 $\mu\text{g/mL}$), PIF NPs (600 $\mu\text{g/mL}$) and PIFC NPs (39.86 $\mu\text{g/mL}$ CQ) for 4 h and NIR irradiation (1.0 W/cm^2 , 5 min), MDA-MB-231 cells underwent staining with DAPI and LysoTracker Green for 30 min at 37°C . Then, cell washes were performed, followed by analysis by confocal laser scan microscopy (CLSM, Olympus, Japan).

2.11 TEM Assessment of Autophagy Inhibition.

MDA-MB-231 cells were administered control medium, CQ, PIF NPs and PIFC NPs in culture medium. After laser irradiation, cell fixation was carried out with 2.5% glutaraldehyde in PBS overnight. Next, the cells underwent washing, further fixation with 1% osmium, dehydration (graded ethanol), epoxy resin embedding, sectioning (70 nm), staining and TEM analysis.

2.12 In Vivo Fluorescence Imaging

BALB/c nude mice (4–6 weeks of age), provided by Xuzhou medical University Laboratory Animal center, underwent housing under specific-pathogen-free (SPF) conditions in the vivarium of the School of Medical Imaging, Xuzhou Medical University. Assays involving animals were conducted in accordance with the "Guiding Opinions on the Care and Use of Laboratory Animals", with approval from the Ethics Committee of Xuzhou Medical University (Ethics Number: L20210226341). MDA-MB-231 cells (1×10^6) in 150 μL PBS were administered into the mouse flank by subcutaneous injection for animal model establishment. When the tumor volume reached $70 \sim 100 \text{ mm}^3$, one mouse was intravenously administered 200 μL of a solution containing PIFC NPs. Respectively at 0 h, 1 h, 2 h, 4 h, 6 h, 8 h, 12 h and 24 h, fluorescence was examined with an in vivo imaging system.

2.13 In Vivo Biodistribution of PIFC Nanoparticles

Nude mice were intravenously administered 200 μL of PIFC NPs, and randomly sacrificed at 0 h, 1 h, 2 h, 4 h, 6 h, 8 h, 12 h, and 24 h post-treatment, respectively. Then, and the main organs (heart, liver, spleen, lung and kidney) were extracted.

2.14 In Vivo Antitumor Studies

Female BALB/c mice administered MDA-MB-231 cells constituted an animal model. When the tumors reached about 70 $\sim 100 \text{ mm}^3$, MDA-MB-231 tumor bearing animals were randomly assigned to 6 groups of five: (1) control, (2) NIR irradiation only (808 nm), (3) PIFC NPs injection only, (4) PIF NPs plus 808-nm irradiation (45°C), (5) PIF NPs plus 808-nm irradiation (55°C) and (6) PIFC NPs plus 808-nm irradiation (45°C). The animals underwent irradiation with an 808-nm laser (1.0 W/cm² for 5 min). Among them, the 5th group underwent irradiation with an 808-nm laser (1.0 W/cm² for 5 min) and treatment with PIF NPs (750 $\mu\text{g/mL}$). Totally 8 h post-injection, tumor temperature recording was carried out with an infrared thermal imaging camera. Throughout the study, body weights were recorded using an electronic balance, and a digital caliper was utilized for tumor measurements every two days. Tumor volume was derived as $\text{width}^2 \times \text{length} / 2$.

2.15 In Vivo Toxicity Analysis

Mice intravenously administered 200 μL of PBS and PIFC NPs (20 mg/kg), respectively, underwent euthanasia at 0, 1, 7 and 21 days, respectively. Then, blood collection was carried out by cardiac puncture for blood biochemistry and complete blood count by the Affiliated Hospital of Xuzhou Medical University. Next, heart, liver, spleen, lung and kidney specimens underwent 10% formalin fixation, paraffin embedding, sectioning and H&E staining. An inverted microscope was utilized for analysis.

