Step-by-step Guided Nanoplatforms for Efficiently Sensitizing Phototherapy via Mitochondrial Metabolic Reprogramming

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**Research Article**

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Abstract

Background

Phototherapy, a promising chemotherapy alternative, has been widely utilized for cancer treatment. However, heat shock protein (HSP70) mediated-heat tolerance in photothermal therapy (PTT), and the attenuated therapeutic effect of photodynamic therapy (PDT) in the hypoxic tumor microenvironment still limit its clinical applications, where both processes are intimately linked to energy metabolism.

Results

We designed a site-specific nanoplatform (CS@ATO/CHC/T780 NPs) modulating energy metabolism to down-regulate the expression of HSP70 and alleviate hypoxia for enhanced phototherapy by integrating a mitochondria-targeted triphenylphosphine (TPP) derivative (T780), mitochondrial electron transport chain inhibitor atovaquone (ATO) and monocarboxylic acid transporter 1 (MCT1) inhibitor α-cyano-4-hydroxycinnamate (CHC), and modified with chondroitin sulfate (CS). Mechanistically, CS and TPP-tailored IR780 were used to achieve site-specific delivery to tumor tissues and mitochondria in tumor cells, respectively. After efficient cellular internalization, the nanoplatform could effectively disassemble to release cargoes in the acid tumor microenvironment. ATO, a mitochondrial electron transport chain complex III inhibitor, could reduce the production of intracellular adenosine triphosphate (ATP) and further downregulate the expression of HSP70 to overcome the thermo-resistance of PTT. Meanwhile, CHC could reduce the uptake rate of lactic acid (LA) to save oxygen consumption for improving the effectiveness of PDT.

Conclusion

The CS@ATO/CHC/T780 NPs exhibits excellent anticancer activity and good biosafety in vitro and in vivo, providing a prospective strategy for efficient phototherapy by manipulating energy metabolism.

Introduction

Phototherapies, including photothermal therapy (PTT) and photodynamic therapy (PDT), have recently been identified as promising alternatives to chemotherapy\(^1\). However, some drawbacks with phototherapy still limits its clinical application. For example, heat stress always induces a defense mechanism to promote the survival of tumor cells by overexpressing ATP-dependent HSP70 during the PTT, which can cause significant thermo-resistance and reduce the PTT efficacy. Furthermore, energy metabolism-mediated oxygen consumption weakens the efficiency of PDT. Thus, manipulating the intracellular ATP level of tumor cells to impaire heat-induced expression of HSPs and alleviate oxygen consumption may provide a prospective strategy to reverse the resistance of phototherapy.
Mitochondria are the vital organelles supplying energy and regulating cancer cell apoptosis, which are more vulnerable to hyperthermia and oxidative damage. Thus, developing various types of new mitochondrial targeting agents would further improve the efficiency of phototherapies. Atovaquone (ATO) is a mitochondrial electron transport chain complex III inhibitor, which has been shown to decrease ATP levels and has been approved by the FDA to treat malaria. Interestingly, the reduction of ATP synthesis will lead to downregulated expression of HSP70, suggesting a great potential to overcome the challenge of tumor heat tolerance in PTT. In the metabolic process of cancer cells, the produced lactic acid (LA) enters tumor cells from the tumor microenvironment via monocarboxylic acid transporter 1 (MCT1) and is rapidly oxidized to produce ATP by consuming oxygen. It has been reported that the MCT1 inhibitor α-cyano-4-hydroxycinnamate (CHC) can make available more intracellular oxygen for PDT, facilitating PDT in cancer treatment. Therefore, mitochondria-targeted phototherapy integrated ATO and CHC may be a promising method to overcome potential defects of phototherapy.

Herein, we designed a multifunctional nanoplatform (CS@ATO/CHC/T780 NPs) which can target mitochondria and regulate ATP and LA metabolism to further enhance PTT and PDT efficacy. As illustrated in Scheme 1, CS@ATO/CHC/T780 NPs was loaded with the TPP-tailored IR780 derivative (T780), atovaquone (ATO), and α-cyano-4-hydroxycinnamate (CHC), and further modified with chondroitin sulfate (CS). In this nanoplatform, CS, a primary CD44-binding molecule frequently overexpressed in cancer, was used for cancer-specific targeting. Further, T780 enhanced the anti-tumor capability of PDT and PTT by targeting mitochondria. ATO lowered ATP synthesis and downregulated HSP70 to overcome the heat tolerance of cancer cells during PTT. In addition, CHC blocked LA uptake via MCT1 inhibition to relieve tumor hypoxia, thereby greatly enhancing the efficacy of the PDT. This integrated strategy significantly improved the therapeutic effects and paves a new way to avoid phototherapy resistance.

