NIR-responsive cancer cytomembrane-cloaked carrier-free nanosystems for highly efficient and self-targeted tumor drug delivery

Ning Zhang a, Minghui Li a, Xuetan Sun a, Huizhen Jia a,b,**, Wenguang Liu a, *

a School of Materials Science and Engineering, Tianjin Key Laboratory of Composite and Functional Materials, Tianjin University, Tianjin, 300350, China
b State Key Laboratory of Molecular Engineering of Polymers, Fudan University, Shanghai, 200433, PR China

A R T I C L E   I N F O

Article history:
Received 30 September 2017
Received in revised form 8 December 2017
Accepted 3 January 2018
Available online 4 January 2018

Keywords:
Cancer cell membrane
Carrier-free
NIR-responsive
Self-reorganization
Chemo-/photothermal therapy

A B S T R A C T

Cell membrane-camouflaged nanoparticles for cancer therapy have received a burgeoning interest over the past years. However, the low drug loading and intratumoral release efficiency, and lack of precise targeting remains a big challenge; in addition, foreign carriers used may pose an expected burden in the course of metabolism. In this study, we designed and fabricated a novel NIR-responsive highly targeted carrier-free nanosystem by coating the exactly identical source of cracked cancer cell membranes (CCCMs) specifically derived from the homologous tumors onto the surface of the co-assembly nanoparticles of doxorubicin (DOX) and FDA-approved photothermal agent, indocyanine green (ICG). The nanosystems exhibited a high drug loading capacity (89.8%), cancer cell self-recognized ability and immune escape function. Further, the nanodrugs could be efficiently released for the membrane disturbance triggered by photothermal effect of ICG under NIR irradiation. The tumor-bearing mice model demonstrated that the self-carried DOX NPs@ICG@CCCM nanoparticles possessed a strong synergistic chemo-/photothermal therapeutic efficacy against tumors in vivo. The present strategy could be developed as a universal approach for designing and constructing carrier-free theranostic nanovehicles by intentionally selecting specific cancer cell membrane and the inner loading cargoes.

© 2018 Elsevier Ltd. All rights reserved.

1. Introduction

Cancer is the leading cause of death worldwide, and conquering malignant tumors is a major challenge mankind is facing [1]. The booming nanotechnology has brought about innovative ideas and approaches to fight against deadly tumors [2–4]. An expectation is that nanotechnology can eventually revolutionize the preparation of carriers which is critical for loading therapeutic and cargoes for treating cancers. However, there are only a few clinically successful application of nanodrugs to date [5]. The critical hurdles to the clinical translation are the poor targetability, very low delivery efficiency of the therapeutic agents as well as the foreign carriers used, most of which lack biofunctions and may pose unexpected risk in the course of metabolism and excretion [6–11]. Recently, a novel class of biologically derived membrane-camouflaged drug delivery systems have received considerable attention [12–18]. It has been increasingly recognized that the cancer cell membrane coated nanoparticles exhibited a unique ability to evade immune system surveillance and self-target the homotypic tumor in vivo [19–21]. This biospired strategy is promising to precisely treat diverse sources of tumors. However, most of drugs are encapsulated in an additional carrier with a low drug loading capacity (<10%, typically). And these inert carriers would lead to the ineffective tumor accumulation and raise the above mentioned concerns.

Recently, self-assembly technology of small molecules offers a new and simple strategy to fabricate carrier-free drug delivery systems. Normally, hydrophobic drug such as paclitaxel or docetaxel or doxorubicin assembled into small nanocrystals which were then modified with hydrophilic polymers or target ligands to increase blood circulation time and tumor homing ability [22–25]. So far, a series of self-carried nanodrugs with a high drug loading (>78 wt%) and multifunctions of therapy and real-time self-monitoring of release and distribution of drug have been reported [26–28]. However, the release rate of these nanodrug systems was too fast before delivery into tumors, severely impairing antitumor
eficacy and bringing about the unwanted side effects. In addition, the target specificity to tumor cells of these carrier-free nano-systems needs to be further improved since their tumor-targeting is achieved by passive targeting which strongly depends on the varying degrees of tumor vascularization and permeability associated with tumor types and development stages or the receptor density expressed in the target sites [19,29–31].

