Mitochondrion- and nucleus-acting polymeric nanoagents for chemo-photothermal combination therapy

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ABSTRACT Developing intrinsically mitochondria-targetable nanosystems for subcellular structure-oriented precise cancer therapy is highly desirable. Here, we conjugate the cluster determinant 44 (CD44)-targetable hyaluronic acid (HA) with cholesterol-poly(ethylene glycol)_{2k}-NH₂ and mitochondria-acting IR825-NH₂ (a cyanine dye) to construct a self-assembled nanostructure (abbreviated as HA-IR825-Chol) for photothermal therapy. The HA-IR825-Chol exhibits improved photostability and desirable photothermal properties, and can rapidly and substantially enter CD44-overexpressed cancer cells and selectively accumulate in the mitochondria of the cells. Upon near-infrared laser irradiation, it can induce severe mitochondrial damage, which causes cytochrome c release and triggers cell apoptosis. Furthermore, we demonstrate the feasibility of loading the chemotherapeutics 10-hydroxycamptothecin (HCPT) into the hydrophobic cores of HA-IR825-Chol NPs for combined chemo-photothermal therapy. HCPT encapsulated within HA-IR825-Chol achieves significantly increased cellular uptake and simultaneous mitochondrial and nuclear localization, leading to the release of cytochrome c from mitochondria and upregulation of cleaved caspase-3, both of which contribute to the cell apoptosis/death. *In vivo* experiments reveal the excellent tumor-targeting ability of HA-IR825-Chol/HCPT, ensuring the efficient tumor eradication by the chemo-photothermal therapy. This work exemplifies the development of an intrinsically mitochondria-targetable nanocarrier for precise subcellular structure-localized drug delivery, and the Chol-mediated rapid and massive endocytosis of the nanoagents may represent a robust strategy for enhancing the efficacies of nanomedicines.

Keywords: combination cancer therapy, drug delivery, apoptotic pathway, cytochrome c, mitochondrial targeting

INTRODUCTION

Cancer as one of the most lethal diseases has severely threatened human health. As an essential organelle that participates in many cellular behaviors, mitochondria are indispensable for energy production in cells [1]. Notably, the number and metabolic activity of mitochondria in cancer cells have been unraveled to be higher than those in normal cells, and therefore targeting and destructing mitochondria in cancer cells represents an effective means for tumor therapy [2]. To date, a number of mitochondria-targeting nanomedicines or nanoparticles (NPs) have been developed [3–6]. Current ligands for the selective delivery of anticancer agents to the mitochondria of tumor cells mainly include lipophilic cations [7,8], peptides [9,10], aptamers [11], and intrinsically targetable nanoparticles [12]. In addition, among the different alternative strategies for anti-tumor treatments [13], photothermal therapy (PTT) plays an essential role as a non-invasive method for cancer therapy. The therapeutic efficiency of PTT usually depends on the photothermal agents (PTAs) that can convert light energy into thermal energy for cancer cell killing. Numerous PTAs have been developed, including metal-based PTAs [14–18], carbon-based PTAs [19–22], melanin-like NPs [23,24], conjugated polymers [25–27], black phosphorus [28,29], metal-organic frameworks [30], covalent organic frameworks [31], cyanine-based PTAs [32], and so on. However, some of these PTAs have shortcomings like tedious synthesis, non-degradable property, high cytotoxicity, and low photothermal conversion efficiency. Therefore, developing a suitable PTA that can address some or all of the above shortcomings is highly desirable. It has been reported that some cyanine-based molecules or composite

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materials possess a strong near-infrared (NIR) absorption character and can realize not only phototherapy but also NIR fluorescence imaging under a single wavelength excitation. Cyanine-based agents mainly include heptamethine cyanine-based agents like indocyanine green [33–42], phthalocyanine-based agents [43–46], and others [47,48]. IR825, a heptamethine cyanine dye, shows high photothermal conversion efficiency, good biodegradability, mitochondria-targeting property, and fluorescence imaging capability, which are beneficial for cancer theranostics [49,50]. More importantly, mitochondria in cells are highly sensitive to hyperthermia [51], and mitochondria-targeted PTT can effectively influence the activity of enzymes, disturb mitochondrial respiration, induce mitochondrial thermal damage, and further activate mitochondrial apoptosis pathways [52,53]. Hence, mitochondria-targeted PTT represents a promising organelle-targeted phototherapy to fight against cancer. However, many previously developed mitochondrial targeting nanoagents involve the use of the cationic triphenylphosphonium (TPP) ligand. However, the TPP modification strategy has many practical problems such as unsatisfied repeatability, potential toxicity, and tedious chemical conjugation [49]. Therefore, developing a nanosystem with intrinsic mitochondrial targeting capability is highly desired.

