Red blood cell membrane-camouflaged melanin nanoparticles for enhanced photothermal therapy

Qin Janga, Zimiao Luob, Yongzhi Menc, Peng Yanga, Haibao Penga, Ranran Guoa, Ye Tian a, Zhiqing Pangb, *, Wuli Yanga, **

State Key Laboratory of Molecular Engineering of Polymers & Department of Macromolecular Science, Fudan University, Shanghai, 200433, PR China

Key Laboratory of Smart Drug Delivery, Ministry of Education & PLA, Department of Pharmaceutics, School of Pharmacy, Fudan University, Shanghai, 201203, PR China

Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, 200433, PR China

**Corresponding author.
***Corresponding author.

E-mail addresses: zqpang@fudan.edu.cn (Z. Pang), wlyang@fudan.edu.cn (W. Yang).

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Abstract

Photothermal therapy (PTT) has represented a promising noninvasive approach for cancer treatment in recent years. However, there still remain challenges in developing non-toxic and biodegradable biomaterials with high photothermal efficiency in vivo. Herein, we explored natural melanin nanoparticles extracted from living cuttlefish as effective photothermal agents and developed red blood cell (RBC) membrane-camouflaged melanin (Melanin@RBC) nanoparticles as a platform for in vivo antitumor PTT. The as-obtained natural melanin nanoparticles demonstrated strong absorption at NIR region, higher photothermal conversion efficiency (~40%) than synthesized melanin-like polydopamine nanoparticles (~29%), as well as favorable biocompatibility and biodegradability. It was shown that RBC membrane coating on melanin nanoparticles retained their excellent photothermal property, enhanced their blood retention and effectively improved their accumulation at tumor sites. With the guidance of their inherited photoacoustic imaging capability, optimal accumulation of Melanin@RBC at tumors was achieved around 4 h post intravenous injection. Upon irradiation by an 808-nm laser, the developed Melanin@RBC nanoparticles exhibited significantly higher PTT efficacy than that of bare melanin nanoparticles in A549 tumor-bearing mice. Given that both melanin nanoparticles and RBC membrane are native biomaterials, the developed Melanin@RBC platform could have great potential in clinics for anticancer PTT.

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1. Introduction

As a noninvasive cancer treatment, photothermal therapy (PTT) has earned much attention in recent years due to its localized tumor ablation and minimal heating damage to adjacent normal tissues [1]. The mechanism of PTT is that photothermal agents strongly absorb and covert near infrared (NIR) light into thermal energy after their adequate accumulation within tumors [2]. Currently, various materials have been explored as effective photothermal agents, including inorganic nanomaterials (e.g. gold-, carbon-, palladium-, and magnetic nanoparticles) [3–6], NIR dye (indocyanine green, IR825) [7,8], polymer nanoparticles (e.g. polydopamine, polyaniline, polypyrrole, poly(-ethylenedioxythiophene) (PEDOT)) [9–12] and other nanomaterials (e.g. porphysomes, Prussian blue) [13,14]. Despite their extraordinary photothermal effect, the long-term safety of these synthetic nanomaterials in vivo may still limit their further clinical application in cancer treatment. For instance, metallic nanoparticles, especially those non-degradable metallic nanoparticles, would retain in the biological system for a very long time and cause metal-related cytotoxicity [15], while carbon-based nanomaterials have been widely reported to induce serious toxicities such as oxidative stress and lung inflammation after administration [16]. Therefore, it will be of great clinical value to develop biocompatible and biodegradable photothermal nanomaterials with high photothermal efficiency for in vivo cancer PTT.

Melanin, a ubiquitous natural biopolymer, which is widely distributed in many organisms including human skin [17]. It has
been a new class of biomaterials for biomedical application because of its intrinsic properties and various biological functions [18]. For example, Lu et al. synthesized melanin-like colloidal nanoparticles as effective photothermal agents for in vivo cancer therapy and achieved as high as 40% photothermal conversion efficiency [19]. Fan et al. synthesized water-soluble melanin nanoparticles and developed a melanin-based theranostic platform for multimodality imaging of melanoma and tumor drug delivery by loading sorafenib on their surfaces through strong binding interaction between melanin and aromatic structures of sorafenib [20,21]. Compared with those synthetic melanin nanoparticles described above, the natural melanin nanoparticles extracted from living organisms attracted more alluring attention for cancer PTT because of their native biocompatibility and biodegradability, which could effectively eliminate the side-effects as well as default metabolism in biological system [22]. Chu et al. successfully extracted natural melanin nanoparticles from black sesame seeds for lymph node imaging and cancer PTT [23]. After intratumoral injection into tumor-bearing mice, the as-obtained natural melanin nanoparticles presented appealing performance in tumor growth inhibition with considerable PTT efficacy and low toxicity. However, although the “enhanced permeation and retention (EPR)” effect of tumors could provide passive targeting, like other nanoparticles, melanin nanoparticles still could be easily recognized and rapidly cleared by the reticuloendothelial system as exogenous invaders when injected via tail-vein, and eventually resulted in poor tumor accumulation and limited therapeutic effect [24]. Therefore, it will be of great significance in strategies that can promote the accumulation of melanin nanoparticles at target sites as well as improve their PTT performance for in vivo cancer therapy.

Recently, functionalization of nanoparticles through a top-down naturally derived bio-membranes coating approach represents an emerging strategy to construct biomimetic system [25–27]. Red blood cell (RBC), of which surface makeup composed of many “self-makers” (e.g. CD47 proteins, peptides, glycans, acidic sialyl moieties), is a native long circulating carrier that enables nanoparticles to effectively escape immune recognition in the body [28]. RBC membrane-camouflaged nanoparticles have been strongly verified as promising candidates for biomedical application, which benefit from the prolonged blood circulation, high biocompatibility, immune-evasion, and reduced accelerated blood clearance effect endowed by RBC membrane [29,30]. For example, Zhang and co-workers have engineered RBC membrane with various functional nanoparticles for in vivo drug delivery, blood detoxification, antibacterial vaccination and anticancer therapies [31–34]. Moreover, our group previously fabricated RBC membrane-coated Fe3O4 particles as a platform for cancer PTT, and in vivo studies revealed that Fe3O4 particles coated with RBC membrane performed superior PTT efficacy than bare Fe3O4 particles [35]. Very recently, Liu et al. achieved outstanding synergistic combination therapy in vivo by cloaking doxorubicin (DOX)-loaded hollow mesoporous Prussian blue (HMPB) nanoparticles with RBC membrane (DOX@HMPB@RBC) [36]. The developed DOX@HMPB@RBC nanoparticles exhibited excellent immune evading capacity and longer blood retention compared with bare HMPB nanoparticles. These studies indicated that erythrocyte membrane coating strategy could be a facile and effective way to enhance tumor accumulation and tumor ablation effect of natural melanin nanoparticles.

In this work, we extracted natural melanin nanoparticles from living cuttlefish and firstly explored them as effective photothermal agents. The photothermal property, biocompatibility as well as degradability of as-obtained natural melanin nanoparticles were investigated. Afterwards, RBC membrane-camouflaged melanin nanoparticles (Melanin@RBC) were developed as a platform for in vivo cancer PTT (Scheme 1). With the inherited photoacoustic imaging (PAI) characteristics from melanin nanoparticle core [37] and promoted immune evasion capability and enhanced tumor accumulation from RBC membrane, the developed Melanin@RBC was employed as a PAI contrast for tumor imaging and its in vivo PTT efficacy in A549 tumor-bearing mice after intravenous administration was investigated.