Materials and methods

Materials

IR-780 iodide (cat. no. 425311), atovaquone (cat. no. 1044651), and α-cyano-4-hydroxycinnamate (cat. no. 70990) were purchased from Sigma-Aldrich. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Millipore Sigma. Primary antibodies used for immunoblotting in this study included anti-β-actin (cat. no. sc-8432, Santa Cruz Biotechnology), anti-HSP70 (cat. no. MA3-007, Affinity BioReagents), anti-MCT1 (cat. no. ab85021, Abcam), and Ki67 (cat. no. bs-23103R, Bioss).

Synthesis of (3-Aminopropyl) TPP Bromide (TPP-NH₂) and TPP-IR780 (T780)

TPP-NH₂ and T780 were synthesized using a previously reported method. 1.3112 g triphenylphosphine and 1.0946 g 3-bromopropylamine hydrobromide were dissolved in 20 mL anhydrous acetonitrile, and the
suspension was refluxed at 85°C for 15 h. After the mixture was cooled down to room temperature, the acetonitrile was removed via vacuum rotary evaporation. Then add 10 mL acetonitrile, 30 mL n-hexane, 100 mL isopropanol, and 50 mL ethyl ether to crystallize from the mixture of TPP-NH₂ at 4°C.

Next, 0.2006g TPP-NH₂ and 0.1667g IR780 iodide were dissolved in 2.5 mL dichloromethane and 5 mL anhydrous N, N-dimethylformamide (DMF), and then 137 µL triethylamine (TEA) was added to the mixture. The mixed solution was stirred magnetically (600 rpm) at 85°C under nitrogen gas protection for 4 h, and the solvent was removed by rotary evaporation. Afterwards, the T780 crude products was separated and purified by silica gel column chromatography and eluted with dichloromethane and methanol (from 100:1 to 20:1, V/V). The final products obtained were stored in the darkness at -20°C.

**Preparation of ATO/CHC/T780 NPs**

ATO/CHC/T780 NPs was synthesized using the nanoprecipitation method. Briefly, 5.1598 mg T780, 1.8342 mg ATO, and 0.9459 mg CHC (molar ratio 1:1:1) were separately dissolved in 150 µL methyl alcohol, and the mixture was added into 5 mL deionized water with quick stirring (1000 rpm, 20 min). The remaining methyl alcohol was removed by rotary vacuum evaporation to prepare ATO/CHC/T780 NPs.

**Preparation of CS@ATO/CHC/T780 NPs**

1 mL ATO/CHC/T780 NPs (NPs) solution dropwise to CS solution (the weight of CS: T780 is 1:1, w/w) under 1000 rpm magnetic stirring for 5 min at room temperature. Then, the CS@ATO/CHC/T780 NPs (CS@NPs) was obtained.

**Characterizations**

The size distribution and zeta potential of CS@NPs were examined using a Zetasizer Nano ZS analyzer (Malvern Instruments, UK). The morphology of nanoparticles was observed using a transmission electron microscopy (TEM, HT7800) in Electron Microscope Room, West China School of Basic Medical Sciences & Forensic Medicine, Sichuan University. The absorption spectra was recorded with a UV/Vis spectrophotometer (UV-2700, Shimadzu, Japan). The stability of CS@NPs was evaluated based on the visual appearance of the nanoparticle solution (Tyndall effect) for 14 days.

**In vitro photothermal performance**

IR780-containing nanoparticles at different concentrations (0 µM, 10 µM, 20 µM, 50 µM, and 100 µM) were added to a 24-well plate. Then, each well was irradiated by 808 nm (for IR780) or 660 nm (for T780, NPs, and CS@NPs). During the near-infrared (NIR) laser irradiation (1 W/cm², 5 min), the temperature was recorded by a digital thermometer at 30 s intervals.

**Detection of singlet oxygen**

DPBF was used as a singlet oxygen (¹O₂) trapping reagent. In brief, 1 mL of IR780, T780, NPs, and CS@NPs (5 µM for IR780 and T780) containing 15 µg/mL DPBF were irradiated using 808 nm (for IR780)
or 660 nm (for T780, NPs, and CS@NPs) laser (1 W/cm²). The characteristic absorbance curves for each sample at 420 nm during different periods of time were measured via a UV-vis spectrophotometer (UV2700, Shimadzu, Japan). The production efficiency of $^{1}\text{O}_2$ was determined by the remaining DPBF (%), which was calculated as $A_t/A_0 \times 100\%$. $A_t$ represented the remaining DPBF absorbance after being irradiated for t min, while $A_0$ represented the absorbance without irradiation. The decrease of only DPBF under irradiation was set as a control.

