Targeted polydopamine nanoparticles enable photoacoustic imaging guided chemo- photothermal synergistic therapy of tumor

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Abstract

Near infrared light responsive nanoparticles can transfer the absorbed NIR optical energy into heat, offering a desirable platform for photoacoustic (PA) imaging guided photothermal therapy (PTT) of tumor. However, a key issue in exploiting this platform is to achieve optimal combination of PA imaging and PTT therapy in single nanoparticle. Here, we demonstrate that the biodegradable polydopamine nanoparticles (PDAs) are excellent PA imaging agent and highly efficient for PTT therapy, thus enabling the optimal combination of PA imaging and PTT therapy in single nanoparticle. Upon modification with arginine-glycine-aspartic-cysteine acid (RGDC) peptide, PDA-RGDC can successfully target tumor site. Moreover, PDA-RGDC can load a chemotherapy drug, doxorubicin (DOX), whose release can be triggered by near-infrared (NIR) light and pH dual-stimuli. The *in vitro* and *in vivo* experiments show that this platform can deliver anti-cancer drugs to target cells, release them intracellular upon NIR irradiation, and effectively eliminate tumors through chemo-photothermal synergistic therapeutic effect. Our results offer a way to harness PDA-based theranostic agents to achieve PA imaging-guided cancer therapy.

1. Introduction

Theranostic nanoparticles (TNPs), defined by the combination of diagnostic and therapeutic agents on a single platform, are emerging as a promising therapeutic paradigm in cancer treatment [1-3]. The resulting nanoplatform enables precise diagnosis of disease, individualized selection of treatment modality and real-time monitoring of therapeutic outcomes [4-6]. For clinically relevant applications in oncology, TNP with absorption in the near infrared (NIR) (650–900 nm) is more attractive, because the transparency window for biological tissues allows for deeper light penetration, relatively low absorption/scattering and could efficiently transfer the absorbed NIR optical energy into heat [7-9]. It is reported that nanoparticles capable of simultaneous heating, thermal sensing and fluorescence imaging are one of the most promising TNP nanoparticles[10-11], but tissue is a highly scattering medium for luminescence in the optical spectral range, and thus optical imaging modalities suffer from either shallow penetration depth or poor spatial resolution. Fortunately, photoacoustic imaging (PAI) detects much less scattered ultrasonic waves [12]. Photon energy absorbed by nanoparticles is partially or completely converted into heat, which thermoelastically induces ultrasonic waves. The induced ultrasonic waves are detected by ultrasonic detectors to form 2D or 3D images of the targeted areas [13-15]. Therefore, PAI solves the resolution drawback of pure optical imaging and the contrast shortcoming of pure ultrasonic imaging, demonstrating many superiorities in terms of low signal scattering in tissues, high resolution and sensitivity, rich optical contrasts and deep imaging depth in the absence of ionizing radiation [16-18]. At the same time, the heat generated by the penetrating NIR radiation can not only be used as the basis of PAI, but also be envisaged to kill the targeted cancer cells, which is

the principle for photothermal therapy (PTT). Therefore, it is natural to combine PAI with PTT in a single nanoparticle [19-20]. PTT has been increasingly regarded as a highly selective and minimally invasive approach for cancer treatment [21-23]. Therefore, NIR-light adsorbed nanoparticles combining the advantage of PAI and PTT (TNP-PAI/PTT) are expected to play a significant role in the dawning era of personalized medicine.

To promote the clinical translation of TNP-PAI/PTT, several requirements to clinical standards have to be met, (i) Good biocompatibility and biodegradability, which is the principal motivation for developing theranostic nanoparticles [24] (ii) Facile and controllable synthesis, by which the theranostic nanoparticles can be large-scaled produced [25]. (iii) Powerful functionalization capacity, making theranostic nanoparticles easy to be modified with some target moieties and beneficial drug delivery. (iv) Last but not the least, high diagnosis and therapy efficiency, which is always pursuit of the clinical application [2]. Up to now, the reported Au-, Ag-, Cu-, Co-, and other metal based, carbon-based TNP-PAI/PTT [26-31] can effectively produce high contrast intensity of photoacoustic imaging and PTT efficiency, but they suffer from complex multicomponent system and poor biocompatibility and biodegradability. Consequently, it has become increasingly urgent to address these existing issues for the clinical translation of TNP-PAI/PTT.