2. Materials and methods

2.1. Materials

Fresh cuttlefish (~400 g) were purchased from Tongren Fishes Wholesale Market (Shanghai, China). Dopamine hydrochloride (DA-HCl) was ordered from Sigma-Aldrich (St. Louis, USA). Ethylene glycol, sodium hydroxide (NaOH), nitric acid (HNO3), hydrogen peroxide (H2O2, 35%) and ammonia (NH₃·H₂O, 25%–28%) were from Aladdin (Shanghai, China). Ethyl alcohol was purchased from Shanghai Heqi Chemical Co. Ltd (Shanghai, China). Roswell Park Memorial Institute (RPMI–1640) medium and Dulbecco’s modified

![Scheme 1. Schematic illustration of red blood cell membrane camouflaged melanin nanoparticles for enhanced photothermal therapy.](image-url)
Eagle medium (DMEM/HIGH GLUCOSE 1X) were purchased from GE Healthcare Life Science (Hyclone™, Pittsburgh, USA). Fetal bovine serum (FBS) and Trypsin-Ethylene Diamine Tetraacetic Acid (Trypsin-EDTA, 0.05%) were purchased from Life Science (Gibco™, Pittsburgh, USA). Cell Counting Kit-8 (CCK-8) was purchased from Beyotime Institute of Biotechnology (Nanjing, China). Live-dead viability/cytotoxicity assay kit for animal cells was purchased from KeyGEN BIOTECH Co. Ltd. (Nanjing, China). Annexin V-FITC/propidium iodide (PI) apoptosis detection kit was purchased from 7Sea Pharmarech Co. Ltd. (Shanghai, China). Cyanine5 amine dye (expressed as Cy5) was purchased from ApexBio Technology (USA). Raman spectra were performed on XploRA laser Raman fluorescence lifetime spectrometer (Photo Technology International, Inc., USA). Raman spectra were performed on an ESCALab 200i-XL spectrometer (Ultra 55, Zeiss, Germany) operates at 5 kV. X-ray photoelectron spectroscopy (XPS) was performed on an ESCALab 200i-XL spectrometer (Ultra 55, Zeiss, Germany) operates at 5 kV.

Male Balb/c nude and ICR (Institute of Cancer Research) mice (20–22 g) were purchased from Shanghai BK Lab. Animal Research Center and raised under a standard condition at 25 ± 2 °C and 60% ± 10% humidity environment with 12-h light/dark cycle. All procedures for animal experiments were handled under the guidelines approved and supervised by the ethics committee of Fudan University.

2.2. Instrumentation

Dynamic light scattering (DLS) and zeta potential were measured at 25 °C on a Zetasizer Nano ZS90 analyzer (Malvern Instrument Ltd, UK). UV–vis spectra were recorded at 25 °C on PerkinElmer Lambda 750 spectrophotometer. Fourier transform infrared (FT-IR) spectra were conducted on Thermofisher Nicolet 6700 fourier transform infrared spectrometer with KBr pellets. Fluorescence measurements were conducted on QM40 fluorescence lifetime spectrometer (Photo Technology International, Inc., USA). Raman spectra were performed on XploRA laser Raman spectrometer (HORIBA JobinYvon, France) with excitation of 638 nm. Transmission electron microscope (TEM) images were taken on a transmission electron microscope (Tecnai G2 20 TWIN, FEI, USA) operated at an acceleration of 10 kV by dropping solution onto a carbon-coated copper grid. Scanning electron microscopy (SEM) images were recorded on a scanning electron microscopy (Ultra 55, Zeiss, Germany) operates at 5 kV. X-ray photoelectron spectroscopy (XPS) was performed on an ESCALab 200i-XL spectrometer electron spectrometer from VG Scientific using 300 W Al Kx radiation (Perkin-Elmer, USA). The base pressure was about 3 × 10−9 mbar. The data were acquired with −10 bias. Confocal laser scanning microscopy (CLSM) images were performed on a Nikon C2™ laser scanning confocal microscope (Nikon, Japan). Flow cytometry analysis was operated at 37 °C on a Beckman Coulter Gallios flow cytometer. Both photoacoustic & ultrasound images were recorded on a high resolution pre-clinical PAI system (Vevo LAZR, FujiFilm VisualSonics Inc, USA) with consequent excitation of 680–970 nm and focus depth of 2 cm. Photothermal effect were tested on an 808 nm consequent NIR laser with spot size of 5 mm × 6 mm (Changchun New Industries Optoelectronics Technology Ltd, China). Thermal images were taken on an infrared thermal imaging camera thermographic system with accuracy of 0.1 °C (Infra Tec, VarioCAM®hr research, Germany).

2.3. Preparation and characterization of natural melanin nanoparticles

Natural melanin nanoparticles were prepared according to the published method [38] with a few modifications. Briefly, ink sac was obtained by dissection of fresh cuttlefish brought from market. Natural melanin nanoparticles was extracted from the freshly dissected ink sac, followed by centrifugation (18000 rpm) at 4 °C for 20 min and wash with deionized water for three times. The resulting melanin nanoparticles were suspended in water for further TEM, SEM, size & zeta potential characterization and analysis. Before FT-IR, Raman and XPS measurement, the as-obtained melanin nanoparticles were first centrifuged at 18000 rpm for 20 min, and dried in the oven (40 °C) over night.

2.4. Synthesis and characterization of polydopamine nanoparticles

The melanin-like polydopamine (PDA) nanoparticles were synthesized according to the procedures of Lu et al. [19] with a few modifications. Briefly, ammonia solution (NH4OH, 25%–28%, 3 ml) was mixed with ethanol (CH3CH2OH, 40 mL) and deionized water (H2O, 90 mL) under mild stirring at 30 °C for 30 min, and then dopamine hydrochloride aqueous solution (0.5 g, 10 mL) was injected into the above mixture solution. Immediately the color of solution turned to pale yellow and gradually changed to dark brown. After reaction for 24 h, the PDA nanoparticles were obtained by centrifugation (18000 rpm, 20 min) and washed with deionized water for three times. The resulting PDA nanoparticles were suspended in water for further TEM, SEM, size and zeta potential characterization. Before FT-IR, Raman and XPS measurement, the as-obtained PDA nanoparticles were first centrifuged at 18000 rpm for 20 min, and dried in the oven (40 °C) over night.

2.5. Preparation of red blood cell membrane-derived vesicles

Red blood cell (RBC) membrane-derived vesicles were prepared according to the published protocol [31] with a few modifications. Briefly, the whole blood (~2 mL) was collected from ICR mice (male, 20–22 g), suspended in 10 mL of heparin-containing phosphate saline buffer (1 × PBS) solution, and centrifuged at 720 × g for 5 min at 4 °C to collect RBCs. Afterwards, RBCs were washed with ice cold 1 × PBS containing 1 mM EDTA-2Na for five times and recollected by centrifugation to remove serum and buffy coat. The resulting RBCs were then subjected to the hypotonic solution (0.25 mM EDTA-2Na) (RBCs/hypotonic solution volume ratio of 1/4) for hemolysis and centrifuged at 20000 × g for four times to remove the hemoglobin. The pink pellet (RBC ghosts) was collected, suspended in water, and stored at −80 °C for further use. For preparation of 200-nm RBC membrane-derived vesicles, the collected RBC ghosts were extruded serially through 1-μm, 400-nm and then 200-nm polycarbonate porous membranes (Avanti Polar Lipids Inc). The resulting RBC membrane vesicles were subsequently re-suspended in water for further TEM, SEM, and DLS & zeta potential characterization.