3. Results And Discussion

3.1 Synthesis and Characteristics of PIFC Nanoparticles.

The formation process for PIFC NPs is depicted in Fig. 2a. TEM revealed PIFC NPs had an evident core-shell structure and spherical outline (Fig. 2b and c). The remarkably high electron density of QDs potentiates their direct visualization within PLGA nanoparticles, indicating that CdSe/ZnS QD were successfully embedded in PLGA nanoparticles. PIFC NPs were $247 \pm 4.1 \text{ nm}$ (Fig. 2d); their PDI was 0.127 (Fig. 2f), zeta potential was $-30.4 \pm 0.09 \text{ mV}$ (Fig. 2e), suggesting it is a stable nanoplatform due to electrostatic repulsion among circulating.^[37] The as-prepared PIFC NPs were highly stable and had elevated dispersity in DI water, PBS, FBS, DMEM and L15, with a hydrodynamic average diameter of 240 nm (Fig. 2f). The spheres appeared smaller under TEM compared with DLS because of shrinking after drying.

The absorption properties of PIFC NPs and constituents were assessed by UV-Vis spectrophotometry. Figure 3a shows IR820 had an absorption peak at about 680 nm, and an overt peak was found at 344 nm, which was attributed to CQ and absent in PLGA NPs. PIFC NPs containing IR820 and CQ showed two small peaks at 810 nm and 344 nm, respectively. These findings confirmed the successful loading of CQ and IR820 into PLGA nanoparticles. Based on respective calibration curves, the loading rates of CQ (Fig. 3b) and IR820 (Fig. 3c) were approximately 39.86% and 41.40%, respectively.

PIFC NPs exhibit remarkable fluorescence stability, owing to the powerful protection of QDs by thick polymer matrices. The corresponding Energy Dispersive Spectrometry (EDS) data revealed that the elements sulfur (S), zinc (Zn), selenium (Se) and cadmium (Cd) were homogenously distributed over the entire nanoparticles (Fig. 3d). Figure 3e presents the photoluminescence spectra of ZnCdSe/ZnS QDs and PIFC NPs, alongside those of empty PLGA NPs. It was noticed PIFC NPs have similar shape and peaks as ZnCdSe/ZnS QDs, with the exception of peaks resulting from the scattering of PIFC NPs. After confirming that PLGA was successfully loaded with ZnCdSe/ZnS QDs, we used a fluorescence

spectrophotometer to characterize its fluorescence performance. As shown in Fig. 3g, elevated solution concentration resulted in stronger fluorescence intensity of PIFC NPs, indicating that fluorescence intensity is related to concentration. In addition, in order to verify the fluorescence stability of PIFC NPs, we tested the changes of fluorescence intensity in different media. PIFC NPs were stable for several weeks in DI Water, PBS, FBS, DMEM cell culture medium and L-15 cell culture medium (Fig. 3f). Obviously, the protection provided by PLGA NPs is sufficient to prevent any photobleaching of the QDs. Therefore, we can consider using PIFC NPs as an optical bioimaging agent and drug carrier.

3.2 Release of CQ

The release of CQ was examined based on its calibration curves at pH 7.4 and pH 5.0 (Fig. 3h). Within the measurement time (120 h), under normal physiological conditions (pH 7.4) the release of CQ in PIFC NPs was 15.99%, while under acidic conditions (pH 5.0) it was significantly enhanced (66.41%) (Fig. 3i). After the rapid release in the first 10 hours, CQ was still released slowly in the subsequent time. The above results suggest PIFC NPs constitute an effective drug carrier that can promote the release and accumulation of CQ in the acidic microenvironment of tumors.