Recognizing these problems, we designed and constructed a novel NIR responsive carrier-free nanosystem coated with cracked cancer cells membranes (CCCMs) with an aim to achieve highly tumor-targeting and intracellularly rapid drug release for significantly enhancing therapeutic efficacy against the tumors. As shown in Fig. 1, hydrophobic chemotherapeutic doxorubicin (DOX) and amphipathic indocyanine green (ICG), an FDA-approved clinically used photothermal agent, were co-assembled in distilled and deionized water into nanoparticles (termed as DINPs) [15,32,33]. Then the cracked cancer cell membranes (CCCMs) derived from HeLa cells were coated onto the surface of the DINPs by simple extrusion method (Fig. 1A). The DOX NPs@ICG@CCCM nanoparticles formed were defined as DICNPs. It is anticipated that the DICNPs would have a high drug loading efficiency with the cell membrane protecting DOX and ICG from leaking during blood circulation. Further, the cancer-derived membrane-coated nano-drugs can achieve highly specific self-recognition to homotypic cancer cells. Once transported to tumor regions, the cell membrane could be thermally-disrupted under the NIR laser irradiation and the loaded cargoes could be rapidly released, thus enhancing the drug concentration in tumors, and eventually boosting the chemo-/photothermal therapy efficacy (Fig. 1B). In light of the simplicity of this assembly, and multi-choice of drugs and intentionally selected cancer cell membranes, the bioinspired strategy could offer a very simple and universal way for the design of clinically translatable nanosystems to achieve highly efficient and targeted therapy and/or diagnose of various diseases.

2. Materials and methods

2.1. Materials

Doxorubicin hydrochloride (DOX HCl, >98%) and Indocyanine Green (ICG, >94%) were purchased from Meilun Bio, China. Dimethyl sulfoxide (DMSO), trimethylamine (TEA), Hoechst 33342 and penicillin-streptomycin were supplied by Sigma. Dulbecco’s Modified Eagle Medium (DMEM), Dulbecco’s phosphate buffered saline (PBS) and fetal bovine serum (FBS) were purchased from Giboco. Calcein-AM and Propidium Iodide (PI) were obtained from Invitrogen Corp. 3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyl tetrazolium bromide (MTT) was purchased from Fluka (Buchs, Switzerland). Membrane and Cytosol Protein Extraction Kit, PMSF and LIVE/DEAD Viability/Cytotoxicity Kit were obtained from Beyotime Institute of Biotechnology. Ultrapure water was used to prepare solutions. All the other reagents were analytical grade and
used without further purification.

2.2. Preparation of the DOX NPs@ICG nanoparticles (DINPs)

DINPs were prepared according to the reported method with a minor modification [25]. Briefly, DOX·HCl was firstly converted into hydrophobic DOX molecules by removing hydrochloric acid in an alkaline trimethylamine (TEA) dissolved in dimethyl sulfoxide (DMSO). Then, 100 µL of the as-prepared DOX-DMSO solution (1 mg mL⁻¹) was added dropwise into 5 mL of deionized water under stirring at a rate of 1000 rpm away from light. After 30 min, 200 µL of ICG in water (1 mg mL⁻¹) was added dropwise into the above solution and stirred at room temperature overnight. The product was further purified by water-phase filter (450 nm).

2.3. Preparation of cracked cancer cell membrane (CCCM) fragments

To obtain the cracked cancer cell membrane fragments, human cervix carcinoma (HeLa) cells were incubated in cell culture dishes with diameter of 10 cm, and then the cells were collected by a cell scraper and centrifuged at 700 g for 5 min. The cell precipitation was resuspended in precooled PBS buffer (pH = 7.4) followed by centrifugation at 600 g for 5 min. In order to remove the residual PBS buffer, further centrifugation for 1 min was performed. The obtained cell pellets were suspended in a hypotonie lysing buffer containing membrane protein extraction reagent and phenylmethylsulfonyl fluoride (PMSF). After that, the cells were incubated in an ice bath for 10–15 min, based on the manufacturer’s instructions. Thereafter, repeated freeze-thawing was carried out to break the cells in the above solution and then centrifuged at 700 g for 10 min at 4 °C. The collected supernatant was further centrifuged at 14,000 g for 30 min to collect the CCMCs. The membrane products were lyophilized overnight, weighed and stored at −80 °C referring the reported protocol. The lyophilized membrane fragments were rehydrated in ultrapure water prior to use.

2.4. Fabrication and characterization of CCMC-coated DOX NPs@ICG nanoparticles (DICNPs)

The DICNPs were prepared by a top-down and a reported extrusion approach [20,34]. Briefly, the DINPs prepared above were mixed with the CCMCs dispersion under vortex stirring and then extruded through a series of water-phase filters with decreasing pore sizes of 1.0 µm, 850 nm and 450 nm for at least 3 cycles. DICNPs were prepared with different membrane-to-DINP mass ratios ranging from 0.5 to 4.

Dynamic Light Scattering (DLS) was used to determine the hydrodynamic size and zeta potential of DICNPs at 25 °C by Nano-ZS903600 (Malvern Instruments). Data were shown as mean ± standard deviation (SD) based on three independent measurements.

The morphologies of nanoparticles dispersed on a carbon-coated copper grid were characterized using a transmission electron microscope (TEM) (JEOL JEM-2100F, Japan) with an accelerating voltage of 80 kV. The morphology of the specimen was placed on a copper grid with formvar film prior to visualization.