Recently, increasing attention is being drawn towards polydopamine nanoparticles (PDAs) due to their good biocompatibility and strong NIR absorptivity [32-34]. PDAs are structurally similar to naturally occurring melanin [35]. Moreover, they can be produced from dopamine with a simple and effective strategy. At the same time, their surface can

be easily modified with thiol- and amino-terminated molecules through Michael addition or Schiff base reaction, and thus facilitates the loading of conjugates with other interesting motifs. Importantly, PDAs can load drugs through π - π stacking to realize synergistic therapeutic [36]. It is evident that PDAs can meet the above demands for clinical use of TNP-PAI/PTT. However, the reported PDAs' applications have been restricted as light-absorbing agents for either PAI or PTT so far [37-42]. Thereof, it is very interesting to endow the cost-effective polydopamine nanoparticles alone with multi-function including PAI and synergistic therapeutic, which may lead to the breakthrough in the field of theranostic.

With this in mind, here smart PDAs were designed as both PA imaging contrast agent and a chemo-thermotherapy therapy agent for tumor. To endow PDAs with targeting functionality, arginine-glycine-aspartic-cysteine acid (RGDC) peptide that has high affinity with the $\alpha_v\beta_3$ integrin over-expressing in tumor [43] was grafted onto the surface of PDAs to form composite nanoparticles (PDA-RGDC). Experimental results show that PDA-RGDC has a better target-to-background ratios PA imaging than non-modified PDAs *in vivo*. Further, chemotherapeutic molecules, doxorubicin (DOX), were effectively loaded through π - π stacking and hydrogen bonding interactions with PDAs [39]. The PDA-RGDC/DOX demonstrated strong NIR absorption and high photothermal conversion efficiency. The release of DOX could be selectively stimulated by NIR-light, enabling intracellular drug accumulation and thereby enhancing the efficiency to kill cancer cell. This finding may help the development of PDA-based nanoplatform for PA imaging-directed synergistic therapy of tumor in clinic.

2. Experimental

2.1 Materials

Dopamine hydrochloride was purchased from Alfa Aesar. Ammonia solution (28.0-30.0% NH_3 by weight), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), 4',6-diamidine-2-phenylindole dihydrochloride (DAPI) and calcein-AM were obtained from Sigma-Aldrich. Maleimide-PEG- NH_2 (MAL-PEG- NH_2) was purchased from Yare Chemical Technology Co., Ltd., (China). RGDC was purchased from Shanghai Biotech Bioscience&Technology Co., Ltd., (China). Doxorubicin hydrochloride (DOX) was purchased from Sangon (Shanghai, China). 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) and N-Hydroxysuccinimide (NHS) were obtained from shanghai kayon biological technol and GL biochem (Shanghai) Ltd, respectively. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco (New York, USA). All chemicals were analytical grade and used as received without further purification. Water used in the experiment was purified by a Millipore system.

2.2 Apparatus

Dynamic light scattering (DLS) and Zeta potential measurements were carried out on Zetasizer Nano ZS (Malvern Instruments Ltd., UK). Fourier transform infrared (FTIR) spectroscopic analysis was performed on a Bruker Vertex 70 spectrometer. UV-vis-NIR spectra were recorded on a VARIAN CARY 50 UV-vis-NIR spectrophotometer. Matrix-assisted laser desorption-ionization time-of-flight mass spectroscopy (MALDI-TOF-MS) was performed on a Bruker autoflex III mass spectrometer in linear, positive ion mode.