On the other hand, hyaluronic acid (HA) is a linear, biodegradable, and biocompatible polysaccharide material, which is promising for drug delivery application [54]. HA is rich in carboxyl groups and it can recognize HA receptors (such as cluster determinant 44, CD44) overexpressed in various types of tumor cells [55] and thus is widely used for targeting tumor tissues [56,57]. Till date, a variety of HA-based nanomaterials have been rationally designed for precise cancer therapy [58–62]. Therefore, using HA polymer to construct photothermal nanoagents holds great potential to realize tumor-selective PTT.

In this work, using the reaction between the carboxyl groups of HA and the amine-containing molecules, we conjugate HA with IR825-NH₂ and cholesterol-poly(ethylene glycol)_{2k}-NH₂ (Chol-PEG-NH₂) to fabricate a self-assembled nanocarrier (termed HA-IR825-Chol) that specifically targets tumor cells and acts on mitochondria (Scheme 1). It should be pointed out that, Chol, a robust lipophilic anchor [63,64], has been introduced to various nanosystems to interact with plasma membranes for enhanced cellular uptake [65–67]. Compared with IR825-NH₂, the as-designed HA-IR825-Chol NPs exhibited improved water-dispersity, photostability, and photothermal properties, and showed satisfying cancer cell

targeting ability *via* the recognition between HA and the CD44 overexpressed in many tumor cells. Upon NIR laser irradiation, the IR825-mediated PTT could damage the mitochondrial structure, resulting in cytochrome c release from mitochondria and tumor cell death. Furthermore, we demonstrated the feasibility of using HA-IR825-Chol NPs to carry a chemotherapeutic drug 10-hydroxycamptothecin (HCPT). It was found that the as-formulated HA-IR825-Chol/HCPT NPs could realize IR825-mediated mitochondrial PTT and HCPT-mediated nucleus- and mitochondrion-oriented chemotherapy.

EXPERIMENTAL SECTION

Synthesis of HA-IR825, HA-IR825-mPEG and HA-IR825-Chol

IR825-NH₂ was synthesized according to a previous report [68]. To prepare HA-IR825, 20 mg HA dissolved in 30 mL of deionized water was added with 94 mg *N*-hydroxy-sulfosuccinimide sodium salt (sulfo-NHS) and 32 mg *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC-HCl) to activate the carboxyl groups of HA. After 2 h, 5 mg IR825-NH₂ dissolved in a small amount of dimethyl sulfoxide (DMSO) was added into the above activated HA solution, and the obtained mixture was stirred at room temperature in the dark for 24 h. After that, the mixture was dialyzed sequentially against “DMSO + deionized water” and deionized water by using a dialysis membrane (molecular weight cut-off (MWCO) = 3500) overnight.

Similarly, after synthesis of HA-IR825, 94 mg sulfo-NHS and 32 mg EDC-HCl were added in the above HA-IR825 suspension for 1 h to activate the unreacted carboxyl groups of HA-IR825. Then, 12.5 mg Chol-PEG-NH₂ dissolved in a small amount of DMSO was added into the above suspension and the mixture was stirred at room temperature in the dark for 24 h. Finally, the mixture was dialyzed sequentially against “DMSO + deionized water” and deionized water using a dialysis membrane (MWCO = 5000) overnight to obtain HA-IR825-Chol. Additionally, HA-IR825-mPEG was prepared with the same procedure except that the 12.5 mg Chol-PEG-NH₂ was replaced by 10.9 mg mPEG-NH₂.

Synthesis of HA-IR825-Chol/HCPT

After the synthesis of HA-IR825-Chol, 10 mg HCPT dissolved in a small amount of DMSO was added into the HA-IR825-Chol suspension. The mixed suspension was subjected to tip sonication for 5 min (25% of the maximal output, 3 s-pulse on, 3 s-pulse off), and then stirred at

Scheme 1 Schematic illustrating the preparation of HA-IR825-Chol/HCPT NPs and their effects on cancer cells.

room temperature in the dark for 24 h. The resultant mixture was centrifuged at 5000 rpm for 5 min to remove the precipitates formed by unencapsulated HCPT molecules and dialyzed against deionized water using a dialysis membrane (MWCO = 5000) for 24 h to obtain HA-IR825-Chol/HCPT NPs.

RESULTS AND DISCUSSION

Preparation and characterization of HA-IR825-Chol NPs

The HA-IR825 NPs were prepared by conjugating HA with IR825-NH₂. Next, HA-IR825-Chol NPs were formed by reaction between HA-IR825 and Chol-PEG-NH₂. The successful synthesis of HA-IR825-Chol NPs was confirmed by Fourier-transform infrared (FTIR) spectroscopy (Fig. S1). The two peaks at 1616 and 842 cm⁻¹ in the FTIR spectrum of HA-IR825-Chol come from the remaining carboxyl groups of the HA moieties and the PEG segment, indicating the successful conjugation be-

tween Chol-PEG-NH₂ and HA. We also determined the molar ratio of HA:IR825 (from IR825-NH₂):Chol (from Chol-PEG-NH₂) is 1:36:23 by using weighing and ultra-violet–visible (UV–vis) spectroscopy.