The fluorescence spectra and the absorbance spectra of DICNPs were obtained by Fluorescence spectrometer (Edinburgh F920, England) and UV–vis spectrometer (Purkinje TU-1810, China).

The photothermal effect in vitro was measured by recording the thermal profiles of ICG, DINPs and DICNPs upon irradiation with NIR laser at the power density of 3 W cm⁻² for 6 min with a thermal infrared imaging camera (FLIR E8, USA). The temperatures at different time points were also recorded. PBS and DOX NPs were used as negative controls.

2.5. Determination of drug loading efficiency (DLE)

The Fluorescence intensity of DINPs and DICNPs dispersion in DMSO solution (FL(DINPs)) and (FL(DICNPs)) was measured after the separation by centrifugation. By comparison with the fluorescence intensity of DOX NPs (FL(DOX)) in DMSO solution, the DLE was calculated according to the formula: 
\[
DLE\% = \frac{FL(DINPs) - FL(DOX)}{FL(DICNPs) - FL(DOX)} 
\]

The fluorescence absorbance intensity of DOX was measured at 600 nm with excitation wavelength of 488 nm, using a F920 fluorescence spectrophotometer (Edinburgh).

2.6. In vitro drug release test

The DOX release behavior from the different formulations including the DINPs, DICNPs and DICNPs with NIR irradiation (808 nm) at 3 W cm⁻² for 5 min was determined by dialysis method in PBS at 37 °C. Briefly, 3 mL of the DINPs or DICNPs solution was transferred into a dialysis tube (MWCO:3500 Da) and immersed in 5 mL of buffer solution (pH = 7.4) with shaking at 200 rpm. The release medium containing DINPs or DICNPs (the concentration of DOX was 10 µg mL⁻¹). After incubated for 4 h in the presence or absence of NIR laser irradiation (808 nm, 3 W cm⁻², 5 min), the cells were washed with PBS for several times after removing the medium. Then 1 mL of DMEM containing 10 µL Hoechst 33342 was added and the cells were incubated for another 15 min at 37 °C. Prior to the imaging under a confocal laser scanning microscopy (Nikon C1-si TE2000, Japan, excitation filter 488 nm and emission cut-off filter 515–530 nm for green light), the cells were washed thrice by 500 µL of PBS. The exposure to strong light should be avoided in the whole process to protect the fluorescent dyes. The CLSM observations in COS7, L929 and HepG2 were served as controls.

2.7. In vitro homotypic targeting assay

Confocal laser scanning microscopy was used to determine the in vitro homotypic targeting effects of DICNPs on HeLa cells. The HeLa cells were seeded in a single dish at a density of 2.5 × 10⁴ cells at 37 °C for 24 h. Then the medium was replaced with fresh medium containing DINPs or DICNPs (the concentration of DOX was 10 µg mL⁻¹). After incubated for 4 h in the presence or absence of NIR laser irradiation (808 nm, 3 W cm⁻², 5 min), the cells were washed with PBS for several times after removing the medium. Then 1 mL of DMEM containing 10 µL Hoechst 33342 was added and the cells were incubated for another 15 min at 37 °C. Prior to the imaging under a confocal laser scanning microscopy (Nikon C1-si TE2000, Japan, excitation filter 488 nm and emission cut-off filter 515–530 nm for green light), the cells were washed thrice by 500 µL of PBS. The exposure to strong light should be avoided in the whole process to protect the fluorescent dyes. The CLSM observations in COS7, L929 and HepG2 were served as controls.

2.8. Flow cytometry analysis

Two-color flow cytometry was used to quantitatively determine the cellular internalization of the payloads. Briefly, the HeLa cells were seeded in the 24-well plates with a density of 6 × 10⁴ cells/well and incubated for 24 h. After that, the cells were treated with 1 mL DMEM containing DINPs and DICNPs respectively. Then the cells were incubated for 4 h with or without NIR irradiation. After removing the medium, the cells were washed by PBS and trypsinized with 0.25% trypsin for 2 min at 37 °C. Subsequently, the cells were collected, washed and resuspended in 500 µL PBS to detect the fluorescence intensity by a flow cytometer (BD LSRFortessa, USA). The blank cells served as a negative control.

2.9. In vivo homologous-targeting and ex vivo fluorescence imaging of DICNPs

The in vivo homologous-targeting of DICNPs was performed in
male BALB/c nude mice bearing HeLa tumors on right hind limb and two-tumors on opposite hind limbs, respectively. 300 μL of prepared DINPs and DICNPs formula at an ICG dose of 5 mg kg⁻¹ was intravenously injected into the mice bearing tumors via tail vein. After 24 h post-injection, the in/ex vivo fluorescence images of ICG were detected by a single-filter set with the excitation wavelength of 740 nm and emission wavelength of 780–850 nm in the absence of autofluorescence produced by the skin and blood vessels. Tumors and major organs were dissected, washed with cold saline and then subjected to Xtreme imaging system to obtain the fluorescence images. Then the tumors and organs were stored at −80 °C for further use.