3.3 Photothermal Features of PIFC Nanoparticles.

To explore the photothermal features of PIFC NPs, aqueous solutions with various particle amounts (0, 150, 300, 450, 600 and 750 $\mu\text{g/mL}$) were irradiated with different powers using an 808-nm laser (0.5, 1.0 and 1.5 W/cm^2 , respectively, for 5 minutes). Then, heating monitoring and quantitation were performed at 1-min intervals with an infrared thermal imaging camera. Under 0.5 W/cm^2 laser irradiation, the highest concentration of nanoparticles only rose the temperature to $\sim 39^\circ\text{C}$ (Fig. 4a). However, under 1.0 W/cm^2 laser irradiation, the PIFC NPs solution (600 $\mu\text{g/mL}$) reached 45°C after irradiation for 5 min (Fig. 4b and c), while pure water was not significantly heated by irradiation, which indicates the PIFC NPs solution can quickly and effectively convert light into heat. Obviously, the heating effect highly depended upon particle levels and irradiation time. As expected, it was also found that a higher laser power density amplitude intensified the heating phenomenon (Fig. 4e and f).

Photostability is considered another key parameter in evaluating potential applications of photothermal agents in cancer treatment. For this purpose, a PIFC aqueous solution (600 $\mu\text{g/mL}$) was irradiated circularly using an 808-nm laser (1.0 W/cm^2 for 5 min). Interestingly, peak temperature change was found to be smaller (Fig. 4d). Both phenomena indicate that PIFC NPs have good photostability as expected. Overall, these unique photothermal features indicate PIFC NPs constitute a promising platform for PTT.

3.4 Cellular Uptake of PIFC Nanoparticles

Compared to other surface-modified QDs,^[38–40] PIFC NPs show an evident advantage in the photoluminescence of individual PIFC NPs, since many QDs are encapsulated in every nanoparticle, with relatively strong protection by the large polymer matrix without aggregation. Therefore, PIFC NPs could constitute a great probe for real-time optical cell imaging, which may be extremely useful for diagnosing and treating cancers. Using the optical properties inherited from the packaged QDs, the cell internalization and intracellular distribution of PIFC NPs were observed by CLSM.

Figure 5 shows fluorescent lysosomes as well as DAPI and overlay images of MDA-MB-231 cells treated for different times. In the fluorescent image, PIFC NPs entered the cytoplasm in small amounts at 0.5 h. After 3 hours, a large number of PIFC NPs entered the cytoplasm and fused with lysosomes. These findings indicate PIFC NPs enter cells via endocytosis and are concentrated in lysosomes.

In addition, the majority of the bright spots occupied the cytosol, and fluorescent signals showed uneven or random distribution in cells. The above findings, alongside punctuate fluorescence appearing along the cells suggest the cellular internalization of PIFC NPs.

3.5 In vitro Anticancer Properties.

Using cell counting kit-8 to evaluate the cyto-compatibility of PIFC NPs to MDA-MB-231 cells, we demonstrated that PIFC NPs solutions at 0 to 750 $\mu\text{g/mL}$ had no cytotoxicity following 24 h of incubation (Fig. 6a). However, after exposing PIF NPs (600 $\mu\text{g/mL}$) to NIR irradiation (1.0 W/cm^2 for 5 min), cell viability was decreased by 50% within 24 h. Upon CQ loading, PIFC NPs induced remarkable cell death, and under the same NIR irradiation, 80% cell death was observed within 24 h (Fig. 6b). What's more, PIF NPs and PIFC NPs solutions at various levels (0, 150, 300, 450, 600 and 750 $\mu\text{g/mL}$) were administered to cells submitted to irradiation at different times (0, 1, 2, 3, 4 and 5 min). As expected, cell viability overtly decreased with increasing nanoparticle amounts and irradiation time. Even at low IR820 level (80 $\mu\text{g/mL}$), cell viability was reduced by 50% after 24 h for PIF NPs (Fig. 6c). Strikingly, PIFC NPs resulted in a more speedy decrease of cell viability compared with PIF NPs under the above conditions (Fig. 6c and d). Fluorescence microscopy of live and dead cells indicated overtly increased in vitro PTT efficiency of PIFC NPs conferred by the delivered CQ molecules (Fig. 6e).