**Loading efficiency of T780**

The loading efficiency (LE) of T780 was calculated based on the following formulas: loading efficiency (%) = $W_E/W_T \times 100\%$. $W_E$ represented the encapsulated amount of T780 in CS@NPs and $W_T$ represented the total amount of T780. The concentration of T780 was quantified by UV- vis spectrum.

**Cell lines and cell culture**

Human cervical cancer cells (Hela and SiHa) were obtained from the American Type Culture Collection (ATCC). The cells were maintained in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% fetal bovine serum (BI) and 1% antibiotics (penicillin/streptomycin, 100 U/mL) and incubated at 37°C under a 5% CO₂ atmosphere.

**Cellular internalization**

The cellular uptake on both Hela and SiHa cells were analyzed to evaluate the targeting specificity and internalization efficiency of nanoparticles. The cellular uptake of T780, NPs, CS@NPs, and CS + CS@NPs (equivalent to T780 at 10 µM) were visualized using a fluorescence microscope (Nikon, Japan). To explore the uptake process further, we evaluated the cellular uptake of CS@NPs at intervals of 1, 2, 4, and 6 h using fluorescence microscopy. Afterward, cells were washed and resuspended with PBS. The time-dependence of uptake was quantitatively measured by flow cytometry (FCM) using a BD FACS Aria III (BD Bioscience, USA).

**Cytotoxicity and proliferation assays**

Hela and SiHa cells were seeded in 96-well plates ($3 \times 10^4$ cells per well) and incubated for 24 h. Then, the cells were incubated with different concentrations of ATO, CHC, T780, NPs, and CS@NPs for 4 h. After discarding the supernatant and washing with PBS, the cells (with or without 660 nm laser irradiation for 1 min, 1 W/cm²) were incubated for another 24 h. Cell viability was determined by the MTT assay. The absorbance of each well was measured at 570 nm.

For the colony formation assay, Hela and SiHa cells were incubated in 12-well plates (2500 cells per well) for 1 week until small colonies could be seen. The cells were then treated with 5 µM of ATO, CHC, T780, NPs, and CS@NPs (with or without 660 nm laser irradiation for 1 min, 1 W/cm²) for 4 hours and continuously cultured for 1 week. Colonies were fixed with 4% paraformaldehyde for 30 min and then
stained with 0.1% crystal violet for 10 min and washed with ddH₂O. The colony numbers of each well were counted.

The EDU assay was utilized to detect cell proliferation. We seeded treated Hela and SiHa cells in 96-well plates for overnight. And then the cells were exposed to 50 µM/L of EdU (Ribobio, China) for an additional 4 h at 37°C. Afterwards, we applied 4% formalin for 30 min and used 0.5% TritonX-100 to permeabilize for 20 mins at room temperature. After being washed with PBS, we added Apollo reaction solution (100 µL/well) to stain the EdU. Subsequently, the nuclei were stained with Hoechst 33342 (100 µL/well) for 30 mins, and used a fluorescent microscope to observe the proliferation of cells. The EdU incorporation rate was expressed as the ratio of EdU-positive cells (red cells) to total Hoechst 33342 positive cells (blue cells).

**Mitochondria co-localization**

Hela and SiHa cells were seeded in 6-well plates (1 × 10⁶ cells each well) for overnight. After treatment with equivalent dose of T780, NPs, and CS@NPs (10 µM for T780) for 4 h, cells were incubated with 200 nM Mito-Tracker Green for 30 min at 37°C. Fluorescence microscopy was used to observe the co-localization of CS@NPs with mitochondria.

**ROS assay**

Cells were plated into 6-well plates (1 × 10⁶ cells per well) overnight and then subjected to different treatments. Then cells were washed twice with PBS, and incubated with 1 µM dichlorofluorescin diacetate (DCFH-DA) for 20 min at 37°C. The fluorescence of oxidized DCFH was measured immediately by fluorescence microscopy and FCM.

**Determination of mitochondrial membrane potential**

The mitochondrial membrane potential (MMP) was evaluated using the JC-1 fluorescent probe (cat. no. C2006, Beyotime Biotechnology). JC-1 exists as a green fluorescent monomer at low MMP, while at higher MMP, JC-1 forms red fluorescent aggregates. Briefly, the cells were cultured in 6-well plates and exposed to different treatments. After being incubated with 10 µg/mL JC-1 in the dark for 20 min at 37°C, cells were washed twice with PBS and subjected to fluorescence microscopy.