2.10. In vivo photothermal ablation of tumor

The mice bearing HeLa tumor were intravenously injected with free ICG, DINPs or DICNPs. After 24 h, the mice were anesthetized by trichloroacetaldehyde hydrate (10%) with a dosage of 40 mg kg⁻¹ body weight at normal body temperature and the tumors were exposed to NIR laser of 808 nm at a power density of 3 W cm⁻² for 6 min. The thermal profile images and the photo-thermal temperatures at different points were recorded with a thermal infrared imaging camera. After that, the TUNEL apoptosis assay and hematoxylin and eosin (H&E) staining were further characterization. The mice treated by the same volume of DOX NPs and PBS were used as control groups.

2.11. In vivo antitumor effect

Mice bearing HeLa tumor were subcutaneously injected with 100 μL of HeLa cells (1 × 10⁶ cells per mouse) on the right hind limbs. After two weeks when the tumor volume reached 150 mm³, the mice were randomly separated into ten groups (n = 5) and injected intravenously with 300 μL of ICG and DINPs via tail vein at DOX dose of 2.5 mg kg⁻¹ per mouse every 3 days for 2 times. The mice injected with PBS, DOX NPs, ICG and DINPs served as control groups. For photothermal treatment, the tumors were treated by NIR laser irradiation (808 nm, 3 W cm⁻²) for 5 min at 1 day and 4 day, respectively. The tumor volume was defined as V = W²L/2, where the W and L are the shortest and longest diameters respectively. The relative volume was calculated as V/V₀, where V₀ and V are the tumor volumes before and after treatment, respectively. After the mice were sacrificed, the tumor tissue and major organs of heart, liver, spleen, lung and kidney were collected for histological examinations by hematoxylin and eosin (H&E) staining. The tumors and major organs were fixed by 4% formalin, embedded in paraffin, and then sectioned with 5 μm thickness. The sections were then placed on the polylysine-coated slides and finally stained with hematoxylin and eosin (H&E). The stained slices were imaged with microscope (CKX41, Olympus, Japan) at 200 × magnifications.

2.12. Statistical analysis

The data were expressed as mean ± SD on the basis of at least three independent experiments. Statistical analysis was performed by Student’s t-test. The differences were considered to be statistically significant for * p < 0.05.

3. Results and discussion

3.1. Synthesis and characterization of DICNPs

The DOX/ICG nanoparticles (DINPs) were first synthesized via a reprecipitation method [25]. To prepare the DICNPs, human cervix carcinoma cells (HeLa) were used as a cell model to produce the cracked cancer cell membranes (CCCMs) with membrane protein extraction kit and differential centrifugation. The assembly proceeded by mixing them with an excess mass ratio of cell membrane versus DINPs. To simplify the research, the mass ratio was first optimized according to the dynamic light scattering (DLS) assay. As shown in the Fig. S1, the DLS data proved that mixing of CCCMs and DINPs at 3:1 and 4:1 led to the generation of nanoparticles with a mean hydrodynamic diameter (Dh) of 220 nm, and there was a minimal change of the Dh after standing at room temperature for 3 days, suggesting that the CCCMs were almost completely coated on the surface of DINPs. To avoid the excessive CCCMs existence, the ratio of CCCMs/DINPs at 3:1 termed as DIC3NPs was used for the further study.

The size and zeta potential of the DINPs, CCCMs and DIC3NPs were shown in Fig. 2A. The hydrodynamic sizes of the DINPs and CCCMs were 100 and 400 nm respectively; after adding the CCCMs, the hydrodynamic size of DIC3NPs was 220 nm with a polydispersity of 0.100–0.180. The zeta-potential of DIC3NPs was −24.7 ± 0.5 mV, which was comparable with that of CCCMs (−24.9 ± 0.7 mV), lower than that of DINPs (−20 ± 1.6 mV). Transmission electron microscopy (TEM) revealed the superficial coverage of the CCCM layer. The Dh of DIC3NPs was fairly close to the mean particle size measured by TEM in dry state (Fig. S2 and Fig. 2B), indicating the compact coverage of CCCM in aqueous medium.