The fluorescence imaging was performed on a reconstructive Nikon Ti-S fluorescent microscope (Nikon, Tokyo, Japan).

2.3 Preparation of PDA-RGDC

PDA_s were synthesized as the reported method [34]. Ammonia aqueous solution (3 mL, NH₄OH, 28-30%) was mixed with ethanol (40 mL) and deionized water (90 mL) under mild stirring at 30 °C for 30 min. Dopamine hydrochloride (0.5 g) was dissolved in deionized water (10 mL) and then injected into the above mixture solution. After reaction for 24 h, PDA_s were obtained by centrifugation and washed with water for three times.

Synthesis of PEG-RGDC: The thiol-terminated of RGDC was incubated with the NH₂-PEG-MAL overnight, dialyzed for seven more days in water and froze to dry for use.

Synthesis of PDA-RGDC: The PDA and PEG-RGDC were stirring in 10 mM Tris-HCl (pH 8.5) for 12h, and then were centrifuged and washed with water by three times.

2.4 Photothermal conversion experiments

To measure the photothermal conversion performance of the PDA-RGDC (or PDA-RGDC/DOX), solutions of the PDA-RGDC (PDA-RGDC/DOX) (1.0 mL) with different concentrations was placed in a quartz cell and exposed to the 808 nm laser source at a distance of 2 cm (wavelength, 808 nm; power density, 2 W/cm² or 1 W/cm²) for different time periods. The original temperature was maintained at 22 °C. As a control, the temperatures of distilled water (1.0 mL) irradiated by the same laser as described above were measured.

2.5 Drug loading and release

For DOX loading, solutions of PDA-RGDC (1 mg mL^{-1}) were mixed with different concentration of DOX in PBS at pH 7.4. After stirring at room temperature for 24 h, excess DOX was removed by centrifugation and calculated concentration by calibration curve of DOX. The PDA-RGDC/DOX was washed with PBS several times and stored at 4°C in the dark for future use.

To study the drug release, a 3 mL solution of PDA-RGDC/DOX was placed in a centrifuge tube in PBS with different pH values (7.4 or 5.0) under 37°C water bath. 0.5 mL of solution was centrifuged, and the amount of released DOX in the supernatant was determined using UV-vis spectrometry.

At designed time intervals, the samples were irradiated by the 808 nm laser with a power intensity of 2 W/cm^2 for 5 min.

2.6 Cell viability and cell uptake assay

To evaluate the cytotoxicity of the nanoparticles, the MTT assay was used on the HeLa cells. Briefly, HeLa cells were seeded in 96-well plates at a density of 10000 cells per well and cultured with 10% FBS DMEM under a humidified 5% CO_2 at 37°C for 12 h. After that, various concentration of PDA-RGDC was added into the culture wells and the cells were further incubated for 24 h. Subsequently, the cells were washed with PBS three times and treated with $10 \mu\text{L}$ MTT (5 mg mL^{-1} in PBS) at 37°C for 4 h in an incubator at the same condition. Finally, the supernatant was removed carefully, and dimethyl sulfoxide (DMSO) was added ($200 \mu\text{L}$ per well) and shaken for 15 min to thoroughly mix the formazan into the solvent. The optical density at 570 nm was measured by the

microtiter plate reader. The untreated HeLa cells were used as control. The percentage of cell viability was calculated by using the optical densities with respect to the control values.

The MTT assay of different loading capacity PDA-RGDC/DOX was carried out by adding NPs into the culture wells. After 4 h incubation, the cells were washed with PBS, and then irradiated by an 808 nm NIR laser at power densities of 2 W/cm^2 for 5 min. The relative cell viabilities were then measured by the MTT assay.

For cell uptake analysis, the cells were incubated in fresh DMEM medium (2 mL) containing PDA-RGDC/DOX or PDA/DOX (200 μg) for a specific time (i.e., 1, 2, 4, 6, and 10 h), respectively. Finally, the stained cells were washed with PBS (pH 7.4, three times), and directly subjected to fluorescence imaging. Those cells were also analyzed by flow cytometry after trypsinization and washing with PBS for 3 times. The cellular DOX fluorescence was analyzed by flow cytometry (FACS Calibur from Becton, Dickinson Company).