2.6. Preparation of RBC membrane-coated melanin nanoparticles

RBC membrane-coated melanin nanoparticles (Melanin@RBC) were prepared by mixing and repeatedly extruding RBC membrane and melanin nanoparticles through 200-nm polycarbonate porous membranes at the surface area ratio of RBCs/Melanin of 1:1, according to the surface area analysis (See Methods in Supplementary Data). Afterwards, the mixtures were centrifuged at 18000 rpm for 10 min to remove excessive free RBC membrane vesicles and washed with deionized water for three times. The resulting Melanin@RBC nanoparticles were re-dispersed for further TEM, DLS & zeta potential and UV–vis characterization.

2.7. RBC membrane protein characterization

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to characterize the membrane protein. Briefly, RBC membrane vesicles and Melanin@RBC nanoparticles...
were lysed with RIPA lystate (Beyotime, China) and the total protein concentration was determined by the bicinchoninic acid (BCA) protein assay kit (Piez, China). All the samples mixed with SDS-PAGE sample loading buffer (Invitrogen, USA) were heated at 100°C for 10 min. Afterwards, samples with equivalent protein amount (40 μg/well) were loaded on 10% SDS-PAGE gel (Beyotime, China) and were run at 120 V for 2 h. The resultant PAGE gel were stained in Coomassie Blue for 2 h and washed overnight for subsequent imaging with GBOX gel documentation system (Syngene, UK).

Western blot was used to analysis CD47 protein, a typical self-marker of RBC membrane. The resulting gel was transferred to a polyvinylidene difluoride membrane. The membrane was probed with a primary rabbit anti-mouse CD47 antibody (Abcam, ab108415, 1:1000) at 4°C overnight, followed by incubation with horseradish peroxidase-labeled goat anti-rabbit IgG (H + L) cross-adsorbed secondary antibody (Invitrogen™, G-21234, 1:2000) for 1 h at 37°C. Beta-tubulin loading control antibody (Invitrogen™, MA5-16308, 1:3000) was used as an internal control. The membrane was then subjected to enhanced chemiluminescence detection kit (BeyoECL Plus, Beyotime, China) and the signals were recorded on a ChemiDoc™ XR+ System (Bio-Rad, USA).

2.8. Measurement of photothermal performance

A consecutive 808-nm NIR laser with spot size of 5 mm × 6 mm was used to evaluate photothermal effect. The power density was 2 W/cm². The distance between laser and sample was fixed at 10 cm. An infrared thermal imaging camera with accuracy of 0.1°C was used to record temperature variation. Before irradiation and record, samples were dissolved in deionized water to achieve a final concentration of 0, 25, 50, 100 μg/mL, respectively. 200 μL of each sample was used for photothermal measurement. Temperature was recorded after irradiation at every 20 s intervals.

2.9. Biodegradation experiment

For biodegradation experiment, hydrogen peroxide (H₂O₂) with various concentrations (0, 1, 2, 3, 4, 5 and 10 mM) were added to nanoparticle suspension (100 μg/mL) and stirred for reaction for 24 h, respectively. Afterwards, samples were subjected to TEM characterization, size and zeta potential analysis, and UV−vis absorption measurement. The absorbance at 808 nm was used for biodegradability evaluation.

2.10. Cell culture

Human lung adenocarcinoma cell line (A549 cells, tumor cells) and human embryonic kidney cell line (HEK-293T cells, normal cells) were supplied by Chinese Science Academy (Shanghai, China) and KeyGEN BIOTECH Co. Ltd. (Nanjing), respectively. A549 cells were cultured in RPMI-1640 supplemented with 10% (v/v) FBS and KeyGEN BIOTECH Co. Ltd. (Nanjing), respectively. A549 cells were cultured in high-glucose DMEM supplemented with 10% (v/v) FBS and human embryonic kidney cell line (HEK-293T cells, normal cells) were cultured in RPMI-1640 supplemented with 10% (v/v) FBS and 1% antibiotics (penicillin/streptomycin, 100 U/mL). HEK-293T cells were cultured in high-glucose DMEM supplemented with 10% (v/v) FBS and 1% antibiotics (penicillin/streptomycin, 100 U/mL). Culture condition was at 37°C and under 5% CO₂ atmosphere.

2.11. Cytotoxicity assay

The cytotoxicity of nanoparticles was tested on A549 cells and 293T cells using a standard CCK-8 assay as previously described [39]. Cells were seeded at a density of 1 × 10⁴/well in 96-well plate at 37°C and under 5% CO₂ atmosphere for 24 h. Then cells were treated with various concentrations (0, 25, 50, 100, 200, 400, 800 and 1000 μg/mL) of nanoparticles (100 μL/well) for 48 h. Afterwards, CCK-8/culture medium (10 μL/100 μL) was added to each well for additional 1-h incubation under the same condition. An enzyme-linked immunosorbent assay (ELISA) reader (Epoch2 Microplate Spectrophotometer, BioTek Instruments Inc., USA) was used to measure the absorbance value at 450 nm (OD 450) of each well. The following formula was used to calculate the cell viability: Cell Viability (%) = [(A_{t}-A_{0})/(A_{c}-A_{0})] × 100%, where A₀, Aₜ, and Aₑ represent the OD 450 of treatment group, control group and blank well, respectively. The experiments were performed in triplicate.

2.12. In vitro PTT assay

A549 cells were cultured at a density of 1 × 10⁴/well in 96-well plate at 37°C and under 5% CO₂ atmosphere for 24 h. Then cells were randomly divided into four groups: Control group, Nanoparticles group, Laser group, and Nanoparticles + Laser group. For Control group, cells were treated with serum-free culture medium. For Nanoparticles group, cells were treated with varying concentration of nanoparticles for 4 h without irradiation. For Laser group, cells were irradiated with an 808-nm laser under 2 W/cm² for 5 min in the absence of nanoparticles. For Nanoparticles + Laser group, cells were treated with varying concentration of nanoparticles for 4 h, washed with PBS for three times, and then irradiated with an 808-nm laser under 2 W/cm² for 5 min. Afterwards, cells were subjected to viability test by CCK-8 assay.

2.13. Confocal laser scanning microscopy (CLSM) imaging & flow cytometry analysis

Melanin nanoparticles were suspended in serum-free culture medium (RPMI-1640 for A549 cells, DMEM for 293T cells) to yield 100 μg/mL of nanoparticle suspension. Prior to CLSM imaging or flow cytometry analysis, cells were cultured in 35-mm glass-bottomed dishes at a density of 1.0 × 10⁴/dish for 24 h, and then randomly divided into four groups: Control group, Melanin group, Laser group, and Melanin + Laser group. For Control group, cells were treated with serum-free culture medium. For Melanin group, cells were treated with 100 μg/mL of melanin nanoparticle suspension for 4 h without irradiation. For Laser group, cells were irradiated with an 808-nm laser under 2 W/cm² for 5 min in the absence of melanin nanoparticles. For Melanin + Laser group, cells were treated with 100 μg/mL of melanin nanoparticle suspension for 4 h, washed with PBS for three times, and then irradiated with an 808-nm laser under 2 W/cm² for 5 min. Afterwards, cells were stained with calcein acetomethoxy (Calcein-AM, 10 μM in PBS) and propidium iodide (PI, 2 μM in PBS) for 15 min at 37°C under 5% CO₂ atmosphere. For CLSM imaging, cells were subjected to imaging analysis with a confocal microscope after replacing the staining solution with free DMEM. Calcein-AM was excited by a 488-nm laser and the green emission (520 nm) was collected with a band pass-filter within the range 500–550 nm. PI was excited by a 564 nm laser and the red emission (620 nm) was collected with a band pass-filter within the range 600 nm–700 nm. For flow cytometry analysis, after cells were stained with Annexin V-FITC/PI apoptosis detection kit, cells were directly digested and analyzed by a Gallios flow cytometry with excitation at 488 nm and emission at 525 ± 40 nm and 620 ± 30 nm, respectively. WinMDI software (version 2.9) was used to calculate cell apoptosis rate.