Next, transmission electron microscopy (TEM) was utilized to characterize the size and morphology of HA-IR825-Chol dispersed in water. The TEM image in Fig. 1a demonstrated the formation of uniform nanospheres of HA-IR825-Chol with an average size of ~43.5 nm. The hydrodynamic size of HA-IR825-Chol NPs in a phosphate-buffered saline (PBS) solution was 64.9 ± 2.9 nm, as measured by dynamic light scattering (DLS), which was slightly larger than that (62.5 ± 3.2 nm) of HA-IR825 (Fig. 1b), suggesting the successful conjugation of Chol-PEG-NH₂ with HA-IR825 to form HA-IR825-Chol. The zeta potential values of HA-IR825 and HA-IR825-Chol were measured to be -34.7 ± 1.4 and -23.9 ± 1.9 mV, respectively (Fig. 1c). The altered zeta potential of HA-IR825-Chol compared with that of HA-IR825 indicated

Figure 1 (a) TEM image and corresponding size distribution histogram (inset) of HA-IR825-Chol NPs. Scale bar = 200 nm. (b) DLS results and (c) zeta potentials of HA-IR825-Chol and HA-IR825 NPs in PBS solutions. (d) UV-vis absorption spectra of IR825-NH₂ (in PBS and DMSO), HA-IR825 (in PBS), and HA-IR825-Chol (in PBS) at the same IR825 concentration (5 μg mL⁻¹). (e) Photothermal heating curves of IR825-NH₂, HA-IR825, and HA-IR825-Chol at the same IR825 concentration (5 μg mL⁻¹) under the continuous 808 nm laser irradiation (1.0 W cm⁻²) for 4 min. PBS was set as the control group. (f) Plots of relative absorbance values *versus* time for evaluating the photostability of IR825-NH₂, HA-IR825, and HA-IR825-Chol at the same IR825 concentration (5 μg mL⁻¹).

the consumption of the carboxyl groups by Chol-PEG-NH₂. Further, the UV-vis absorption spectra of IR825-NH₂ (dissolved in DMSO and PBS), HA-IR825 (dispersed in PBS), and HA-IR825-Chol (dispersed in PBS) were measured. It was observed that the absorption peak of IR825-NH₂ in DMSO was much sharper than that in the PBS solution (Fig. 1d), indicating the better dispersity of the dye in DMSO. Similarly, the sharper absorption band of HA-IR825-Chol than that of HA-IR825 suggested the better dispersity of HA-IR825-Chol, as proved by its smaller polydispersity index (PDI) (Fig. 1b).

To investigate whether HA-IR825-Chol NPs possess desirable photothermal properties, we recorded the temperatures of PBS solution, IR825-NH₂ solution, HA-IR825 suspension, and HA-IR825-Chol suspension under continuous laser irradiation at 808 nm for 4 min (Fig. 1e). The results revealed that the temperature elevations of the

three samples were ranked as follows: HA-IR825-Chol > HA-IR825 > IR825-NH₂, suggesting the improved photothermal conversion ability of HA-IR825-Chol compared with the other two samples. Furthermore, the photostabilities of IR825-NH₂, HA-IR825, and HA-IR825-Chol were also investigated *via* UV-vis spectroscopy (Figs S2-S4), and the calculated relative absorbance values were presented in Fig. 1f. The photodegradation process of IR825 moieties in HA-IR825 or HA-IR825-Chol NPs was remarkably slowed down. The results showed that HA-IR825 and HA-IR825-Chol NPs own better photostability than IR825-NH₂, indicating the protective role of HA and Chol-PEG moieties. In addition, the photothermal conversion efficiencies of IR825-NH₂ and HA-IR825-Chol were calculated to be 16.0% and 24.3%, respectively, according to a previously reported method [20,49,50], revealing the improved photothermal property of HA-IR825-Chol compared with that of IR825-NH₂.