To study the NIR light triggering drug releasing in vitro, HeLa cells were cultured in confocal dish containing PDA-RGDC/DOX for 4 h, washed with PBS for three times, then irradiated by an 808 nm NIR laser at power densities of 2 W/cm^2 for 5 min. Cells were labeled with DAPI and then imaged by a laser scanning confocal fluorescence microscope.

For cellular location study, HeLa cells were cultured in confocal dish containing PDA-RGDC/DOX or PDA/DOX for 4 h, washed with PBS for three times. Cells were labeled with DAPI and then imaged by a laser scanning confocal fluorescence microscope.

For combination therapy, HeLa cells in 96-well plates were incubated with different ratio of DOX and PDA-RGDC, PDA, PDA-RGDC/DOX for 4 h, washed with PBS, and then irradiated by an 808 nm NIR laser at different power densities and duration time. After additional incubation for 24 h, the relative cell viabilities were then measured by the MTT assay and stained with calcein AM (green fluorescence from calcein AM indicates live cells). Then, the images were captured using a laser scanning confocal fluorescence microscope.

2.7 The biocompatibility of PDA-RGDC *in vivo*

The biocompatibility of PDA-RGDC *in vivo* was administrated by intravenously injected the PDA-RGDC NP (20 mg kg^{-1}) into the mice with average weight of 20 g. The body weight was recorded for one month, and then the mice were sacrificed. As for blood analysis, the blood was collected by extracting the eyeball blood for blood biochemistry assay and hematology analysis. For histology studies, the main organs (heart, liver, spleen, lung, and kidney) were collected from two groups (control and test groups), which were stained with hematoxylin and eosin (H&E) following the standard protocol for histopathological analysis. All sections were examined under a Leica microscope.

2.8 *In vivo* imaging

For IR thermal imaging, mice injected with various materials were anesthetized and imaged under an IR thermal camera (Flir C2) with or without irradiation by the 808 nm laser at power densities of 2 W/cm^2 for 5 min.

For PA imaging, an MSOT inVision 128 small animal imaging system (iThera Medical GmbH, Munich, Germany) was used [44]. Briefly, a tunable optical parametric oscillator (OPO) pumped by an Nd:YAG laser provides excitation pulses with a duration of 8 ns at wavelengths from 680 nm to 980 nm at a repetition rate of 10 Hz with a wavelength tuning speed of 10 ms and a peak pulse energy of 100 mJ at 730 nm. For ultrasound detection, 128 cylindrically focused ultrasound transducers with a center frequency of 5 MHz (60% bandwidth), organized in a concave array of 270 degree angular coverage and a radius of curvature of 4 cm, are used. Laser exposure was adapted to 6 mJ/cm^2 for all the experiments. Regions of interests (ROIs) were selected and the PA signal was analyzed using ViewMOST^{TM} software.

For *in vitro* PA imaging, agar gel cylinders with a diameter of 3 mm containing PDA-RGDC at varied concentration were fabricated and PA signals of these cylinders were obtained at three different positions near the middle of the cylinder at 808 nm, and their intensities were calibrated and quantitatively analyzed based on the reconstructed images.

For *in vivo* PA imaging, the Balb/c nude mice bearing tumor were injected intravenously by tail vein with PDA and PDA-RGDC (20 mg kg^{-1}) respectively. Mice were anaesthetized with 2% isoflurane throughout the experiments, and placed in a horizontal position in a holder surrounded by a thin polyethylene membrane to prevent direct contact with water and allowed acoustic coupling between mouse and transducer array. The light fibers and ultrasonic transducer array were in a fixed position for all data acquisitions, whereas the mouse can be translated through the imaging plane using a linear stage. A series of sequential PA images at specific time (0, 4, 8, 24, 48, and 72 h)

were acquired to cover the subcutaneous tumor regions. Regions of interests (ROIs) were selected and the PA signal was analyzed using ViewMOST^{TM} software.