2.14. Pharmacokinetics and biodistribution studies

Before pharmacokinetics and biodistribution studies, Cy5-modified melanin nanoparticles were prepared by mixing 20 mg...
of melanin with 5 mg of Cy5 in dimethyl sulfoxide (DMSO)/PBS (v/v, 1/99, 10 mM, pH = 7.4) solution by magnetic stirring in the dark for 24 h. Then, the mixture was centrifuged (18000 rpm, 10 min) and the pellet was washed three times with DMSO/PBS (v/v, 1/99) to remove free Cy5. The actual loading amount of Cy5 on the surface of melanin nanoparticles was 1/10 (m/m), quantified by reading the fluorescence intensity of free Cy5 ($\lambda_{ex} = 640$ nm, $\lambda_{em} = 665$ nm) in the supernatant. Afterwards, the obtained Cy5-modified melanin nanoparticles were subjected to size and zeta potential measurement.

For pharmacokinetics study, eight Balb/c nude mice (male, 20–22 g) were randomly divided into two groups: Melanin group and Melanin@RBC group, in which mice received 100 $\mu$L of melanin nanoparticles and Melanin@RBC nanoparticles (2 mg/mL) intravenously, respectively. At different time points post injection (i.e. 1, 2, 4, 6, 8 and 24 h), 100 $\mu$L of blood were collected from mouse orbit and diluted to 1000 $\mu$L with PBS buffer (10 mM, pH = 7.4). The blood samples were centrifuged at 1200 rpm for 5 min, and the supernatant were collected for fluorescence intensity measurement ($\lambda_{ex} = 640$ nm, $\lambda_{em} = 665$ nm).

For biodistribution study, sixteen A549 tumor-bearing Balb/c mice were prepared by subcutaneous injection of $5 \times 10^6$ A549 cells (100 $\mu$L) into the right fore leg of each mouse. When tumor volume reached approximately 100 mm$^3$, mice were randomly divided into two groups: Melanin group and Melanin@RBC group, in which mice received 100 $\mu$L of melanin nanoparticles and Melanin@RBC nanoparticles (2 mg/mL) intravenously, respectively. At 4 h and 24 h post injection, four mice from each group were sacrificed, and their tumors and major organs including heart, liver, spleen, lung and kidney were harvested, weighted and homogenized in lysis buffer (Beyotime Biotech, China). To explore the possible metabolism pathway of Melanin@RBC nanoparticles, the major organs and tumors of mice at varying time points (i.e. 1, 4, 8, 12, 24 and 48 h) post intravenous nanoparticle injection were collected, weighted and homogenized in lysis buffer. Afterwards, the lysate of each tissue sample was centrifuged at 1200 rpm for 5 min and the
supernatant was subjected to fluorescence intensity measurement. The nanoparticle concentration in tissues or blood was expressed as percentage of injected nanoparticle dose per gram of tissue (% ID/g) (See Methods in Supplementary Data).

2.15. In vitro & in vivo PA imaging

For in vitro PA imaging, varying concentrations (0, 5, 12.5, 25, 50, 100 and 200 μg/mL) of melanin nanoparticle and Melanin@RBC dispersion were filled into polyethylene capillaries, laid on the coupling gel, and then fixed in the PA imaging system. Both PA and ultrasound (UA) signals were acquired with the laser wavelength set at 680 nm. The maximum absorption wavelength of melanin nanoparticle was within the range of 680 nm—970 nm in the system. ImageJ software was used to process the PA images after acquisition. For quantification analysis, ROIs (region of interests) were drawn over the sample and their average values of PA intensity were recorded.

For in vivo PA imaging, A549 tumor-bearing mice were prepared by subcutaneous injection of 5 × 10⁶ A549 cells (100 μL) into the right fore leg of each mice. After 13 days, when tumor volume reached approximately 100 mm³, mice were randomly divided into two groups: Melanin group and Melanin@RBC group, in which mice received 100 μL of melanin nanoparticles and Melanin@RBC (1 mg/mL) intravenously, respectively. After anesthetization with 5% iso-flurane, tumor-bearing mice were subjected to PA imaging and PA signals at tumor area were recorded at different time points before (pre) and after (1, 2, 4, 6 and 24 h) nanoparticle injection. The PA signal of melanin nanoparticle was acquired using the unmixing spectra modules of the PA imaging system to exclude signals from hemoglobin and oxygerated homolobin in vivo. Quantification analysis of PA signals at tumors was performed as described in the in vitro study.

2.16. In vivo PIT efficacy

A549 tumor-bearing mice were prepared as described above. When tumor volume reached about 100 mm³, mice were randomly divided into five groups: Control group, Laser group, Melanin-2 (2 W/cm² Laser group), Melanin@RBC-2 (2 W/cm² group), and Melanin@RBC-1 W/cm² group. For Control group, normal mice did not receive any treatment. For Laser group, tumor-bearing mice were irradiated by an 808-nm laser (2 W/cm²) for 5 min. For Melanin-2 W/cm² Laser group and Melanin@RBC-2 W/cm² group, tumor-bearing mice received 100 μL of melanin nanoparticles and Melanin@RBC (2 mg/mL) intravenously, respectively, and then were irradiated by an 808-nm laser (2 W/cm²) for 5 min 4 h post injection. For Melanin@RBC +1 W/cm² group, tumor-bearing mice received 100 μL of Melanin@RBC (2 mg/mL) intravenously, and then were irradiated by an 808-nm laser (1 W/cm²) for 10 min 4 h post injection. The temperature elevation at tumors was recorded using an NIR camera. Body weight and tumor size of each group were measured every other day. The following formula was used to calculate the tumor volume (V): V = ab²/2, where a, b represent the maximum diameter and the minimum diameter of tumors, respectively.

When the tumor volume reached more than 1500 mm³ (approximately 13 days post nanoparticle injection), mice were dissected and their tumors and major organs including heart, liver, spleen, lung and kidney were harvested, fixed with 4% para-formaldehyde, sectioned into slices, stained with hemotoxylin and eosin (H&E) for histological analysis.

2.17. Blood biochemistry and hematology test

To test the in vivo biosafety, thirty-two ICR mice (male, 20—22 g) were randomly divided into four groups: Melanin-2 group, Melanin@RBC-2 group, Melanin-6 group and Melanin@RBC-6 group. In Melanin-2 and Melanin@RBC-2 groups, mice received a single injection of 0.2 mg of melanin and Melanin@RBC nanoparticles (2 mg/mL, 100 μL) intravenously (single-dose), respectively. In Melanin-6 and Melanin@RBC-6 groups, mice received an injection of 0.2 mg of melanin and Melanin@RBC nanoparticles (2 mg/mL, 100 μL) intravenously every 2 days for 3 times (multiple-dose), respectively. At 24 h and 48 h post injection, four mice from each group were sacrificed and their blood samples were collected. The whole blood panel data from control mice without any treatment and nanoparticle-treated mice were tested (i.e. WBC: white blood cell, RBC: red blood cell, HGB: hemoglobin, HCT: hematocrit, MCV: mean corpuscular volume, MCH: mean corpuscular hemoglobin, MCHC: mean corpuscular hemoglobin concentration, RDW: red blood distribution width, PLT: platelets). Three important hematologic indicators (i.e. ALT: alanine aminotransferase, AST: aspartate aminotransferase, ALP: alkaline phosphatase) and two renal function-related indicators (i.e. CREA: creatinine, UREA: urea nitrogen) were measured using a 7080 blood biochemical auto analyzer (HITACHI, Japan).