3.6 Mechanisms.

To evaluate the increased cytotoxicity of CQ-loaded PIFC NPs, immunoblot and confocal microscopy were performed. The LC3II protein is considered the gold standard for detecting autophagy. In the process of autophagy, the LC3 precursor molecule is cut at the C-terminal 5 amino-acid peptide by ATG4B and cleaved to form cytosolic LC3 (i.e., LC3I). Subsequently, LC3I is induced by APG7L/ATG7, transferred to ATG3 and combined with fatty acid ethanolamine (PE). This coupling is transformed into the membrane type LC3 (i.e., LC3II), which attaches to the autophagosome membrane to form the structural protein of the autophagosome.^[41] In MDA-MB-231 cells incubated with PIF NPs and irradiated with an 808-nm laser, LC3II was overtly upregulated compared with the non-NIR irradiation group (Fig. 7a and b). The above data demonstrated PTT overtly induced autophagy, corroborating a recent report.^[21] Both cells administered CQ and PIFC NPs under NIR irradiation had elevated LC3II protein amounts (Fig. 7c and d). These findings suggest CQ loaded on PLGA NPs does not prevent LC3 I conversion into LC3 II. Next, LysoTracker Green was utilized for staining acidic vesicles (lysosomes) in cells.

Upon NIR irradiation, PIFC NPs induced remarkably enhanced accumulation of autophagic vesicles (green) in cells in comparison with other groups (Fig. 7g). TEM images indicated that MDA-MB-231 cells administered PIF NPs followed by NIR irradiation generated more autophagic vesicles (both autophagosomes and autolysosomes) in comparison with controls. At the same time, in comparison with MDA-MB-231 cells administered PIF NPs, those administered both PIFC NPs and NIR radiation showed more autophagic vesicles, especially autolysosomes (high density of the content; Fig. 7h). This marked elevation of autophagic vesicles in cells after treatment with PIFC NPs may be attributed to CQ, preventing autolysosome degradation. In order to further assess whether autophagic vesicle elevation results from increased autophagy or its suppressed degradation by CQ, P62 degradation levels were examined. P62 is a ubiquitin-binding protein, which is tightly associated with protein ubiquitination. It regulates various cell signal transduction and autophagic processes.^[42] During the process of autophagy, P62 protein interacts with ubiquitinated proteins, forming a complex with LC3-II on the inner membrane of the autophagy body and being degraded together in the autophagic lysosome.^[43] Therefore, during autophagy, cytosolic P62 protein is continuously degraded; in case of reduced or defective autophagy, the P62 protein continuously accumulates in the cytosol, with its amounts indirectly reflecting autophagosome clearance.^[44] As depicted in Fig. 7e and f, in comparison with control cells, P62 amounts were increased in CQ and PIFC NP-treated cells, indicating that the observed increase in autophagic vesicles was due to suppressed autophagy-lysosomal degradation mediated by CQ.

Generally, the occurrence of autophagy needs to go through the following four stages: (1) the intracellular cargo is engulfed to form a double membrane structure; (2) autophagosome formation; (3) autophagosome and lysosome fusion to produce an autophagolysosome; (4) autophagolysosome degradation.^[45] Autophagy removes injured organelles,

resists cell stresses and induces resistance to therapeutic agents^[36]. As shown in Fig. 7i, CQ molecules delivered by PIFC NPs had no negative effect on LC3 I conversion into LC3 II, likely because the autophagy inhibitor CQ acts on the last stage of autophagy to prevent the degradation of autophagic lysosomes and does not affect LC3I conversion into LC3II. However, it markedly suppressed the degradation of autolysosomes by lysosomes, accumulating autophagic vesicles in cells. The observed in vitro PTT efficacy was therefore highly increased after treatment with PIFC NPs.