It is well known that the carrier-free nanodrugs can be formed by the aggregates of drug molecules and precipitate due to the abrupt change in the solvent quality [23,35]. Herein, the DINPs were formed by the strong hydrophobic interactions and π-π stacking between the ICG and carrier-free DOX nanoparticles fabricated from the aggregation of hydrophobic DOX molecules [36]. The DIC3NPs were formed by the crossing of DINPs to the lipid bilayers of CCCM through a top-down assembly [37]. The larger-scale TEM image clearly showed that the DINPs were originated from the aggregation of multicompartment nanoparticles (the smaller dark points were marked with red arrow) and the superficial coverage of CCCM layer (marked with red arrow) (Fig. S2). One or more DINPs can be packaged by CCCM, so the average size of DIC3NPs was relatively larger than that of DINPs.

DIC3NPs dispersion exhibited high stability and there was a minimal size change even after standing at room temperature in PBS containing 10% FBS (Fig. 2E). We noted that the application of ICG was limited by its poor stability in water [33,38]. In our work, the ICG entrapped in the DIC3NPs remained 90% fluorescence intensity after 5 days, while the fluorescence intensities of free ICG and the ICG in DINPs degraded to 50% and 80% of the initial value, respectively (Fig. 2F). These data suggested that the optical stability of ICG can be significantly improved by encapsulation into the DIC3NPs. The data also mirrored indirectly that the membrane was well coated on the surface of DINPs.

Next, gel electrophoresis and Western blotting were used to examine the transfer of the membrane protein within the HeLa cell membrane to the shells of the DIC3NPs. It turned out that the DIC3NPs and CCCMs possessed almost the same protein profile compared with HeLa cell lysate (Fig. 2C and D). Obviously, the proteins from the cell membrane could be well retained in the DIC3NPs after a series of treatments (Fig. 2D). Based on the Western blotting analysis, several key membrane and intercellular proteins were confirmed. HeLa cells, CCCMs and DIC3NPs with an equal amount of protein were separately loaded in each lane. It is seen that Glycoprotein 100 (gp100), as a tumor-associated transmembrane protein, and Cadherins and Na(+)/K+-ATPase as plasma membrane-specific markers were well maintained after treatments. Whereas intracellular protein markers of COX IV, Histone H3 and GAPDH respectively for the nucleus, mitochondria and cytosol
were poorly retained on the cracked cancer cell membrane and final nanoparticles, demonstrating the selective retention of membrane fragments after the treatment (Fig. 2C).

3.2. NIR-driven rapid drug release from DIC3NPs

The drug loading efficiency and rapid intracellular release are critical for the application of the nanoparticles. The loading efficiencies (LE) of DOX and ICG were estimated to around 93.3% and 91.4%, respectively (Fig. S3). It was reported that ICG could generate heat upon NIR light irradiation, thus leading to the disruption of the membrane [33,39]. Therefore, the temperature of DIC3NPs was expected to increase and thus contributed to a rapid release of the loaded cargoes upon exposure to NIR light irradiation. The absorbance and fluorescence spectra of the encapsulated DOX and ICG were shown in Fig. S4 and Fig. 3A and B. Compared with free ICG, the emission peak wavelengths of ICG both in DINPs and DIC3NPs remained almost unchanged; nevertheless their emission intensities were increased significantly and their UV-absorption spectra were slightly red-shifted possibly due to the changed
molecular conformation after ICG was complexed with cell membrane or DOX [40,41]. The characteristic peaks of DOX were also observed in DINPs and DIC3NPs, consistent with the spectra of DOX NPs. The fluorescence emission of DOX in DINPs and DIC3NPs was obviously quenched due to the aggregation of the entrapped DOX NPs [42]. After irradiation by NIR laser (808 nm), the fluorescence intensity of DIC3NPs returned to the same level as the free DOX (Fig. 3B). These results indicated that both the ICG and DOX could be successfully encapsulated into DIC3NPs and maintained their optical properties.

The photothermal conversion experiments were performed to characterize the photothermal effects of ICG (Fig. 3D and E). The ICG, DINPs and DIC3NPs exhibited similar temperature rising profiles, indicating that the cell membrane coating and the DOX nanodrugs had negligible effects on the photothermal effects of ICG. The temperature of free ICG, DINPs and DIC3NPs aqueous solutions showed a quick increase trend within 5 min, and the maximal temperature at 6 min increased to 65.6°C, 69.7°C and 73.3°C, respectively. However, the maximal temperature of PBS and free DOX NPs solution only increased to 31.6°C and 31.8°C, respectively, under the same laser irradiation (808 nm, 3 W cm⁻²). The photothermal effects of ICG encapsulated in DINPs and DIC3NPs were enhanced slightly [33], which was consistent with previous reports that ICG-containing NPs were more efficient in triggering laser-dependent temperature increase than free ICG.