2.9 Combination therapy *in vivo*

Balb/c nude mice (~20 g) were injected subcutaneously with the cell suspension containing 10⁶ HeLa cells. When the size of tumors reached to 3-4 mm in diameter, the mice were randomly divided into six groups (6 mice per group), minimizing weight and tumor size differences. The mice were injected intravenously by tail vein with 0.1 mL of PBS (control group), DOX, PDA, PDA-RGDC, PDA-RGDC/DOX (2 mg mL⁻¹) respectively. After designed times, the tumors were treated with or without NIR light (2 W/cm², 808 nm) for 5 min. Tumor sizes were monitored every 2 days for 2 weeks. The length and width of the tumors were measured by a digital caliper. The tumor volume was calculated according to the following formula: width²* length/2. At day 14, three mice were sacrificed and tumors were excised for H&E staining and the remains were observed for 50 days.

3. Results and discussion

3.1 Synthesis and characteristic of PDA-RGDC

At present work, PDAs were readily synthesized through the oxidation and self-polymerization of dopamine according to our previously reported method [34]. Typical TEM image revealed that the resultant PDAs were spherical in shape with an average

diameter of 120 nm, and had good dispersity in water (Figure. 1D). On account of the importance of integrin $\alpha_v\beta_3$ to tumor angiogenesis and metastasis, we conjugated PDAs to RGDC peptide, a potent integrin $\alpha_v\beta_3$ antagonist, aiming at *in vivo* targeting of integrin $\alpha_v\beta_3$ -positive tumors in mice via RGD-integrin $\alpha_v\beta_3$ binding [43,45,46]. RGDC was introduced to the termini of the PEG chains to form PEG-RGDC (Figure. 1B, Figure. S2A), which was then bound onto the surface of PDA via Schiff/Michael reaction through the amine and catechol/quinine (Figure. 1A, 1C, Figure. S1). The bands at 2868 cm^{-1} and 2941 cm^{-1} were assigned to the asymmetric and symmetric stretching vibrations of $-\text{CH}_2$ in the alkyl chain respectively. Therefore, the FTIR results confirmed that PEG-RGDC has been successfully grafted onto the surface of PDA. Because of the PEG-RGDC coating, the average hydrodynamic diameter of PEG-functionalized PDA became larger (Figure 1F). It was noted that there was little change in the morphology of PDA before and after PEG-RGDC modification (Figure. 1E). Furthermore, the as-prepared PDA-RGDC nanoparticles dispersed well in NaCl, PBS solution, serum or DMEM cell culture medium and remained stable for one week without any detectable agglomeration (Figure. S3, S4). In addition, the as-prepared PDA-RGDC exhibited broad absorbance in the NIR region (Figure. 1G), and the molar extinction coefficient ϵ_{808} was calculated to be $9.8 \times 10^8 \text{ M}^{-1} \text{ cm}^{-1}$, which was higher relative to many PTT agents reported elsewhere (Supporting Information Table S1). To confirm the photothermal effect, temperature changes of the PDA-RGDC dispersion in PBS were measured upon irradiation using an 808 nm laser. As expected, when irradiated by an 808 nm NIR laser (1 W/cm^2 or 2 W/cm^2), the PDA-RGDC solution exhibited an obvious power density and concentration-dependent heating effect (Figure. 1H, Figure. S2B). The photothermal conversion

efficiency (η) was also calculated to be 39.3 % (Figure. S5) according to the thermal balance as described in previous reports [34], which proves PDA-RGDC nanoparticles to be a promising PTT agent.