Cellular uptake behavior and cytotoxicity of HA-IR825-Chol NPs

Using murine mammary cancer cells (4T1 cells) as the model cells, we then investigated the cellular uptake behaviors of IR825-NH₂, HA-IR825, and HA-IR825-Chol by using confocal microscopy and flow cytometry. Meanwhile, to prove the effect of Chol on the cellular internalization of HA-IR825-Chol, we also synthesized HA-IR825-poly(ethylene glycol)_{2k}-methoxy (termed HA-IR825-mPEG) by conjugating HA-IR825 with methoxy-poly(ethylene glycol)_{2k}-NH₂ (mPEG-NH₂) and evaluated its cellular uptake behavior. As shown in Fig. 2a and c, HA-IR825-Chol exhibited the largest cellular uptake efficiency among all the groups, showing the crucial role of Chol in the cellular internalization of HA-IR825-Chol. Besides, we investigated the intracellular location of HA-IR825-Chol by co-staining the 4T1 cells with HA-IR825-Chol and one of the following commercially available organelle-selective trackers: rhodamine 123 (Rhod 123, a commercial mitochondrial dye), ER-Tracker Green (ER-Tracker, for staining endoplasmic reticulum, ER), Golgi-Tracker Green (Golgi-Tracker, for staining Golgi apparatus), and LysoTracker Green (LysoTracker, for staining lysosomes). Fig. 2b revealed that the green fluorescence of Rhod 123 well overlapped with the red fluorescence of the IR825 in HA-IR825-Chol, and the corresponding Pearson's correlation coefficient (PCC) and overlap coefficient (OLC) were 0.98 and 0.98, respectively. In contrast, HA-IR825-Chol did not stain ER well, and the corresponding PCC and OLC were 0.74 and 0.74, respectively. Further, a

Figure 2 (a) Confocal fluorescence images of 4T1 cells after incubation with IR825-NH₂, HA-IR825, HA-IR825-mPEG, or HA-IR825-Chol (2 μg mL⁻¹ IR825 in each sample) for different time periods. Scale bar = 20 μm. (b) Confocal fluorescence images of 4T1 cells incubated with HA-IR825-Chol (2 μg mL⁻¹ of IR825) for 2 h and then stained by Rhod 123 (100 nmol L⁻¹) for 10 min, ER-Tracker (1 μmol L⁻¹) for 30 min, Golgi-Tracker (5 μmol L⁻¹) for 30 min, or LysoTracker (1 μmol L⁻¹) for 30 min. Scale bar = 20 μm. (c) Fluorescence (FL) intensities of 4T1 cells after incubation with IR825-NH₂, HA-IR825, HA-IR825-mPEG, or HA-IR825-Chol (2 μg mL⁻¹ IR825 in each sample) for different time periods as measured by flow cytometry. (d) Effects of various endocytosis inhibitors and low temperature (4°C) on the endocytosis of HA-IR825-Chol by 4T1 cells evaluated *via* flow cytometry by using the allophycocyanin (abbreviated as APC in the figure) channel. (e) Relative viabilities of 4T1 cells after various treatments. **P* < 0.05, ***P* < 0.01.

poor overlap was seen in the cases of Golgi apparatus and lysosome, with a PCC of 0.22 and 0.22, respectively. These results suggested that HA-IR825-Chol has an excellent mitochondrial targeting capability, which may possibly be attributed to the excellent mitochondrial targeting property of IR825.

Furthermore, we investigated the cell endocytic pathways of HA-IR825-Chol by using 4T1 cells. Chlorpromazine (CPZ) hydrochloride, genistein, 5-(*N,N*-dimethyl)-amiloride (amiloride), methyl-β-cyclodextrin (MβCD), and 4°C treatment were used to inhibit clathrin-mediated, caveolae-mediated, macropinocytosis-dependent, lipid raft-mediated, and energy-dependent pathway, respectively [69]. The flow cytometric results in Fig. 2d and Fig. S5 showed that genistein, amiloride, and 4°C treatment evidently decreased the internalization of HA-IR825-Chol, indicating that the HA-IR825-Chol NPs

were internalized mainly through caveolae-mediated, macropinocytosis- and energy-dependent endocytosis.

Meanwhile, the dark toxicity and phototoxicity of HA-IR825-Chol were tested by using 4T1 cells. The results in Fig. 2e indicated that free IR825-NH₂ did not elicit evident dark toxicity to the cells at the tested concentrations, while HA-IR825-Chol NPs exhibited significantly higher phototoxicity than IR825-NH₂ due to their remarkable endocytosis by cancer cells.

Characterization and cellular experiments of HA-IR825-Chol/HCP NPs

We next explored the feasibility of using the HA-IR825-Chol NPs as the nanocarriers of chemotherapeutics. HCP, a DNA topoisomerase I inhibitor [70,71], has extensively entered clinical trials; however, its extremely poor water-solubility severely hampers the efficient nu-

clear delivery of the drug. Because HCPT owns an excellent ability to act on DNA in the nucleus and mitochondria [72], we anticipate that the HCPT-loaded HA-IR825-Chol NPs (termed HA-IR825-Chol/HCPT NPs) can realize combined chemo-photothermal therapy for improving the final therapeutic outcomes.