2.18. Statistical analysis

GraphPad Prism 5 software was used for statistical analysis. One-way analysis of variance (ANOVA) followed by Tukey’s post hoc test was used to determine differences between groups. The asterisks (*p < 0.05, **p < 0.01, ***p < 0.001) was considered statistically significant and n.s. represented no significance.

3. Results and discussion

3.1. Preparation and characterization of natural melanin nanoparticles

In this study, natural melanin nanoparticles were extracted from the ink sacs of living cuttlefish (Fig. 1a) with relatively simple separation procedures. TEM image (Fig. 1b) demonstrated that the obtained melanin nanoparticles had an average diameter of 128 ± 22 nm, with spherical morphology as well shown by SEM image (Fig. 1c). The hydrodynamic diameter of melanin nanoparticles measured from DLS was approximately 216 nm (Fig. 1d), bigger than that from TEM and SEM probably due to the interaction between surrounded water molecules and nanoparticles. The low polydispersity index (PDI) of 0.097 and ζ potential of −18 mV (Table S1, Supplementary Data) further supported the good dispersibility of melanin nanoparticles in water. As shown in Fig. 1e, melanin nanoparticles had strong and broad FT-IR transmission...
peak at about 3300 cm\(^{-1}\), which corresponded to the O–H and N–H stretching vibration of hydroxyl- and amino-group. The consecutive aromatic bending (C=C, C==N) at about 1600 cm\(^{-1}\) and carbonyl stretching (C=O) at about 1350 cm\(^{-1}\) indicated the melanin nanoparticles from cuttlefish might be composed of aromatic structures like other biopolymers through the strong π-π interaction. In addition, the weak peaks between 800 and 600 cm\(^{-1}\) were also observed, suggesting part of aromatic rings of melanin nanoparticles possibly substituted by aromatic hydrogen [40]. Moreover, Raman spectra of melanin nanoparticles (Fig. 1f) showed two strong Raman signals at 1350 cm\(^{-1}\) and 1580 cm\(^{-1}\). The vibration signal at 1350 cm\(^{-1}\) was attributed to sp\(^2\) carbon of 2D hexagonal structure lattice (D band) while the vibration signal at 1580 cm\(^{-1}\) corresponded to sp\(^2\) carbon of in-plane aromatic
structure ring (G band) [41]. Interestingly, the Raman intensity of D band versus that of G band was very close to 1, indicating that the obtained melanin nanoparticles may have a graphene sheet-like structure with high-density vacancy defects, which was further verified by the XPS spectrum of melanin nanoparticles. The peaks at 396.5 eV corresponded to N1s of graphene, revealing the presence of the formation of graphene (Fig. 1h). Considering that two key factors of absorbance in NIR region and photothermal conversion efficiency determine photothermal effect, UV–vis spectra of melanin nanoparticles in water was initially investigated. As shown in Fig. 1g, melanin nanoparticle suspension has broad and strong absorption in the NIR region from 600 nm to 850 nm, thus making them promising photothermal agents.

3.2. Photothermal effect of melanin nanoparticles

Melanin nanoparticles with varying concentrations were exposed to 808-nm consecutive NIR laser with the power density of 2 W/cm². As shown in Fig. 2a, the temperature of melanin nanoparticle suspension was elevated to 41.5, 54.3 and 61.6 °C, respectively, when melanin nanoparticle concentration increased from 25, 50 and 100 μg/mL while pure water had slight temperature elevation (<4 °C) after irradiation for 500 s. To investigate the photothermal efficiency of melanin nanoparticles, melanin-like polydopamine (PDA) nanoparticles with similar size (124 ± 24 nm) was synthesized through typical oxidative polymerization (TEM, SEM, DLS, zeta potential, FT-IR and XPS of PDA nanoparticles were packed in Table S1, Fig. S1 and S2).

Fig. 4. a) Schematic illustration of RBC-membrane vesicles extraction and the preparation of RBC membrane-coated melanin nanoparticles. TEM images of melanin nanoparticles before (b) and after (c) RBC membrane coating. Inset were representative TEM images of a melanin and a Melanin@RBC nanoparticle negatively stained with uranyl acetate, respectively. Scale bars = 100 nm. d) UV–vis spectra of melanin nanoparticles, RBC-membrane vesicles and Melanin@RBC nanoparticles at an equivalent concentration of 100 μg/mL. e) Hydrodynamic size of melanin nanoparticles and Melanin@RBC nanoparticles measured from DLS. f) Zeta potential of melanin nanoparticles and Melanin@RBC nanoparticles. g) Colloidal stability of Melanin@RBC nanoparticles in water (100 μg/mL) over one week. Data were means ± s.d. N = 3.
Similarly, PDA nanoparticles with varying concentrations (25, 50 and 100 µg/mL) were exposed to 808-nm NIR laser with the same power density and increases in temperature were recorded by an infrared thermal camera (Fig. 2b). Although having similar absorbance at 808 nm (Fig. S3, Supplementary Data), melanin nanoparticles exhibited significantly higher temperature increment (ΔT = 37.2 °C), than PDA nanoparticles (ΔT = 24.5 °C) at equivalent concentration (Fig. 2c), suggesting that natural melanin nanoparticles have better photothermal performance over melanin-like PDA nanoparticles. Furthermore, the photothermal conversion efficiency (η) of melanin nanoparticles was calculated according to formula described by Roper et al. [42] (See Methods in Supplementary Data) (Fig. 2d). The η value of melanin nanoparticle was calculated to be 40%, remarkably higher than that of synthesized PDA nanoparticles (29%), as well as reported common photothermal agents [3,5,6,43] (Table S2, Supplementary Data). Meanwhile, the photostability of melanin nanoparticles was tested by successive exposure to NIR.

![Graphs](image-url)
laser (808 nm, 2 W/cm²). Negligible changes were observed after irradiation for 60 min at 808 nm (Fig. S4, Supplementary Data), indicating good photostability of melanin nanoparticles. Therefore, the superior photothermal performance of melanin nanoparticles might owe to the superior photothermal conversion ability and good photostability, which can rapidly convert more optical energy into heat during the same irradiation time. In addition, the biodegradability of melanin nanoparticles was evaluated in the presence of hydrogen peroxide (H₂O₂), which was widely distributed in macrophages and major organs [44]. The absorption at 808 nm of melanin nanoparticles declined along with increasing concentration of H₂O₂ (Fig. 2e), and the half maximum degradation concentration (DC₅₀) value was determined according to the sigmoidal relationship (Y = 192.42/(1 + exp [(X+8.14)/1.32]), R² = 0.9992) between the absorbance at 808 nm of melanin nanoparticles (Y value) and H₂O₂ concentration (X value) (Fig. 2f). The DC₅₀ value of H₂O₂ was calculated to be 0.86 mM, which was much lower than that of synthetic melanin-like PDA nanoparticles (4.55 mM) (Fig. 2g and h), indicating the good biodegradability of melanin nanoparticles. Moreover, TEM characterization of melanin nanoparticles after being treated with H₂O₂ demonstrated that the size of melanin nanoparticles decreased with increasing concentration of H₂O₂ (Fig. S5, Supplementary Data). In addition, the DLS results also revealed that after treatment with increasing concentration of H₂O₂ for 24 h, melanin nanoparticles has smaller size compared with pristine melanin nanoparticles (Table S3, Supplementary Data). Taken together, these results indicated that the natural melanin nanoparticles with excellent photothermal property as well as favorable biodegradability hold great potentials for cancer PTT treatment.