It was well established that the photo-heat could lead to the destruction of the membrane shell, thus rapid release of the encapsulated drugs [33,39]. Herein, the DOX release from DIC3NPs before and after NIR laser irradiation was further tested. As shown in Fig. 3F, about 80% DOX in DINPs exhibited a burst release during the first 12 h. After coating DINPs with cell membranes, there was only about 20% cumulative release of DOX within 12 h, indicating that the CCCMs coating efficiently inhibited the release of DOX, meanwhile enhancing the stability of DIC3NPs in the physiological condition. Upon irradiation with 808 nm laser, the DOX release was dramatically accelerated and showed the similar release profile as DINPs without NIR irradiation. It is evident that the photothermal effects could destroy the cell membranes shielding the DINPs and thus facilitate the diffusion of DOX into the solution. The TEM images in Fig. 2B showed that the gray shell was clearly damaged after NIR irradiation, and new signals representing larger particles of CCCMs and/or DOX NPs aggregates (>500 nm) and smaller particles of DOX NPs emerged (Fig. 3C), suggesting that the thermal destruction from ICG against the cell membrane of DIC3NPs occurred. These results demonstrated that NIR laser irradiation could boost the DOX release by opening the cell membrane coated on the nanodrug itself.

3.3. In vitro targeting of DIC3NPs to homotypic cells

To prove our assumption that CCCMs coating would be self-recognized by homotypic cancer cells, the cell internalization of DIC3NPs was evaluated in four different cell lines including HeLa cells, COS7 cells, L929 cells and HepG2 cells upon 4 h co-incubation. An amazing outcome was found that the fluorescence intensity of DOX and ICG from DIC3NPs was much higher in the HeLa cells than that in heterotypic cells (Fig. 4A). The fluorescence intensity of DOX and ICG in HeLa cells was approximated to 3.3–5 fold stronger than that in other cell lines by the quantitative flow cytometry analysis (Fig. 4B and C). Additionally, in HeLa cancer cells, compared with the much stronger fluorescence intensity originating from DIC3NPs, DINPs group displayed weaker fluorescence intensity of both red and green, suggesting the highly specific self-recognition affinity of DIC3NPs to the homologous cells and offset the repulsion effect of negative charged surface due to the homotypic binding properties. These results demonstrated that the cell membrane coated on the surface of DINPs played a critical role in cellular uptake of the DINPs. After NIR irradiation, the fluorescence intensity of DOX from
DIC3NPs exhibited a dramatic increase. As aforementioned, the hyperthermia from ICG in DIC3NPs under NIR irradiation (808 nm) could result in a thermal perturbation and accelerated the release of DOX from DIC3NPs. These results were consistent with those of Figs. 2 and 3, corroborating that cancer cell membrane shells could remarkably increase the cellular internalization to the same source of cancer cells since the residual proteins and surface molecules on the cracked HeLa cancer cell membrane still remained the primary targeting ability.

An ideal delivery system should be designed with excellent immunocompatibility. Antiphagocytosis is one of the most important indicators that can reflect the degree of foreign body reaction [43]. Thus, the RAW264.7 cells were selected to verify the favorable immunocompatibility of DIC3NPs. As shown in Fig. S5, compared to the DINPs, the DIC3NPs exhibited negligible macrophage engulfment after incubation with the RAW264.7 cells for 4 h. The CLSM data manifested that the nanoparticles coated with cancerous cell membranes have the similar immune-evasion efficacy as the normal cells.

The homologous-targeting of DIC3NPs to HeLa cells was further verified by MTT assay. The cells were incubated with the samples for 4 h; after that, the medium was replaced and suffered to NIR laser irradiation (808 nm) for 5 min at 3 W cm⁻². As shown in Fig. 5B, the viability of cells treated with DINPs + NIR containing 20 μg mL⁻¹ of ICG decreased to 40%, while DIC3NPs + NIR containing the same concentration of ICG induced up to 82% cell death after co-incubation for 24 h due to the enhanced cellular uptake for the homologous targeting of DIC3NPs as proved above (Fig. 5B). It is noted that the cytotoxicity of ICG in NPs was higher than that of the free ICG, owing to the greatly enhanced photothermal efficacy induced by the increased intracellular ICG concentration. In the meantime, the cell viability of free ICG, DINPs and DIC3NPs was respectively 82%, 67% and 55% without NIR irradiation. The growth tendency of the cells was almost the same with that of the cells

---

**Fig. 4.** (A) Confocal fluorescence images of four cell lines including HeLa, L929, COS7 and HepG2 after incubation 4 h with DINPs and DIC3NPs. The nuclei were stained by Hoechst 33342 (blue). Scale bars: 25 μm. (B, C) Flow cytometric profiles of four cell lines and fluorescence intensities inside four cells after treated by different samples for 4 h. The concentration of DOX and ICG was fixed at 10 μg mL⁻¹ and 20 μg mL⁻¹, respectively. a: negative control, b: DINPs, c: DIC3NPs and d: DIC3NPs + NIR. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)
treated by NIR laser (Fig. 5A). There was no obvious difference in the DOX NPs-treated groups with or without irradiation, revealing that the NIR laser alone could not cause an evident effect on the growth of cells. To visually evaluate the phototoxicity, Live/Dead Cell Staining was further conducted in the HeLa cells (Fig. 5C). It can be found obviously that when the cells were treated with DIC3NPs + NIR, almost all the cells were destroyed to death, which was consistent with the results of MTT assay.