**Intracellular ATP measurement**

The intracellular ATP levels were determined using the ATP assay kit (cat. no. S0026, Beyotime Biotechnology). Briefly, the cells were seeded in 6-well plates (1 × 10⁶ cells per well) for overnight, then left in different treatments before the subsequent test. According to the manufacturer's instructions, 20 µL of harvested samples was added to each testing well, followed by the addition of 100 µL of ATP working solution, quickly mixed and measured the value of relative light unit (RLU) by a Chemiluminometer (luminometer).

**LA concentration assays**
The lactate acid (LA) concentration was detected using the LA assay kit (cat. no. BC2235, Solarbio) according to the manufacturer’s instructions. In brief, after different treatments, the cells were scraped off and resuspended, then homogenized at 4°C with an ultrasonic sonicator (CIENTZ). Following centrifugation at 10,000 g for 10 min at 4°C, the individual supernatant was mixed with the reaction reagent and kept for 15 min at 37°C, then transferred the mixture to a 96-well plate. Finally, the LA concentration was detected by measuring the absorbance at 450 nm using a multifunctional microplate reader (PerkinElmer, USA).

**LDH release assay**

The LDH release assay kit (cat. no. C0016, Beyotime Biotechnology) was used to detect LDH levels, which was based on the reaction that LDH can catalyze lactate to form pyruvate. The samples were incubated with LDH release reagent for 1 h and then the supernatant was collected after centrifugation. Following this, the samples were incubated with LDH detection solution for 30 min in dark, and the levels of LDH were determined by measuring the absorbance of each sample at 490 nm.

**Western blot analysis**

Proteins were separated on SDS-PAGE gels. Then, the proteins were transferred to polyvinylidene difluoride (PVDF) membranes. The PVDF membranes were then blocked with 5% BSA diluted in TBS for 2 h at room temperature. Primary antibodies against HSP70, MCT1, and β-actin were then added according to the manufacturer’s protocols. The samples were gently shaken at 4°C overnight. HRP-conjugated rabbit anti-mouse IgG (Abcam) and mouse anti-rabbit IgG (Abcam) secondary antibodies were added and incubated at room temperature for 2 h. Densitometric analysis was performed using the ChemiDoc Touch Imaging System (Bio-Rad Laboratories, USA).

**Animals and tumor models**

Female BALB/c nude mice (6 – 8 weeks) were ordered from Jiangsu Jicui Yaokang Biological Technology Co. Ltd. and bred in a specific pathogen-free (SPF) laboratory. All animal procedures performed in studies were in accordance with recognised ethical standards. Hela tumor-bearing models were obtained by subcutaneously injecting Hela cells (2 × 10⁶) dispersed in 100 µL of PBS into the right side of the back of each mouse.

**In vivo tumor imaging and biodistribution**

The tumor-bearing mice were injected via the tail vein with free IR780, T780, NPs, and CS@NPs (2 mg/kg T780). Whole-body images were acquired by the IVIS Lumina III Imaging System (CLS136334, PerkinElmer) under 2.5% isoflurane anesthesia at different preset time points of post-injection (2, 4, 6, 8, 12, and 24 h). After the final imaging, the mice were sacrificed, and major organs and tumors were collected and subjected to ex vivo imaging.

**In vivo infrared thermography**
After the tumor volume grew to about 100 mm³, normal saline, free IR780, T780, NPs, and CS@NPs solutions (2 mg/kg T780) were intravenously injected into Hela tumor-bearing mice. After 6 h, the tumors were exposed to a NIR laser (1 W/cm²) for 5 min. During irradiation, the temperature at the tumor region was imaged using an infrared thermal imaging system (Infra Tec. VH-620) with intervals of 1 min.

In vivo antitumor efficacy

Once the tumor volume reached approximately 100 mm³, the mice were randomly divided into 4 groups (n = 5) and injected with normal saline, T780, NPs, and CS@NPs (2 mg/kg T780) via the tail vein every 2 days. All mice in the T780, NPs, and CS@NPs groups received laser irradiation (1 W/cm², 5 min) at 6 h post-injection. Tumor volume and body weight were measured by a digital caliper every 2 days during a 14-day observation period. The tumor volume was calculated according to the following formula: \( V = L \times W^2 / 2 \), where \( L \) and \( W \) respectively mean the longest diameter (mm) and the shortest diameter (mm) of the tumor. All the mice were sacrificed on day 15, and the tumors and major organs (heart, liver, spleen, lung, and kidney) were collected and sectioned for further hematoxylin and eosin (H&E) staining and Ki-67 antibody staining assay for detecting the tumor growth fraction. Tumors were digested and lysed, then the ATP and lactic acid contents were measured.