3.3. Cytotoxicity and in vitro PTT efficacy of melanin nanoparticles

As a new class of photothermal agents extracted from living organisms, the obtained melanin nanoparticles would be biocompatible with less toxicity. To verify our hypothesis, A549 cancerous cells and HEK-293T normal cells were used to test the cytotoxicity of melanin nanoparticles with standard CCK-8 assay. Even if melanin nanoparticle concentration was as high concentration as 1 mg/mL, the cell viabilities of both A549 cells and HEK-293T cells still remained above 90% after incubation for 48 h (Fig. 3a), significantly higher than that of synthesized PDA nanoparticles at equivalent concentration (Fig. S6, Supplementary Data). These results indicated the low cytotoxicity of melanin nanoparticles that would be applicable for in vitro PTT testing. Then in vitro PTT effect of melanin nanoparticles on A549 cells was evaluated by using CCK-8 assay, together with live/dead cell staining. As shown in Fig. 3b, melanin-treated cells retained high viability in the absence of laser. However, after irradiation for 5 min, melanin-treated cells showed concentration-dependent reduced viability. For instance, at nanoparticle concentration of 50 µg/mL and 100 µg/mL, cell viability decreased over 65% and 81% in Melanin + Laser group, respectively, while it decreased only 2% and 6% in Melanin group without laser irradiation, respectively. Moreover, melanin nanoparticles exhibited lower cell viability than synthesized PDA nanoparticles at equivalent concentration (for instance, at concentration of 50 µg/mL and 100 µg/mL, cell viability decreased 50% and 70% in PDA + Laser group, respectively, Fig. S7, Supplementary Data), indicating the excellent photothermal ablation ability of natural melanin nanoparticles. As a contrast, negligible decrease in cell viability was observed for Control group without any treatment as well as Laser group without melanin treatment. The photothermal ablation ability was further confirmed by using CLSM imaging with Calcein-AM and PI staining. Calcein-AM is a cell-permeable dye that labels living cells with green fluorescence, whereas PI is a cell-impermeable dye that only labels dead cells with red fluorescence [45]. As shown in Fig. 3c, cells in Melanin + Laser group exhibited intense red fluorescence, indicative of dead cells, while cells in Melanin group showed bright green fluorescence, indicative of living cells. By contrast, minimal cell death was observed in Control group as well as Laser group. In addition, quantitative analysis of cell apoptosis at each treatment group was achieved by using flow cytometry (Fig. 3d). The apoptosis rate of cells reached as high as 94.69% in Melanin + Laser group, indicating near-total cell damage was induced by melanin nanoparticles via photothermal effect. Taken together, the in vitro cell studies consistently demonstrated that melanin nanoparticles were capable of efficiently ablating cancerous cells via photothermal effect and were little toxic without laser irradiation.

3.4. Preparation of RBC membrane-coated melanin (Melanin@RBC) nanoparticles

The RBC membrane-coated melanin (Melanin@RBC) nanoparticles were prepared by coating RBC membrane vesicles derived from natural RBCs onto the melanin nanoparticle surface through an extrusion approach reported previously [31], as illustrated in Fig. 4a. Firstly, RBCs were extracted from fresh blood and subjected
to hypotonic treatment and extrusion to yield RBC membrane vesicles with diameter of ~200 nm (Fig. 5S, Supplementary Data). Secondly, RBC membrane vesicles and melanin nanoparticles at the surface area ratio (S\text{RBC}/S\text{melanin}) of 1:1 were mixed and extruded repeatedly through 200-nm polycarbonate porous membranes. The extrusion facilitated the fusion of RBC membrane onto nanoparticle surface effectively, resulting in Melanin@RBC nanoparticles. As shown in Fig. 4b and c, the Melanin@RBC nanoparticles had an average diameter of 144 ± 18 nm while the average diameter of uncoated melanin nanoparticles was 128 ± 22 nm. The slightly increased diameter of ~16 nm was well correlated to the thickness of two layers of RBC membrane phospholipid bilayers (~7.8 nm) reported previously [46]. The TEM images of nanoparticles negatively stained with uranyl acetate (Fig. 4b inset, c inset, and Fig. S9, Supplementary Data) displayed that Melanin@RBC nanoparticles had a core-shell structure with the outer shell thickness of approximately 8 nm compared with pristine melanin nanoparticles, which verified the successful coverage of RBC membrane on melanin nanoparticles. UV–vis spectra showed that the RBC membrane coating process barely altered the absorption spectrum at NIR region of uncoated melanin nanoparticles but induced a new absorbance peak at 420 nm (Fig. 4d), which corresponded well to the plasmonic absorption band of RBC membrane, suggesting the successful RBC membrane coating process. Moreover, after RBC membrane coating, the hydrodynamic size of melanin nanoparticles measured from DLS was increased from 216 nm (PDI = 0.097) to 241 nm (PDI = 0.157) (Fig. 4e and Fig. S10, Supplementary Data), which was in consistency with TEM results. The zeta potential of melanin nanoparticles changed from −17.6 ± 0.5 mV to −8.4 ± 1.3 mV (Fig. 4f) after RBC membrane coating, probably owing to the charge shielding effect caused by RBC membrane of which the zeta potential was less negative (−6.2 ± 1.0 mV, Table S4, Supplementary Data). Furthermore, SDS-PAGE analysis demonstrated that Melanin@RBC nanoparticles had a similar protein profile to natural RBC membrane vesicles, suggesting that the majority of membrane proteins were preserved on Melanin@RBC nanoparticles (Fig. S11a, Supplementary Data). Further western blot analysis demonstrated the presence of CD47 protein on the Melanin@RBC nanoparticles (Fig. S11b, Supplementary Data), which validated the successful RBC membrane coating on melanin nanoparticles [28]. Taken together, these results indicated that the RBC membrane had been successfully coated onto the exterior of melanin nanoparticles without significant alteration of their polydispersity and optical property. In addition, the resulting Melanin@RBC nanoparticles also exhibited good colloidal stability in water (Fig. 4g) and in 10% fetal bovine serum (FBS) (Fig. S12, Supplementary Data), suggesting that the RBC membrane coating effectively protected melanin nanoparticles from aggregation.

3.5. The photothermal property of Melanin@RBC nanoparticles

Because the absorption at 808 nm of melanin nanoparticles showed scarce change after RBC membrane coating, the as-prepared Melanin@RBC nanoparticles was expected to retain the excellent photothermal property of melanin nanoparticles. To assess it, Melanin@RBC suspension was exposed to the 808-nm consecutive NIR laser with the power density of 2 W/cm² and pure water was used as control. As expected, at the concentration of 100 µg/mL, Melanin@RBC nanoparticles exhibited prominent temperature elevation from room temperature to about 60.1 °C while the temperature of melanin nanoparticle suspension was increased to 61.6 °C after irradiation for 500 s (Fig. 5a), suggesting that RBC membrane coating has little impact on the photothermal effect of melanin nanoparticles. The photothermal conversion efficiency of Melanin@RBC nanoparticles was determined to be 40% (Fig. 5b), the same as that of uncoated melanin nanoparticles, indicating that Melanin@RBC nanoparticles retain photothermal conversion capability of melanin nanoparticles. Moreover, the photostability of Melanin@RBC nanoparticles was examined by repeated exposure to NIR laser (808 nm, 2 W/cm²) for five cycles. Little loss of thermal fatigue resistance was observed over five cycles of exposure to NIR laser (Fig. 5c). Meanwhile, negligible changes in absorption spectrum were observed after five cycles of irradiation at 808 nm (Fig. 5d), demonstrating the good photostability of Melanin@RBC nanoparticles. These results indicated that Melanin@RBC nanoparticles with good photothermal efficiency and photostability could be used for photothermal ablation of cancer cells.