The as-prepared HA-IR825-Chol/HCPT NPs displayed an average size of 65.5 ± 3.5 nm and their zeta potential was measured to be -18.6 ± 1.0 mV (Fig. 3a and Fig. S6). The encapsulation ratio of HCPT and drug (*i.e.*, HCPT) loading coefficient of HA-IR825-Chol/HCPT NPs were 8.1% and 0.7%, respectively. In addition, we measured the UV-vis absorption spectra of HCPT and HA-IR825-Chol/HCPT in PBS (10 mmol L^{-1} , pH 7.4) containing 0.1% Triton X-100 solution (to dissolve HCPT) and HA-IR825-Chol in PBS (Fig. 3b). The UV-vis results showed that HA-IR825-Chol/HCPT (obtained after centrifugation and dialysis) dispersed in PBS containing 0.1% Triton X-100 had a new absorption peak at 377 nm (a characteristic peak of HCPT) relative to HA-IR825-Chol, indicating the presence of HCPT in the nanocomplexes. Further, the release kinetics of HCPT from HA-IR825-Chol/HCPT NPs at pH 6.5 (tumor microenvironment condition) and pH 7.4 (normal tissue condition) were evaluated (Fig. S7). The results indicated that the nanoagents have a similar drug release behavior in slightly acidic and neutral solutions.

Next, the subcellular fate of HA-IR825-Chol/HCPT NPs after cellular internalization was studied by confocal imaging. In Fig. 3c, the HCPT loaded in HA-IR825-Chol NPs showed enhanced cellular uptake compared with free HCPT in 4T1 cells. Besides, it could be seen that the HCPT in HA-IR825-Chol/HCPT NPs gradually entered the nuclei as the incubation time increased. We also found that the fluorescence signals of HCPT partially matched those of Rhod 123 (Fig. 3d), suggesting the mitochondrial accumulation of HCPT. The two main pathways of chemotherapy-induced tumor cell apoptosis include the mitochondrial pathway and the death receptor pathway [73]. The mitochondrial membrane potential of cells reflects the degree of mitochondrial apoptosis. Hence, to investigate the effect of HA-IR825-Chol/HCPT on the mitochondrial pathway, the mitochondrial membrane potentials of the cells with different treatments (*i.e.*, control (untreated), HCPT, and HA-IR825-Chol/HCPT) were compared *via* measuring the fluorescence intensities of Rhod 123 for 4T1 cells. The results in Fig. 3e showed that HA-IR825-Chol/HCPT could more significantly affect the mitochondrial membrane potential of the cancer cells compared with free

HCPT and the control group, and free HCPT did not cause an evident alteration of the mitochondrial membrane potential of the cells relative to the control group. These results indicated that HA-IR825-Chol/HCPT achieved an enhanced apoptosis-inducing effect on the mitochondrial pathway of the cancer cells.

In addition, to further explore the impact of HA-IR825-Chol/HCPT NPs on mitochondria, we carried out immunofluorescence staining to visualize cytochrome c in the 4T1 cells. Cytochrome c, as an indicator of apoptosis, is located at the mitochondrial inner membrane and cannot penetrate through the outer membrane under normal conditions, while it is released into the cytoplasm during apoptosis as a consequence of the altered mitochondrial membrane permeability [72,74–76]. Rabbit anti-cytochrome c antibody (combined with fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit IgG antibody) and Hoechst 33342 were used to stain cytochrome c and cell nuclei, respectively. As shown in Fig. 3f and Fig. S8, the “HA-IR825-Chol/HCPT + laser” group showed the strongest green fluorescence signals in the cytosol compared with other groups, suggesting that cytochrome c was released into the cytosol from the mitochondrial membrane. Meanwhile, the “HA-IR825-Chol/HCPT” and “HA-IR825-Chol + laser” groups also showed stronger green fluorescence signals in the cytosol than other groups (except the “HA-IR825-Chol/HCPT + laser” group), indicating that after being encapsulated by HA-IR825-Chol, HCPT could destroy the structure of mitochondria and the mitochondria-targeted HA-IR825-Chol could damage the mitochondria with the help of laser irradiation. The above results might indicate that, compared with other groups, HA-IR825-Chol/HCPT plus laser irradiation achieved the highest anticancer efficacy, possibly due to their strongest capability to induce cell apoptosis, as evidenced by the most evident cytochrome c release from mitochondria.

Further, to elucidate the death receptor pathway mechanism of cancer cells induced by various treatments, we investigated the cleaved caspase-3 expression in 4T1 cells *via* immunofluorescence staining. Caspase-3 is a frequently activated death protease, catalyzing the specific cleavage of many key cellular proteins. Caspase-3 is seen as a crucial mediator of programmed cell death and a typical hallmark of apoptosis [74]. More importantly, the pathway of caspase-3 activation was found to be dependent on mitochondrial cytochrome c release [77], and HCPT acting on nuclear DNA was demonstrated to trigger the upregulation of cleaved caspase-3 [78]. The results in Fig. 3g and Fig. S9 revealed that the fluores-