3.7 In Vivo Fluorescence Imaging with PIFC nanoparticles

Through fluorescence signal distribution in internal organs (heart, liver, spleen, lung and kidney), the metabolic pathway of PIFC NPs was explored. As depicted in Fig. 8a, with extended injection time, the fluorescence signals of the kidney and liver also increased. At 2 h, the fluorescence signal of the kidney was strongest, but the signal intensity was always lower than that of the liver. After that, the fluorescence signal of the kidney gradually weakened, while that of the liver continued to increase, reaching a peak at 8 h; the fluorescence signal disappeared after 24 h. These results showed that PIFC NPs were mainly metabolized by the liver, and a small amount was metabolized by the kidney.

Based on in vitro cell internalization data, tumor imaging of PIFC NPs was examined in tumor-bearing mice. PIFC NPs were administered via the tail vein to study time-dependent in vivo fluorescence (Fig. 8b). With increasing time, fluorescence signals were increasingly stronger; within 8 h post-injection, strong fluorescence signals were observed in the tumor region, and the tumor was overtly demarcated from the adjacent non-cancerous tissues. The subsequent 16 h witnessed a steady and slow reduction in fluorescence intensity at the tumor site. Even after 24 hours, fluorescence in the tumor area was weakened, but could still be distinguished from normal tissues. Next, fluorescence intensity of total tumor photons was determined as a post-injection time function. There was overtly elevated concentration of PIFC NPs in the tumor that was maintained within approximately 8 h post-injection, and the results showed that the prepared PIFC NPs can remain in the tumor for a long time and could be utilized as an ideal fluorescence imaging contrast agent.

3.8 In vivo Anticancer Therapeutic Effects of PIFC Nanoparticles

In vivo anticancer effects in various groups with or without laser therapy were examined in mice harboring MDA-MB-231 cell xenografts. When the tumors approximated 100 mm³, the animals were assigned to 6 groups of five, including the (1) control, (2) NIR irradiation only, (3) PIFC NPs, (4) PIF NPs with NIR irradiation at 45°C, (5) PIF NPs with NIR irradiation at 55°C and (6) PIFC NPs with NIR irradiation at 45°C groups. The animals underwent irradiation with an 808-nm laser for 5 min upon administration of NPs for 8 h in groups 2, 4, 5 and 6. An IR thermal camera was utilized to monitor temperature changes at the tumor site under NIR laser irradiation (Fig. 9a). After injection, the tumor site rapidly heated up under near-infrared irradiation, reaching 45°C within 5 minutes. Such temperature could efficiently preserve healthy tissues while exerting overt antitumor effects. Mouse body weights had no overt changes 14 days post-treatment, suggesting no obvious biotoxicity for the tested products (Fig. 9b). Tumor volumes were assessed every other day (Fig. 9c). At study end, mouse euthanasia was performed, and tumors were extracted and weighed. Average tumor weights in various groups after 14 days are depicted in Fig. 9d. The imaged tumors follow the order described in Fig. 9e. Tumors in groups 2 and 3 had rapid growth, similar to control animals. After administration of PIF NPs following NIR irradiation (group 4), tumor growth was inhibited significantly. In addition, we also assessed a high temperature group (group 5), whose tumor growth inhibition was higher than that of the low temperature group (group 4). However, group 6 (PIFC with NIR irradiation at 45 °C) had the most significant tumor inhibition. Tumor volumes were quickly reduced by this treatment and remained low throughout the study.