3.4. In vivo homologous-targeted imaging of DIC3NPs and biodistribution

The encouraging results of in vitro self-recognition to homotypic cancer stimulated us to evaluate the in vivo tumor targeting. Firstly, the mice model with HeLa tumor on the right hind limb was intravenously injected with DIC3NPs and DINPs with an identical DOX dosage of 2.5 mg kg\(^{-1}\) and ICG dosage of 5 mg kg\(^{-1}\) at 24 h, the in vivo living fluorescence imaging (Fig. 6A) and the ex vivo imaging toward major organs (heart, liver, spleen, lung and kidney) and tumors (Fig. 6B) were performed. In the DIC3NPs group, the tumor exhibited much stronger fluorescence compared with other tissues, indicating the efficient tumor accumulation of DIC3NPs. In contrast, a large fraction of ICG was accumulated into the liver, lung and kidney in the control group intravenously injected with DINPs. It turned out that the ICG diffused out of the DINPs in the process of blood circulation, leading to the quick clearance from the body. The higher fluorescent signals of DOX and ICG in DIC3NPs group implied that this nanosystem was much more stable during blood circulation due to the shielding of cell membrane. It was also demonstrated that the DOX and ICG were well co-packed by HeLa cell membrane and could be delivered into the tumors simultaneously on the other hand. Quantitative analysis of fluorescence intensity in HeLa tumors also indicated that the intratumor content of DOX and ICG in DIC3NPs-treated group was 1.4-fold and 2.1-fold higher than in the control and DINPs-treated groups, respectively (Fig. 6C).

To further evaluate the self-recognition ability of DIC3NPs to homotypic tumors in vivo, the mouse model simultaneously bearing two kinds of tumors (HeLa tumor on the Left hind limb and HepG2 tumor on the right) was established. The dual-tumor bearing mice were intravenously injected with DIC3NPs. The in vivo living fluorescence imaging (Fig. 6D) and ex vivo imaging of tumors (Fig. 6E) were conducted after injection for 24 h. The results of fluorescence intensity clearly showed the overwhelmingly preferential accumulation of DOX and ICG in DIC3NPs in the left HeLa tumor compared to that in the right HepG2 tumor. Quantitative analysis (Fig. 6F) on the biodistribution of DOX and ICG fluorescence confirmed that DIC3NPs were capable of uniquely recognizing and homing to the homologous HeLa tumor while bypassed the coexisting heterologous HepG2 tumor.

In cancer therapy, to obtain the effective accumulation of nanoparticles at the tumors, the long circulation time of the nanoparticles was really a primary challenge. Therefore, the blood retention of DIC3NPs after CCCM coating was measured with the bare DOX, DINPs as controls. As shown in Fig. S6, compared with the DOX NPs and DINPs, the DIC3NPs showed a much higher elimination half-life (t\(_{1/2}\)) and area under the curve, suggesting the ability of DIC3NPs to maintain a prolonged circulation time. These results further confirmed that DINPs coated with CCCMs intrinsically carrying “self-recognized” proteins could inhibit the internalization of macrophages, which was in good accordance with in vitro macrophage uptake results.
3.5. In vivo chemo-/photothermal therapy of DIC3NPs against tumor