Statistical analysis

All data were presented as mean ± SD based on at least three independent tests. Comparisons between groups were performed by two-tailed Student’s t-test or one-way ANOVA. The \( P \)-value was calculated using GraphPad Prism 9.0 software. A \( P \)-value < 0.05 was considered statistically significant, the significance is coded as: * for \( P < 0.05 \), ** for \( P < 0.01 \), *** for \( P < 0.001 \). Fluorescence intensity analysis of the images was performed by Image J 2X software. The data of FCM was analyzed by FloJo-V10.

Results and Discussion

Preparation and characterization of CS@ATO/CHC/T780 NPs

The T780 conjugate was prepared in two steps, as described previously[10]. The synthetic pathways for TPP-NH₂ and T780 are shown in Fig. 1A-B. The \(^1\)H NMR spectrum of IR780, TPP-NH₂, and T780 confirmed the successful synthesis of T780 (Fig. 1C-E). CS@ATO/CHC/T780 NPs were constructed with ATO, CHC, T780, and CS by a nanoprecipitation method. Dynamic light scattering (DLS) analysis revealed that average particle size of ATO/CHC/T780 NPs (NPs) and CS@ATO/CHC/T780 NPs (CS@NPs) were 133.86 nm and 134.75 nm, respectively (Fig. 1F-G), which was in good agreement with the TEM images (Fig. 1H-I). The zeta potential of NPs was 34.3 mV, while the zeta potential of CS@NPs was -36.2 mV, indicating the successful modification of CS and good blood circulation stability of CS@NPs (Fig. 1J). According to UV-vis absorption spectra, CS@NPs has a maximum absorption peak at 660 nm (Fig. 1K and Additional file 1: Fig.S1A). Thus, CS@NPs could be activated by NIR laser (660 nm) and serve as
efficient photothermal and photodynamic agents. The drug loading efficiency of T780 in CS@ATO/CHC/T780 NPs was 27.8% as measured by UV-vis spectrophotometer characterization. Moreover, the Tyndall effect of CS@NPs was still clearly visible even after 14 days (Additional file 1: Fig. S1B-C), suggesting its excellent storage stability.

The photothermal and photodynamic capabilities of CS@NPs were further studied. As shown in Fig. 1L and Additional file 1: Fig.S1D-F, CS@NPs exhibited better photothermal capacity in a dose- and time-dependent manner compared to free IR780, T780, and NPs. To estimate the photodynamic effect of CS@NPs, a 1,3-diphenylisobenzofuran (DPBF) probe was used to evaluate singlet oxygen ($^1O_2$) production in vitro. Measurement of UV − vis absorption spectrum of DPBF in IR780, T780, NPs, and CS@NPs during 150 s NIR laser irradiation are shown in Fig. 1M-N and Additional file 1: Fig.S1G-I. The remaining DPBF (%) exhibited an obvious decrease of CS@NPs under irradiation, indicating excellent $^1O_2$ generation of the CS@NPs. Taken together, the CS@NPs exhibited excellent photothermal and photodynamic performances in vitro.

In vitro cellular uptake and cytotoxicity of CS@NPs

Cellular uptake is essential for enhancing the organelle-targeted capacity and therapeutic efficacy of cancer cells. We evaluated the cellular uptake efficacy of CS@NPs by fluorescence microscopy and flow cytometry in two cervical cancer cell lines (Hela and SiHa). Both fluorescence imaging and flow cytometry assay showed that CS@NPs had the capability of cellular internalization and reached a maximum after 4 hours of incubation (Fig. 2A–D). Furthermore, the cellular uptake efficiency of CS@NPs was reduced after pre-treatment with free CS (Fig. 2E-F), which reflected that the enhanced uptake efficiency was due to the CS modification. Consequently, these results suggested that CS@NPs could act as an ideal nanoplatform to efficiently co-deliver ATO, CHC, and T780 into cancer cells, enabling effective PTT and PDT.

We then measured the viability of cervical cancer cells following different treatments using the MTT assay. Under laser irradiation, CS@NPs exhibited significantly enhanced growth inhibition activity of cervical cancer cells compared with free ATO, CHC, T780, and NPs, which followed a dose-dependent manner (Fig. 3A-D). In addition, the inhibitory rate of CS@NPs was slightly higher than that of NPs at the corresponding concentration, due to the enhanced cellular uptake. These results suggested that phototherapy mediated by CS@NPs had superior cytotoxicity against cervical cancer cells. We further evaluated the anti-proliferative potential of CS@NPs, the results of colony formation (Fig. 3E-F) and EdU assays (Fig. 3G-J) were in accordance with the MTT assay. These results indicate that CS@NPs had excellent anti-tumor activity in vitro.