As shown in Fig. 9f, the high temperature of 55°C caused the skin tissue on the tumor surface to be severely injured due to heat, and the scab surface was large. During the treatment, the tumor recurred in the unscabbed area, and the temperature was mild at 45°C. Under photothermal conditions, the wound surface is less damaged and the scab is small. During the treatment, the tumor is effectively ablated due to the presence of the autophagy inhibitor CQ. The experimental results showed that mild photothermal therapy combined with autophagy inhibition can effectively prevent excessive

damage to the skin tissue, while effectively inhibiting tumor growth. The above findings confirmed that PIFC NPs have great potential use in PTT, and autophagy inhibition by CQ could evidently improve the antitumor effect in vivo. Indeed, many studies have recently been conducted to provide the PTT method a functional factor that can achieve a certain degree of autophagy inhibition.^[46, 47] For example, combination of iron oxide nanoparticles with chloroquine efficiently improved treatment effects by inhibiting autophagy suppression.^[48] Autophagy inhibitors (CQ and 3-MA) have been used in chemical-PTT synergistic strategies to obtain excellent results in drug-resistant cancers.^[49] However, this research mostly examined direct injection of autophagy suppressors, resulting in the inability to control the dose in subsequent clinical trials. For this reason, the PIFC NPs manufactured in this study provide a high potential for the PTT method under mild near-infrared conditions.

3.9 In vivo Toxicity Research

To further evaluate PIFC NPs for in vivo safety, routine blood and blood chemistry markers (reflecting renal and hepatic functions) were assessed in mice administered PIFC NPs for 3 weeks, and no marked changes were detected (Fig. 10a and 10b). H&E staining was also carried out, indicating no overt inflammation or damage in the heart, kidney, spleen, liver, lung and intestine within 3 weeks (Fig. 10c). The above findings indicate PIFC NPs at the administered doses are safe for biological application.

4. Conclusion

In summary, the prepared PIFC NPs have excellent fluorescence performance for imaging, and may render NPs suitable for high-efficiency PTT. The results indicated nanoparticle-mediated PTT enhances tumor cell autophagy, whose suppression obviously increases PTT's efficiency in cancer cell inhibition. In addition, PIFC NPs are biocompatible nanoparticles comprising photothermal NPs and autophagy inhibitors, which can be used for systemic delivery and in vivo cancer therapy. In this study, autophagy suppression in tumor cells significantly enhanced PTT's efficacy, thereby completely inhibiting tumors at mild treatment temperatures. This research showed that regulating autophagy in cancer may be promising in assisting PTT for therapy. Finally, the successful preparation of PIFC NPs in one pot provides a simple approach for manufacturing nanotheranostics that may be useful in both fluorescent imaging and PTT in cancer.

Declarations

Conflict of interest

The authors report no conflicts of interest in this work.

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Figures

Figure 1

Schematic illustration of the integrated research on mild photothermal therapy and fluorescence imaging.

Figure 2

Characterization and performance monitoring of PIFC NPs. a) Structural illustration of PIFC NPs. b and c) TEM images of PIFC NPs. d) Hydrodynamic diameters of PIFC NPs assessed by dynamic light scattering. e) Zeta potential of PIFC NPs. f) Size and PDI changes of PIFC NPs in different media such as DI water, PBS, FBS, DMEM and L15 within 7 days; insets are PIFC NPs in different media.

Figure 3

Characterization of PIFC NPs. a) UV-vis spectra of CQ, PLGA NPs, IR820 and PIFC NPs. b) Standard curve of CQ levels in aqueous solution. c) Standard curve of IR820 levels in aqueous solution. d) EDS analysis of PIFC NPs. e) Fluorescence emission spectra of PIFC NPs (blue), ZnCdSe/ZnS QD (dark) and PLGA NPs (red) at λ_{ex} of 450 nm. f) Fluorescence changes in different media such as DI water, PBS, FBS, DMEM and L15 within 7 days; insets show PIFC NPs in different media. g) Fluorescence changes at various concentrations. h) Standard curves of CQ levels in PBS (pH 7.5 and pH.5.0). i) In vitro release profiles of PIFC NPs at pH 5.0 and 7.4.