To confirm that the enhanced tumor accumulation and rapid drug release of DIC3NPs could achieve synergistic chemo-/photothermal therapy in vivo, we carried out the in vivo antitumor experiments of DIC3NPs. First, the photothermal effect of DIC3NPs in vivo was measured. As shown in Fig. 7A, after intravenous injection with 300 μL of PBS, DOX NPs, free ICG, DINPs and DIC3NPs (both containing 5 mg kg⁻¹ ICG) for 24 h, the temperature increase in the tumor region during NIR laser irradiation was recorded. For the groups treated by PBS, DOX NPs, the temperature in the tumor region only increased to 39.9 and 41.0 °C, respectively after irradiation for 5 min. This temperature was not high enough to destroy the tumors (temperature above 42°-47°C was reportedly to contribute to the selective destruction of the tumor cells) [39]. Since the DINPs might achieve a small amount of tumor accumulation via EPR effect, the tumors treated with DINPs + NIR exhibited a temperature rise to 43.9 °C, which was almost the same with the tumors treated with free ICG (44.6 °C). The reason is that the ICG located on the surface of DINPs came out easily as free ICG during blood circulation. Combined with the homologous targeting and passive targeting induced by the EPR effect, DIC3NPs achieved high accumulation in tumor and induced a maximum temperature up to 52.4 °C, much higher than 43 °C, thereby causing irreversible tumor damage. To further determine the photothermal ablation effect of DIC3NPs in vivo, HeLa tumor tissues treated respectively by PBS, DOX NPs, ICG, DINPs and DICNPs for 24 h with NIR irradiation for 5 min were detected by TUNEL apoptosis Assay Kit (Fig. 7G) and stained with hematoxylin and eosin (H&E) (Fig. 7H). Compared with the tumors treated by ICG and DINPs, the group treated by DIC3NPs plus NIR irradiation showed typical features of thermal damage in tumor tissues. The results of TUNEL assay were in accordance with that of H&E—a large number of apoptosis cells were labeled green in the DIC3NPs group, demonstrating its most significant anti-tumor activity under NIR laser irradiation. The photothermal efficacy of DIC3NPs was further evaluated on HeLa tumor-bearing mice. There was no obvious variation in mice weight in all the treated groups, suggesting that the experimental treatments were well tolerated (Fig. 7C). As far as the index of tumor volume was concerned, administration of free ICG without NIR laser irradiation made no difference with the negative control (Fig. S7 and Fig. 7F). And there were no signs of antitumor effects in the groups treated with or without NIR laser irradiation alone, suggesting that the NIR irradiation has no effect on tumor therapy. As expected, obvious antitumor effects were gained for all the DOX-containing formulations and ICG-containing formulations plus NIR laser (Fig. 7D and F). There was no difference between the DOX NPs and the ICG containing DOX NPs in terms of antitumor effect in the absence of the NIR irradiation. While, after NIR laser irradiation,
DINPs exhibited a much better antitumor efficacy than free DOX NPs and DINPs without NIR laser irradiation, reinforcing the superiority of co-loaded DOX and ICG. Of note, the best performance was achieved in the group receiving DIC3NPs formulation, where the tumor volume shrank remarkably along with time. On the contrary, for the other treatments, a different degree of tumor expansion was still observed. Specially, under NIR lased irradiation, the tumor size of the group treated with DIC3NPs formulation declined continuously to 0.2-fold its original volume after 18 days of treatment, whereas the tumor sizes of the groups treated with blank PBS solution, DOX NPs, free ICG and DINPs were increased to around 5-, 3.8-, 4.1- and 3.2-fold the original volume, respectively (Fig. 7E and F). All these results demonstrated that the homotypic targeting to specific tumors, photothermal-triggered fast release of DOX in the tumors as well as the local hyperthermia ablation effect of ICG contributed to an excellent antitumor activity of the DIC3NPs.

On day 18, all mice were sacrificed since the tumors in control groups were too large, and the tumors were excised and weighed. Furthermore, hematoxylin and eosin (H&E) was used to stain HeLa tumor tissues and various major organs to visually evaluate the side effects. Fig. 8 showed that the heart, liver, spleen, lung and kidneys of various groups remained the normal physiological morphologies, and no pathological changes were observed. Taken together, the carrier-free DIC3NPs combining high loading efficiency drugs, homotypic targeting ability and photo-thermal effect achieved a superior chemo-thermotherapy of cancers with a minimal side effect in vivo.

4. Conclusion

In summary, we reported a simple strategy to design and fabricate a safe and potent carrier-free nanosystem integrated with a high drug loading capacity, autologous targeting and in situ NIR-induced rapid drug release. We demonstrated that the DOX loading capacity was as high as 89.8% without using any inert carriers. Coated with cracked cancer cell membrane (CCCMs), the nanoparticles showed an excellent stability and self-recognized targeting to the source tumor cells as well as immune escape function. More importantly, we demonstrated that the CCCMs-cloaked co-assembled nanoparticles of DOX and ICG efficiently inhibited the cargo leakage during blood circulation, and achieved a highly tumor-selective accumulation of nanodrugs due to their unique homologous targetability. Further, the loaded drugs could be delivered in a “bomb-like” manner to the surroundings of tumors due to the membrane disruption induced by the photo-thermal effect of ICG under the NIR irradiation, thus leading to an enhanced chemo-
photothermal therapeutic efficacy. In light of multi-choice of small molecular chemotherapeutic drugs and diverse sources of cancer cell membranes, the bioinspired strategy opens up a new avenue to tailor precise theranostic nanosystems for a specific patient or a disease.

Acknowledgments

This work was financially supported by National Natural Science Foundation of China (Grant No. 51703161), National Natural Science Funds for Distinguished Young Scholar (Grant No. 51325305), Tianjin Municipal Natural Science Foundation (Grant No. 17JCQNJC02900), and National Key Research and Development Program (Grant No. 2016YFC1101301).

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.biomaterials.2018.01.007.

References