The mechanism of CS@NPs for anti-cervical cancer

Mitochondria are critical for regulating cancer cell death and metabolism and are more susceptible to hyperthermia and oxidative damage. Hence, therapeutic strategies that target mitochondria are promising for cancer therapy\textsuperscript{[2]}. Molecules modified by TPP are able to easily cross the lipid bilayer and accumulate
in the mitochondrial matrix owing to their lipophilic and hydrophilic properties for the effective delivery of drugs to mitochondria\cite{11}. To investigate the selective mitochondria-targeting ability of CS@NPs in the cervical cancer cell, the subcellular localization was detected using fluorescence microscopy with Mito-Tracker Green after 4 h uptake of the CS@NPs. As shown in Fig. 4A and Additional file 1: Fig.S2A, the red fluorescent of T780 coincides well with the green fluorescent of Mito-Tracker Green, indicating that the CS@NPs can specifically accumulate in mitochondria.

Mitochondria are involved in the production of reactive oxygen species (ROS) and the generation of excessive ROS to promote cell death\cite{12}. In this study, the intracellular level of ROS were measured with the DCFH-DA probe by using flow cytometry and fluorescent microscopy (Fig. 4B-C and Additional file 1: Fig.S2B-C). Under NIR laser treatment, CS@NPs induced significantly higher ROS production than T780 and NPs due to effective uptake and targeting ability, indicating a potent PDT efficacy.

Oxidative damage to mitochondria has been shown to impair mitochondrial function\cite{13}. We used a 5,5',6,6'-tetrachloro-1,1',3,3'-tetracyanoethylbenzimidazole carbocyanine iodide (JC-1) dye to measure mitochondrial membrane potential (MMP) to evaluate mitochondrial function. Figure 4D and Additional file 1: Fig. S2D show the representative fluorescence images of JC-1 assays. The green/red (G/R) ratio enables quantification of the MMP loss of cells during PDT. Low MMP shifted from high JC-1 red emissive fluorescent aggregates on normal mitochondria to green emissive monomers on depolarized mitochondria. We observed a significant decrease in the MMP caused by the CS@NP treatment, indicating mitochondrial dysfunction.

Furthermore, mitochondria are the main sites for intracellular oxidative phosphorylation (OXPHOS) and ATP formation\cite{14,15}. We measured the concentration of ATP in cervical cancer cells. The results indicated that CS@NPs was able to effectively reduce the intracellular ATP levels compared with ATO and T780 (Fig. 4E and Additional file 1: Fig.S2E), showing the potent inhibitory effects of on ATP production. On the other hand, a decreased intracellular ATP level down-regulates heat shock protein 70 (HSP70) expression (Fig. 4H and Additional file 1: Fig.S2H), which would reduce tumor cells heat resistance and improve the PTT therapeutic effect.

In PDT therapy, inhibiting the transport of lactic acid (LA) into cancer cells can release more intracellular oxygen. Here, we used CHC, an inhibitor of MCT1 expression, to solve the hypoxia issue in PDT by regulating LA metabolism. In order to detect the inhibition efficiency of CS@NPs for the transport of LA, we determined the concentration of LA in cervical cancer cells (Fig. 4F). The result showed that CS@NPs significantly inhibited the uptake of LA. Additionally, we characterized the expression of the MCT1 protein by western blotting (Fig. 4H and Additional file 1: Fig.S2H), demonstrating the effect of CS@NPs on the inhibition of MCT1 protein expression. Furthermore, we also detected an increase in lactate dehydrogenase (LDH) release (Fig. 4G and Additional file 1: Fig.S2G). These findings suggested CS@NPs can impede LA transport into cervical cancer cells by downregulating MCT1 protein expression, freeing up more intracellular oxygen for PDT.
In summary, CS@NP treatment enhanced mitochondrial targeting capacity, produced mitochondrial malfunction, and boosted PTT and PDT efficiency to cause tumor cell death, suggesting that it might be employed as a powerful phototherapy nanoplatform for anti-cervical cancer treatment.

In vivo biodistribution and anti-tumor capacity of CS@NPs

NIR fluorescence imaging was used to monitor the biodistribution of CS@NPs in cervical cancer-bearing mice models at various time points in vivo. As illustrated in Fig. 5A, CS@NPs exhibited higher fluorescence signals than IR780, T780, and NPs. The higher accumulation of CS@NPs could be due to the active targeting mediated by CS. In addition, the fluorescence signal intensity of the tumor region reached a maximum fluorescence at 6 h post-administration. Thus, laser irradiation at 6 h post-administration was chosen as the optimal time point for subsequent phototherapy. The tumors and main organs were separated for ex vivo fluorescence imaging at 24 h post-administration. As shown in Additional file 1: Fig. S3A, CS@NPs were mainly distributed in tumor tissue, indicating excellent tumor-targeting ability and prolonged retention time in tumor sites.