Figure 3 (a) Hydrodynamic diameter of HA-IR825-Chol/HCPPT NPs in a PBS solution (pH 7.4). (b) UV-vis absorption spectra of HCPPT (in PBS with 0.1% Triton X-100), HA-IR825-Chol (in PBS), and HA-IR825-Chol/HCPPT (in PBS with 0.1% Triton X-100). (c) Confocal fluorescence images of 4T1 cells after incubation with HCPPT or HA-IR825-Chol/HCPPT NPs at the same HCPPT concentration of $1.6 \mu\text{g mL}^{-1}$ for different time periods. Before imaging, the HA-IR825-Chol/HCPPT-treated cells were stained by Rhod 123 to visualize mitochondria. The dotted circles in purple indicate the positions of nuclei. Scale bar = $25 \mu\text{m}$. (d) Confocal fluorescence image of a representative 4T1 cell with the same treatment as that in (c). The red color (pseudo-color) stands for the fluorescence of HCPPT and the green color stands for the fluorescence of Rhod 123. The yellow color, the merged color of the red and green colors, indicates the colocalization of HCPPT and mitochondrion. Scale bar = $10 \mu\text{m}$. (e) FL intensities of Rhod 123 (as measured by flow cytometry) in 4T1 cells after different treatments as indicated. Rhod 123 was added to the treated cells to reflect the mitochondrial membrane potentials of the cells. (f, g) Immunofluorescence staining results of 4T1 cells with different treatments as indicated. IR825- NH_2 , HA-IR825-Chol, HA-IR825-Chol/HCPPT: $2 \mu\text{g mL}^{-1}$ of IR825. HCPPT: $1.6 \mu\text{g mL}^{-1}$. Laser irradiation: 808 nm , 1.0 W cm^{-2} , 5 min . For (f), before imaging, the treated cells were stained by “rabbit anti-cytochrome c antibody + FITC-labeled goat anti-rabbit IgG antibody” and Hoechst 33342 (abbreviated as Hoechst in the figure) to visualize cytochrome c and cell nuclei, respectively. Scale bar = $25 \mu\text{m}$. For (g), before imaging, the treated cells were stained by “cleaved caspase-3 rabbit monoclonal antibody + FITC-labeled goat anti-rabbit IgG antibody” and Hoechst 33342 (abbreviated as Hoechst in the figure) to visualize cleaved caspase-3 and cell nuclei, respectively. Scale bar = $25 \mu\text{m}$. (h) Relative viabilities of AT II, A549, MCF-10A, and MDA-MB-231 cells after treatment with different concentrations (based on IR825 or HCPPT) of HA-IR825-Chol/HCPPT NPs for 24 h. (i) Statistical results (obtained *via* flow cytometry) of the early apoptotic and late apoptotic/necrosis cells for different cell lines. Before flow cytometry analysis, the cells were incubated with HA-IR825-Chol/HCPPT ($0.5 \mu\text{g mL}^{-1}$ of IR825) for 24 h. (j) Relative viabilities of 4T1 cells after various treatments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ns: nonsignificant difference.

cence signals from the immunofluorescence-labeled cleaved caspase-3 in the HA-IR825-Chol/HCPPT group

were stronger than those in the HA-IR825-Chol and HCPPT groups, implying that HA-IR825-Chol/HCPPT

could exert a stronger apoptosis-inducing effect. The “HA-IR825-Chol/HCP + laser” led to the highest cleaved caspase-3 level compared with other groups, indicating that the combined chemo-photothermal therapy could result in a higher level of cell apoptosis.

We then evaluated the toxicity of HA-IR825-Chol/HCP toward different cell types *via* 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. The results in Fig. 3h revealed that the viabilities of human normal lung (AT II) cells were evidently higher than those of human lung cancer (A549) cells after treatment with the respective concentrations of HA-IR825-Chol/HCP. The higher cytotoxicity of the NPs to the A549 cells might be due to the higher uptake amount of the NPs in the A549 cells than that in the AT II cells (Fig. S10). Similarly, the NPs also elicited higher toxicities to the human breast cancer (MDA-MB-231) cells than the human normal breast (MCF-10A) cells. The selective cancer cell killing activity of the NPs indicated that the HA-containing NPs could preferentially target the CD44-overexpressed cancer cells *via* HA-CD44 recognition. To prove this, 4T1 cells were pretreated with the HA polymers ($50 \mu\text{g mL}^{-1}$, 2 h) to block the HA receptors, followed by HA-IR825-Chol incubation. The results showed that the cellular uptake of the NPs was significantly weakened (Fig. S11), which further validated the HA-endowed cancer cell targeting property of HA-IR825-Chol.

In addition, we further performed flow cytometric analysis to confirm the above MTT results *via* the annexin V-FITC/propidium iodide (PI) dual staining assay. In Fig. 3i, the rate of apoptotic cells (early and late apoptotic cells) and necrotic cells in the AT II group was lower than that in the A549 group. Similarly, the rate of apoptotic and necrotic cells in the MCF-10A group was lower than that in the 4T1 or MDA-MB-231 group. These results confirmed that the HA-CD44 recognition played an important role in inducing a higher toxicity of the NPs to the cancer cells. Then, the *in vitro* anticancer efficacy of HA-IR825-Chol/HCP was studied by the MTT assay (Fig. 3j). Compared with free HCP, HA-IR825-Chol/HCP showed significantly higher dark cytotoxicity at respective HCP concentrations, indicating that the significantly enhanced HCP internalization of HA-IR825-Chol/HCP relative to the internalization of free HCP (as revealed in Fig. 3c) was responsible for the higher cytotoxicity of the NPs. In addition, “HA-IR825-Chol/HCP + laser” group displayed higher toxicity than the other groups, demonstrating that the combined chemo-photothermal therapy could achieve the best cancer

therapeutic outcome.