Furthermore, in vitro PTT effect of Melanin@RBC nanoparticles was tested by CCK-8 assay. As shown in Fig. 5e, Melanin@RBC nanoparticles exhibited negligible cytotoxicity at different concentrations in the absence of laser irradiation. However, after irradiation for 5 min, cell viability decreased over 63% in Melanin@RBC + Laser group at the concentration of 50 µg/mL while decreased over 80% at the concentration of 100 µg/mL, indicating the excellent photothermal ablation ability of Melanin@RBC nanoparticles. In addition, the UV–vis spectra show that the absorbance of Melanin@RBC nanoparticles at 808 nm rapidly declined after reacting with increasing concentration of H₂O₂ from 0 mM to 5 mM (Fig. 5f), and the DC₅₀ value of H₂O₂ were determined to be 1.09 mM, indicating the good biodegradability of Melanin@RBC nanoparticles as bare melanin nanoparticles. Therefore, the excellent photothermal performance, good photostability and favorable biodegradability of Melanin@RBC make it a promising platform for in vivo photothermal therapy.

3.6. Pharmacokinetics and biodistribution

To assess whether the Melanin@RBC nanoparticles inherited the long blood circulation capability from natural RBC membranes, pharmacokinetics and biodistribution studies were performed by labeling melanin nanoparticles with a near infrared fluorescence dye cyanine5 (Cy5, ex/em, 640 nm/665 nm). Cy5 dye could effectively diminish the background signal from the blood or tissues and label the nanoparticles with a near infrared excitation measurement. As shown in Fig. 6a, at 24 h post intravenous injection, the blood retention of melanin nanoparticles was decreased to 1.09 ± 1.35% ID/mL in contrast, about 11.16 ± 2.82% ID/mL of Melanin@RBC nanoparticles were still in blood circulation.

Fig. 7. a) Plots of in vitro PA signal versus various concentrations of melanin and Melanin@RBC nanoparticles. Inset is the PA images of Melanin@RBC nanoparticles with various concentrations of 0, 5, 12.5, 25, 50, 100 and 200 µg/mL. Unmixed image profiles (top row – ultrasound (UA) images; middle row – PA images; bottom row – merged images of UA and PA) of tumor region before and after intra-tumoral injection of 100 µL of Melanin@RBC (b) and melanin (c) nanoparticles (1 mg/mL) in A549 tumor-bearing mice, respectively. Tumor region was enveloped by black dotted lines. d) Quantitative analysis of in vivo PA signal at tumor region before (Pre) and after intravenous injection of 100 µL of Melanin@RBC and melanin nanoparticles (1 mg/mL) in A549 tumor-bearing mice. The PA intensity from Pre image was defined as 0. *p < 0.05, **p < 0.01, compared with melanin nanoparticles. Data were means ± s.d. N = 3. e) Representative thermal images of A549 tumor-bearing mice exposed to NIR laser irradiation (808 nm, 2 W/cm², 5 min) 4 h post nanoparticle injection. Tumor-bearing mice without any treatment were used as a negative control. Tumor region was enveloped by white dotted lines. f) Tumor temperature elevation curve as a function of irradiation time.
The significant increase of blood retention indicated that RBC membrane-camouflaged melanin nanoparticles exhibited superior blood circulation compared with bare melanin nanoparticles [35], which further verified RBC membrane had been successfully translated onto the surface of melanin nanoparticles.

Furthermore, the in vivo biodistribution of nanoparticles was investigated by collecting the major organs and tumors for fluorescence analysis. As shown in Fig. 6b and c, Melanin@RBC nanoparticles showed significantly enhanced tumor accumulation compared with melanin nanoparticles. For instance, at 4 h and 24 h post injection, the accumulation of Melanin@RBC nanoparticles in tumors were calculated as $8.11 \pm 1.93$ and $3.64 \pm 1.79$ ID/g, while the accumulation of melanin nanoparticles in tumor were $5.64 \pm 1.28$ and $2.51 \pm 1.49$ ID/g, respectively. The Melanin@RBC nanoparticles exhibited 1.44-fold (e.g. 4 h post injection) enhancement in nanoparticle accumulation in tumors compared with melanin nanoparticles, which might benefit from the longer blood circulation of Melanin@RBC nanoparticles than melanin nanoparticles. In addition, accumulation of both Melanin@RBC and melanin nanoparticles could be found in major organs. It should be noted that, Melanin@RBC nanoparticles exhibited significantly lower accumulation in livers than melanin nanoparticles. As liver is an important part of primary reticuloendothelial system (RES) to eliminate exogenous invader [47], these results further validated that RBC membrane coating could enable melanin nanoparticles to effectively escape RES recognition, improve circulation time. Further biodistribution of Melanin@RBC nanoparticles in tumor-bearing mice at different time points showed that Melanin@RBC nanoparticles were mainly eliminated by the liver and the spleen (Fig. S13, Supplementary Data). It agreed with previous reports that the liver could rapidly uptake, degrade, and eliminate nanomaterials [47], and the spleen was a major lymphoid organ which removed and metabolized old red blood cells rapidly [48].

3.7. In vivo photoacoustic imaging and tumor accumulation of Melanin@RBC nanoparticles

As nanomedicine used for in vivo PTT therapy, the “real” therapeutic efficacy toward tumors was not just determined by the photothermal efficiency of nanomaterials, but greatly depended on the effective accumulation of photothermal nanomaterials at target sites through “enhanced permeability and retention (EPR)” effect [49]. Therefore, tumor accumulation of Melanin@RBC nanoparticles were investigated using photoacoustic (PA) imaging technique, which provided preferable spatial resolution compared to conventional optical imaging methods, and thus facilitated clarifying local nanoparticle distribution at tumor region [43]. As shown in Fig. 7a, the PA signal exhibited a linearity enhancement with increasing concentration of Melanin@RBC nanoparticles ($R^2 = 0.9912$) in vitro and similar phenomena were observed on melanin nanoparticles, which further verified the excellent PA property of melanin nanoparticles [37]. Taken into account comparable PA imaging property between Melanin@RBC and melanin nanoparticles, Melanin@RBC and melanin nanoparticle suspension (1 mg/mL, 100 μL) were intravenously injected into A549 tumor-bearing mice and PA signals at tumor area were recorded at different time points before (Pre) and after (1, 2, 4, 6 and 24 h) intravenous administration, respectively. After that, PA signals obtained were processed with the unmixing spectra module of system to exclude signals from hemoglobin and oxygenated hemoglobin in vivo. As shown in Fig. 7b and c, gradually increased PA signals at tumor area were observed within 4 h in both Melanin@RBC and Melanin group and PA signal peaked at 4 h post injection, indicating maximum nanoparticle accumulation in tumors was achieved around 4 h post injection. Quantification analysis of PA signal of Melanin@RBC and melanin nanoparticles at the tumor region was performed by calculating the average PA intensity of ROIs at tumor area. For instance, at 2 h and 4 h post injection, the average PA intensity at tumor area of Melanin@RBC group was calculated as $0.19 \pm 0.028$ and $0.54 \pm 0.035$, while the average PA intensity of Melanin group was $0.12 \pm 0.024$ and $0.40 \pm 0.042$, respectively (Fig. 7d). The Melanin@RBC exhibited 1.35-fold enrichment (e.g. 4 h post injection) at tumor region compared with melanin nanoparticles, suggesting that RBC membrane endowed melanin nanoparticles with “invisibility” to the host immune system and improved tumor nanoparticle accumulation as reported previously [31,34–36]. However, taking a broader look, we found that the average value of PA intensity of Melanin@RBC group had dropped significantly after 24 h post intravenous administration, which was probably due to the partial degradation of Melanin@RBC at tumor sites, where excessive amount of H2O2 was produced for cancer cells proliferation [50]. The Melanin@RBC nanoparticles accumulated in tumors via EPR effect might be partly decomposed by H2O2 into harmless byproducts after 24 post-injection. Altogether, the in vivo PA imaging studies well demonstrated that Melanin@RBC nanoparticles were capable of PA imaging and 4 h after nanoparticles injection shall be considered as a priority option for tumor PTT therapy.