The photothermal effect of CS@NPs was examined on the cervical cancer-bearing mice model at 6 h post-administration. The infrared thermal images and temperature change curves of tumors is presented in Fig. 5B and Additional file 1: Fig. S3B. A negligible temperature increase was observed in the normal saline group under NIR light. There is only a moderate temperature increase in the IR780 and T780 groups, owing to the poor stability and rapid plasma clearance in vivo. Nevertheless, the temperature increase of the tumor sites in the CS@NPs group was higher than NPs, which could be attributed to the CS modification.

Subsequently, the antitumor efficacy in vivo was investigated in Hela tumor-bearing BALB/c mice models (Fig. 5C). At 7 days after inoculation the Hela cell, the mice were randomly divided into four groups and received either normal saline, T780, NPs, and CS@NPs injection every 2 days for 15 days. Free T780 group under laser irradiation exhibited a slight inhibition effect on tumor growth due to the fast blood clearance. CS@NPs–mediated PTT and PDT therapy showed an excellent anti-tumor capability (Fig. 5D-F), which benefited from the preeminent accumulation and retention capability in tumors of CS@NPs. Furthermore, we observed decreased ATP and LA levels in tumor tissues with CS@NPs, and weaker IHC staining of Ki-67, HSP70, and MCT1 (Fig. 5G-I).

Evaluation of the biosafety of CS@NPs

The hemolytic test revealed that the hemolysis ratio of the CS@NPs was less than 5% (Fig. 6A), so it cannot give rise to acute hemolysis. Moreover, no noticeable body weight change was observed among the treated mice, suggesting an excellent biosafety profile of CS@NPs (Fig. 6B). The serum levels of various biochemical parameters in the liver (ALT/AST) and kidney (BUN/CR) were within the normal range (Fig. 6C-F), suggesting negligible side toxicity of CS@NPs. In addition, H&E images showed no noticeable damage or inflammatory lesion in all major organs following CS@NPs treatment (Fig. 6G).
summary, our study revealed that CS@NPs showed good biosafety, making it a promising candidate for biological applications.

**Conclusions**

In conclusion, we have effectively prepared a mitochondria-targeted nanoplatform (CS@ATO/CHC/T780 NPs) that may improve the efficacy of phototherapy. The CS@NPs exhibited high photothermal conversion efficiency and excellent singlet oxygen yield. *In vitro*, the CS@NPs exhibited increased colocalization of mitochondria and efficient cellular uptake. After internalization, CS@NPs showed excellent photocytotoxicity and resistance to proliferation in cervical cancer cells. This was attributed to decreased ATP levels, ATO-mediated inhibition of HSP70 to overcome the thermo-resistance, as well as decreased LA levels due to CHC inhibition of MCT1 to reduce oxygen consumption in cells, resulting in enhanced ROS production and efficient mitochondrial damage. Moreover, CS@NPs played a significantly anti-tumor efficacy and good biosafety *in vivo*. Consequently, our work provides an innovative mitochondrial targeting phototherapy strategy, which can improve the anticancer efficacy of phototherapy without utilizing conventional chemotherapy and has great potential for applications in anticancer treatment.

**Declarations**

**Acknowledgements**

Not applicable.

**Author contributions**

Y.G. and R.X. conceptualized the study. C.H. and H.D. supervised the study. Y.G., T.Z., and K.D. performed the experiments. Y.G., T.Z., and J.L. performed the data analysis. Y.G., B.L., Q.L., and H.T. wrote the manuscript. E.N. proofread and revised the manuscript. All authors read and approved the final manuscript.

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**Ethics approval and consent to participate**

This study was approved by the Animal Ethical Committee of Ningbo University (no. 11114).

**Availability of data and materials**
The data supporting the findings of this study are available from the corresponding author upon reasonable request.

Consent for publication

We give our consent for the manuscript to be published in Journal of Nanobiotechnology.

Competing interests

The authors declare that they have no competing interests.

References


**Scheme**

Scheme 1 is available in the Supplementary Files section.