***In vivo* fluorescence imaging and combination cancer therapy**

The *in vivo* biodistribution of the HA-IR825-Chol/HCP NPs in 4T1 tumor-bearing nude mice was monitored using an *in vivo* fluorescence imaging system. As displayed in Fig. 4a and b, the HA-IR825-Chol/HCP NPs significantly accumulated in the tumor region at 1 h after intravenous injection of the NPs and achieved stable tumor retention for at least 24 h. In contrast, negligible fluorescence signals could be observed at the tumor site in the free IR825-NH₂ group during the observation period. Further, the *ex vivo* fluorescence imaging of the major organs and tumor tissues of the mice sacrificed at 1, 3, and 7 d after the intravenous injection of free IR825-NH₂ or HA-IR825-Chol/HCP was performed. The results in Fig. 4c and d revealed that, for free IR825-NH₂ molecules, they were mainly in the liver, spleen, lung, and kidneys at 1 d postinjection, and at 3 d postinjection, the liver deposition of the molecules became even higher, accompanied by the decreased accumulation in the lung and kidneys; for the HA-IR825-Chol/HCP NPs, they were distributed in all the major organs (except the heart) and tumor tissue at 1 d postinjection, and at 3 d postinjection, evident accumulation in the liver and tumor could still be observed. In addition, it was found that for the HA-IR825-Chol/HCP NPs, they could be cleared out of the mouse body after 7 days, with negligible fluorescence signals in the major organs and tumor; while for the free IR825-NH₂ molecules, evident fluorescence signals could still be seen in the liver and spleen after 7 days. The efficient body clearance of the HA-IR825-Chol/HCP NPs might ensure the *in vivo* biosafety of the nanoagents.

To carry out *in vivo* PTT treatments, we first monitored the temperature changes of the tumors of the mice after intravenous injection of PBS, IR825-NH₂, and HA-IR825-Chol/HCP under laser irradiation (808 nm, 1.0 W cm^{-2}). The results in Fig. 4e and f showed that after irradiation for 10 min, the tumor temperatures of the mice in the PBS, IR825-NH₂, and HA-IR825-Chol/HCP groups reached 34.0, 38.4, and 46.2°C.

Meanwhile, to evaluate the antitumor efficiencies of various treatments in 4T1 tumor-bearing BALB/c nude mice, we carried out hematoxylin and eosin (H&E) staining experiments, and the results shown in Fig. 4g demonstrated the severest damage to the tumor tissue after the “HA-IR825-Chol/HCP + laser” treatment, which was in good accordance with its strongest tumor ablation activity as shown in Fig. 4h.

Figure 4 (a) Time-dependent *in vivo* fluorescence images of 4T1 tumor-bearing nude mice after intravenous injection of IR825-NH₂ or HA-IR825-Chol/Hcpt (7 mg kg⁻¹ of IR825). Green dotted circles indicate the tumor sites of mice. “Pre” indicates a time point before the injection of these drugs. (b) FL intensities of the tumors partially shown in (a). (c) *Ex vivo* fluorescence images and (d) corresponding FL intensities of major organs and tumors excised from mice at 1, 3, and 7 (only for (c)) d after intravenous injection of IR825-NH₂ or HA-IR825-Chol/Hcpt (7 mg kg⁻¹ of IR825). (e) *In vivo* thermal imaging results of mice after intravenous injection of PBS, IR825-NH₂ (7 mg kg⁻¹), and HA-IR825-Chol/Hcpt (7 mg kg⁻¹ of IR825). Before imaging, the tumor sites of the mice were irradiated by a laser (808 nm, 1.0 W cm⁻²) for different time periods. (f) Temperatures of the tumor sites partially shown in (e). (g) Representative optical images of H&E-stained tumor slices from tumor-bearing mice after various treatments. (h) Tumor growth profiles of mice after various treatments. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

Biosafety of HA-IR825-Chol/Hcpt NPs

Encouraged by the excellent antitumor efficiency of HA-IR825-Chol/Hcpt NPs, we then carefully evaluated the biosafety of the NPs in nude mice. First, no evident loss of weight was observed in all the groups with various treatments (Fig. S12). Second, the hematology and blood biochemical analysis were performed for the treated mice at 14 d, and negligible differences were observed in all the blood indexes of the control and “HA-IR825-Chol/Hcpt + laser” groups (Fig. 5a–j). Meanwhile, the hemolysis results revealed that IR825-NH₂, HA-IR825-Chol, and HA-IR825-Chol/Hcpt at the highest tested concentrations of 10 μg mL⁻¹ (based on IR825) presented ignorable hemolytic activity (Fig. 5k and Fig. S13). In addition, the H&E staining results of the major organs of the mice in

the control and “HA-IR825-Chol/Hcpt + laser” groups proved no obvious systemic toxicity of the combined treatment (Fig. 5l). These results collectively indicated the good safety of the HA-IR825-Chol/Hcpt NPs.