Encouraged by the outstanding in vitro photothermal performance of Melanin@RBC nanoparticles, the in vivo antitumor PTT efficacy was further evaluated in A549 tumor-bearing mice. First, the photothermal effect of Melanin@RBC nanoparticles in tumor-bearing mice (tumor volume of ~100 mm$^3$) was investigated. After an intravenous injection 100 μL of Melanin@RBC nanoparticle suspension (2 mg/mL) tumors were completely exposed to 808 nm laser (2 W/cm$^2$) 4 h post injection. As shown in Fig. 7e, upon irradiation for 5 min, the tumor temperature of mice in Melanin@RBC group rapidly increased from 30.9 °C to 57.2 °C ($\Delta T = 26.3^\circ$ C) while the tumor temperature in Melanin group increased from 30.5 °C to 47.7 °C ($\Delta T = 17.2^\circ$ C) (Fig. 7f). As a control, the tumor temperature of mice without any nanoparticle treatment was found to increase no more than 8 °C after the same irradiation. These results together with tumor accumulation studies strongly indicated that the Melanin@RBC had achieved enhanced tumor accumulation and improved in vivo photothermal effect by the RBC membrane coating strategy.

3.8. In vivo antitumor PTT efficacy

A549 tumor-bearing mice with tumor volume of ~100 mm$^3$ were randomly divided into four groups (N = 5) for in vivo PTT. As shown in Fig. 8a, laser irradiation displayed no significant antitumor efficacy compared with Control group, while tumors were almost completely eliminated within 13 days in Melanin@RBC +2 W/cm$^2$ Laser group with no recrudescence. Tumors in Melanin +2 W/cm$^2$ Laser group was found re-growing aftertreatment, suggesting less favorable tumor ablation effect of melanin nanoparticles alone, which might own to their relatively lower tumor accumulation. The digital photographs of tumors on the 13th day after treatment showed that tumor size in Melanin@RBC +2 W/cm$^2$ Laser group was much smaller than other groups (Fig. 8c). For melanin nanoparticles, the antitumor rate calculated from tumor weight was ~78%. When coating with RBC membrane, the antitumor rate of Melanin@RBC nanoparticles was increased to almost 100%. To further confirm the effective ablation of tumor, mice were dissected on the 13th day after treatment, and their tumors and major organs were subjected histological analysis. Apparent tumor tissue lesions were observed in Melanin +2 W/cm$^2$ Laser and Melanin@RBC +2 W/cm$^2$ Laser group (Fig. 8d), and unnoticeable abnormality were found in the heart, liver, spleen, lung
Fig. 8. a) Tumor volume curves of A549 tumor-bearing mice with different treatment (for Laser, Melanin + 2 W/cm² Laser and Melanin@RBC + 2 W/cm² Laser groups, tumors were irradiated by an 808-nm laser (2 W/cm²) for 5 min; for Melanin@RBC + 1 W/cm² Laser group, tumors were irradiated by an 808-nm laser (1 W/cm²) for 10 min). Tumor models without any treatment were used as a negative control. b) Body weight curves of mice of each group. c) Photos of tumors dissected from each group on the 13th day after photothermal treatment and comparison of tumor weight of each group. **p < 0.01, ***p < 0.001 and n.s. represents no significance. Data were means ± s.d. N = 5. d) H&E staining images of major organs (heart, liver, spleen, lung, and kidney) and tumor tissues dissected from each group on the 13th day after photothermal treatment. Tumor-bearing mice without any treatment were used as a negative control. Scale bars = 50 μm.
and kidney region of treatment groups compared to those of Control group. Moreover, even when tumors were irradiated with the laser at the power density of 1 W/cm² for 10 min (Fig. 8a, b, c, d), similar tumor ablation capability was observed on Melanin@RBC nanoparticles-treated mice compared with those irradiated at the power density of 2 W/cm² for 5 min, suggesting the extraordinary photothermal effect of Melanin@RBC nanoparticles could be realized at a lower power density. To this end, these results strongly indicated that Melanin@RBC performed efficiently in ablating cancerous cells with little side effect.

Last but not least, the in vivo biosafety of both the melanin and Melanin@RBC nanoparticles was evaluated by blood biochemistry and hematology test. Hematology analysis revealed no significant differences in the blood parameters between nanoparticle-treated groups and control group at 24 h (Table S6, Supplementary Data) and 48 h (Table S7, Supplementary Data) post injection, respectively. Additionally, compared with control group, both the Melanin@RBC and melanin nanoparticles, with whether a single-dose or a multiple-dose, did not result in obvious increase in hepatic and renal indicator levels at 24 h or 48 h post injection (Fig. S14, Supplementary Data), suggesting that the nanoparticle administration did not induce liver or kidney injury. As reported that most nanoparticles formulations were probably cleared by liver and concentration did not induce liver or kidney injury. As reported that most nanoparticles formulations were probably cleared by liver and kidney [51], it was important that Melanin@RBC nanoparticles could not induce any acute hepatic or renal damage. These results were well consistent with histological analysis with H&E staining. Besides, no significant differences were observed in mouse body weight between groups (Fig. 8b), further verifying that the safety and low toxicity of Melanin@RBC nanoparticles. These results suggested that Melanin@RBC nanoparticles could serve as effective photothermal agents for in vivo cancer PTT with great translational potential in clinics.

4. Conclusion

In summary, a novel PTT platform based on RBC membrane-camouflaged melanin nanoparticles (Melanin@RBC) was successfully developed for in vivo cancer PTT. The natural melanin nanoparticles extracted from living cuttlefish displayed native biocompatibility and biodegradability. After being coated with RBC membrane, the resultant Melanin@RBC nanoparticles effectively enhanced in vivo blood retention and tumor accumulation via EPR effect. With the inherited photoacoustic imaging property and excellent photothermal conversion capability, Melanin@RBC nanoparticles exhibited superior antitumor PTT efficacy in A549 tumor-bearing mice compared to melanin nanoparticles. Altogether, our results highlighted Melanin@RBC nanoparticles as a biocompatible and effective theranostic platform for in vivo cancer PTT.

Notes

The authors declare no competing financial interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biomaterials.2017.07.027.

References