Figure 4

Thermal performance. a) Temperature increases of pure water and PIFC NPs solutions at various levels after irradiation with an 808-nm laser (0.5 W/cm²) for a long time. b) Temperature increases of pure water and PIFC NPs solutions at various levels after irradiation with an 808-nm laser (1.0 W/cm²) for a long time. c) Temperature increases of pure water and PIFC NPs solutions at various levels after irradiation with an 808-nm laser (1.5 W/cm²) for a long time. d) Temperature changes of PIFC NPs in aqueous solution (600 µg/mL) under cyclic irradiation (808 nm NIR, 1.0 W/cm², 300s, 4 cycles). e) Real time thermal images of PIFC NPs solutions under irradiation with an 808-nm laser (1.0 W/cm²). f) Real time thermal images of PIFC NPs solutions under irradiation with an 808-nm laser (1.5 W/cm²). (i) Temperature changes of PIFC NPs in aqueous solution (600 µg /mL) under cyclic irradiation (808 nm NIR, 1.0 W/cm², 300s, 4 cycles).

Figure 5

PIFC NPs uptake by MDA-MB-231 cells upon incubation for 0, 0.5 and 2 h, as assessed by confocal microscopy. Left to right: PIFC NPs, LysoTracker Green-labeled lysosome, DAPI-labeled nucleus and the merged image.

Figure 6

In vitro anticancer effects. a) MDA-MB-231 cell viability after incubation with PIFC NPs at 37°C for 24 h at various concentrations. b) MDA-MB-231 cell viability upon incubation with blank control, CQ, PIF NPs and PIFC NPs, with or without irradiation with an 808-nm laser (1.0 W/cm², 5 min). c) MDA-MB-231 cell viability after incubation with PIF NPs and PIFC NPs at various concentrations upon irradiation with an 808-nm laser (1.0 W/cm², 5 min). d) MDA-MB-231 cell viability after incubation with blank control, PIF NPs and PIFC NPs following irradiation with an 808-nm laser (1.0 W/cm²) for various times. e) Fluorescent images of MDA-MB-231 cells after calcein-AM (green, live cells) and propidium iodide (red, dead cells) staining following various treatments. Scale bar, 200 μm. **p<0.01 and ***p<0.001, obtained by Student's t-test.

Figure 7

Autophagy related mechanisms. a) Immunoblot detection of LC3 I and LC3 II. b) Expression of LC3 II relative to β -actin. MDA-MB-231 cells were administered PIF NPs with or without 808 nm irradiation (1.0 W/cm², 5 min). c) Immunoblot detection of LC3 I and LC3 II. d) Expression of LC3 II relative to β -actin. MDA-MB-231 cells underwent incubation with blank control, CQ, PIF NPs and PIFC NPs with 808 nm irradiation (1.0 W/cm², 5 min). e) Immunoblot detection of P62. f) Expression of P62 relative to β -actin. MDA-MB-231 cells were treated as in (c). g) Confocal images of MDA-MB-231 cells after staining with DAPI and LysoTracker Green. h) TEM images of MDA-MB-231 cells treated as in (c). i) Proposed mechanism of the photothermal antitumor effect enhanced by autophagy suppression. ** $p < 0.01$ and *** $p < 0.001$, determined by Student's t-test.

Figure 8

Fluorescence performance analysis. a) Fluorescence metabolic pathway of PIFC NPs. b) In vivo fluorescence imaging of tumor-bearing mice upon tail injection of PIFC NPs.

Figure 9

In vivo anticancer effect. a) Real time infrared thermal images. b) Body weights of mice following different treatments. c) Relative tumor volumes upon different treatments. d) Tumor weights following different treatments. e) Digital photographs of tumors obtained at day 14 in various mouse groups. f) Wound surfaces of the skin in the tumor area with different temperatures in the laser irradiation area. Scale bar, 100 μm . ** $p < 0.01$ and *** $p < 0.001$, determined by Student's t-test.

Figure 10

In vivo toxicity. a) Blood biochemistry analysis. b) Routine blood analysis. c) H&E-stained organs from mice i.v. administered PIFC NPs. Euthanasia was performed at 0, 1, 7, and 14 days.

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