CONCLUSIONS

In this research, we designed a smart HA-IR825-Chol nanocarrier with CD44 recognition and mitochondria-targetable properties, which could efficiently encapsulate the chemotherapeutic drug HCPT for realizing cooperative photo-chemotherapy. It was found that, in HA-IR825-Chol, the water-solubility of the modified IR825 was significantly improved, and the endocytosis rate and internalization amount of IR825 in cancer cells were considerably increased. In addition, the as-designed HA-

Figure 5 (a–j) Hematology and blood biochemical analysis results collected from the healthy mice (control) or the 4T1 tumor-bearing nude mice after “HA-IR825-Chol/HCPT + laser” treatment. The tested indexes include number of red blood cells (RBC), number of white blood cells (WBC), concentration of hemoglobin (HGB), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and five hepatic and renal function parameters like contents of total protein (TP), glutamate pyruvate transaminase (ALT), alkaline phosphatase (ALP), creatinine (CRE), and blood urea nitrogen (BUN). (k) Photographs of RBC suspensions after treatment with various concentrations (based on IR825) of IR825-NH₂, HA-IR825-Chol, or HA-IR825-Chol/HCPT. 1% Triton X-100 solution and PBS solution were set as positive control and negative control, respectively. (l) Representative optical images of H&E-stained slices from the major organs of a healthy mouse (control) and a tumor-bearing mouse treated with “HA-IR825-Chol/HCPT + laser”.

IR825-Chol NPs owned better photothermal properties compared with IR825-NH₂. Besides, by being encapsulated in the HA-IR825-Chol nanocarrier, HCPT showed significantly higher cellular uptake, and part of the HCPT molecules were located in the mitochondria and part of them entered cell nuclei. Moreover, we have demonstrated that both the HA-IR825-Chol-based mitochondrial PTT treatment and the mitochondria-oriented HCPT could destroy mitochondria and trigger cytochrome c release, and the HCPT in the nuclei could promote cleaved caspase-3 upregulation, both of which contributed to the cell death. The *in vitro* and *in vivo*

experiments both proved the synergistic performance of the chemo-photothermal combination therapy. This work exemplifies the development of an intrinsically mitochondria-targetable nanocarrier for precise subcellular structure-localized drug delivery and may deepen the understanding of the relationship between apoptosis and cancer therapy.

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Author contributions Liu X, Zhu Y, Xu K, and Yu X performed the experiments. Liu X, Jia H, Gao G, Jiang Y, and Wu F analyzed the data. Liu X, Jia H, Zhu Y, Cheng X, and Wu F wrote the manuscript. Wu F supervised the project.

Conflict of interest The authors declare that they have no conflict of interest.

Supplementary information Experimental details and supporting data are available in the online version of the paper.

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作用于线粒体和细胞核的聚合物纳米材料及其在化疗/光热治疗联合抗癌中的应用

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摘要 开发本身即具有线粒体靶向能力的亚细胞精准纳米诊疗试剂对于改善癌症治疗效果具有重要意义. 本文使用可靶向癌细胞表面过度表达的CD44抗原的透明质酸-胆固醇-聚乙二醇-氨基和可作用于线粒体的花菁类染料IR825-NH₂, 构建了一种可实现光热治疗的自组装纳米材料(HA-IR825-Chol). 相较于游离的IR825-NH₂, 该结构具有更好的光稳定性、更高的光热转换效率和对癌细胞的识别能力. HA-IR825-Chol可以有效靶向细胞线粒体, 并可以在近红外激光照射下诱导线粒体损伤. 此外, 我们通过疏水作用包裹了化疗试剂10-羟基喜树碱(HCPT)(所形成的药物命名为HA-IR825-Chol/HCPT). 相关实验结果显示, 包裹于纳米材料后HCPT被细胞摄取效率显著提高, 并能够同时分布于线粒体和细胞核中, 从而诱导线粒体中细胞色素c的释放和细胞中cleaved caspase-3的上调, 最终促进细胞凋亡与死亡. 另外, HA-IR825-Chol/HCPT优异的体内肿瘤靶向能力为光化疗联合治疗消除肿瘤提供了必要保证. 该工作实现了定位于线粒体的精准亚细胞药物递送, 并发展了利用胆固醇提高药物摄取速率和效率的策略, 预期将为提高纳米药物抗癌效果提供借鉴.