**Figures**
Figure 1

Synthesis and Characterization of CS@NPs. (A-B) Synthetic routes to TPP-NH$_2$ (A) and T780 (B). (C-E) $^1$H-NMR spectrum of IR780 (C), TPP-NH$_2$ (D) and T780 (E) in DMSO-$d_6$. (F-G) DLS size distribution of NPs (F) and CS@NPs (G). (H-I) TEM image of NPs (H) and CS@NPs (I). Scale bar: 100 nm. (J) Zeta potential of NPs and CS@NPs. (K) UV–vis absorption spectra of NPs and CS@NPs. (L) Photothermal heating curves
of different concentrations of CS@NPs under laser irradiation (660 nm, 1 W/cm², 5 min). (M) Residual amount of DPBF in CS@NPs under laser irradiation (660 nm, 1 W/cm², 150 s). (N) Generation of \( {^1}O_2 \) for IR780, T780, NPs and CS@NPs under laser irradiation (1 W/cm², 150 s).

Figure 2

The cellular uptake of CS@NPs in vitro. (A) Fluorescence microscopy images and (B) flow cytometric results of cell uptake of CS@NPs in Hela cells at different time points. Scale bar: 100 μm. (C)
Fluorescence microscopy images and (D) flow cytometric results of cell uptake of CS@NPs in SiHa cells at different time points. Scale bar: 100 μm. (E-F) Cellular uptake of IR780, T780, NPs, CS@NPs, and CS+CS@NPs in Hela cells (E) and SiHa cells (F) after incubation for 4 h. Scale bar: 100 μm.

Figure 3
The cytotoxicity of CS@NPs *in vitro*. (A-B) Cell viability of Hela cells co-cultured with ATO, CHC, T780, NPs, and CS@NPs (λ = 660 nm, 1 W/cm², 1 min). (C-D) Cell viability of SiHa cells co-cultured with ATO, CHC, T780, NPs, and CS@NPs with laser irradiation (λ = 660 nm, 1 W/cm², 1 min). (E-F) Colony formation assay of Hela and SiHa cells co-cultured with ATO, CHC, T780, NPs, and CS@NPs with laser irradiation (λ = 660 nm, 1 W/cm², 1 min). (G-J) Quantification of Hela and SiHa cells co-cultured with ATO, CHC, T780, NPs, and CS@NPs in the EdU assay.
The mechanism of CS@NPs for anti-cervical cancer treatment. (A) Fluorescence microscopy images showing subcellular localization in Hela cells of T780, NPs, and CS@NPs. Scale bar: 100 μm. (B-C) Fluorescence microscopy images (B) and flow cytometry analysis (C) for intracellular ROS generation of Hela cells using DCFH-DA as a probe. Scale bar: 100 μm. (D) Fluorescence microscopy images of the mitochondrial membrane potential using the JC-1 dye in Hela cells (JC-1 monomer: green; JC-1 aggregate red). Scale bar: 100 μm. (E) After different treatments, the ATP content assay in Hela cells using a luminescence assay. (F) The LA content assay in Hela cells after different treatments. (G) The LDH release assay of Hela cells following different treatments. (H) Western Blot analysis of HSP70 and MCT1 in Hela cells.
Figure 5

The anti-cervical cancer effect of CS@NPs in vivo. (A) Real-time in vivo imaging of IR780, T780, NPs, and CS@NPs in cervical cancer-bearing mice after intravenous injection. The white circles indicate the location of the tumors. (C-D) Infrared thermal images of tumor-bearing mice at the tumor sites (D) during laser irradiation (1 W/cm² for 1, 2, 3, 4, and 5 min) after intravenous injections of IR780, T780, NPs, and CS@NPs. (C) Schematic illustration of Hela tumor-bearing mice model establishment and procedure of
treatment. (D) Representative images of tumors in various groups receiving normal saline, T780, NPs, and CS@NPs. Scale bar: 1 cm. (E) The volume of tumors in different groups (n = 5). (F) Average tumor weight and corresponding tumor inhibition ratios of mice treated with different formulations (n = 5). (G) ATP levels in tumors with different treatments (n = 5). (H) LA content of tumor tissue in different groups (n = 5). (I) Representative H&E staining images and IHC images of Ki67, HSP70, and MCT1 staining detected in tumors. Scale bar: 40 μm.
Figure 6

The evaluation of biosafety of CS@NPs in vivo. (A) Hemolysis ratio of CS@NPs at different sample concentrations. Each sample was tested three times in parallel. (B) The body weight of mice in different groups (n=5). (C-F) Blood biochemical analysis of ALT (C), AST (D), UREA (E) and CREA (F) in different groups (n=5). (G) H&E stained images of major organs (heart, liver, spleen, lung, and kidney) collected from different groups. Scale bar: 40 μm.

Supplementary Files

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