3.2 *In vitro* DOX loading and release

It was noticed that PDA with the surface of sp^2 -bonded carbon nanostructures had binding capability to the drugs with aromatic structures through hydrophobic interaction and π - π stacking [39, 41]. To demonstrate the drug delivery ability of the PDA-RGDC, DOX was loaded as a guest molecule by soaking PDA-RGDC with DOX in PBS (pH 7.4) overnight. The unbound drug was removed by centrifugation and repeated rinsing. DOX loading on the surface of PDA-RGDC was evidenced by eye due to the reddish color of the PDA-RGDC/DOX suspension. Furthermore, a peak of DOX appears at around 488 nm in the UV-vis spectrum of the PDA-RGDC/DOX suspension (Figure. 2A). All these results demonstrate that DOX molecules have been successfully loaded onto PDA-RGDC nanoparticles. Also, the loading efficiency of DOX on PDA-RGDC was calculated by measuring the concentration of unbound drug according to the characteristic absorption of free DOX in the UV-vis spectra. The DOX loading capacity and the zeta potential of PDA-RGDC/DOX increased with increasing amounts of added DOX (Figure. 3B, Figure. S6). Meanwhile, the strong NIR absorbance and photothermal effect of PDA-RGDC/DOX was not affected after combination with DOX (Figure. 2A, Figure. S7).

To test the drug release profile of PDA-RGDC/DOX, diverse stimuli including pH and NIR light was tested. As shown in Figure 2C, the release amount of DOX at pH 7.0 was

below 5% over 48 h, indicating the high stability of PDA-RGDC/DOX in biological media. Upon exposure of PDA-RGDC/DOX to lower pH environment, a burst release of DOX molecules was noticed, and the amount of cumulative released DOX was NIR-light irradiation enhancement (Figure. 2C, 2D). The increased drug release upon NIR-light irradiation could be attributed to the increased temperature due to the effective photothermal heating property of PDA-RGDC, which may result in the destruction of intermolecular forces and hydrogen bonding. Therefore, the DOX release was reinforced. We investigated the stability or degradation vs temperature of PDA, PDA-RGDC and PDA-RGDC/DOX through TGA. Weight loss of about 6% (PDA-RGDC/DOX&PDA or PDA-RGDC) was ascribed to the DOX absorption on the surface of PDAs (Figure. S8). Consequently, PDA-RGDC/DOX could release the DOX molecule under exogenous NIR irradiation, and the endogenous lower pH in endolysosome, which will enhance antitumor efficacy and minimize the side effect of the drug [47].

3.3 *In vitro* experiments

As discussed above, PDA-RGDC nanoparticle is a synergistic platform for drug carrier and photothermal therapy. To prove the PDA-RGDC capable for bio-medical application, MTT assay *in vitro* was performed to determine the biocompatibility of PDA-RGDC. As shown in Figure 2E, with the concentration of PDA-RGDC increasing, all of the cells retained over 95% viability, demonstrating that PDA-RGDC possessed low cell cytotoxicity and good biocompatibility. DOX loaded PDA-RGDC, on the other hand, was able to kill cancer cells by a loading capacity dependent manner (Figure. 2F, Figure.

S9). Herein, we chose the 1:1 loaded PDA-RGDC/DOX as the model to evaluate the chemo-phototherapy efficacy.

First, to verify the specific interaction between the RGD moieties and integrin $\alpha_v\beta_3$ -positive HeLa cells [45], we compared the internalization process as well as intracellular release behavior of PDA-RGDC/DOX with PDA/DOX. HeLa cells were incubated with PDA-RGDC/DOX and PDA/DOX for 1, 2, 4, 6, and 10 h, respectively. The red fluorescence of DOX inside cells increased dramatically in the two kinds of nanoparticles over time (Figure. 3A). Furthermore, the DOX fluorescence from the HeLa cell measured by flow cytometry confirmed the greater cellular uptake of PDA-RGDC/DOX than that of PDA/DOX (Figure. S10). This indicated that PDA-RGDC was superior to PDA as drug carrier in targeting HeLa cells. More importantly, their distribution in cell was significantly different. The PDA/DOX was distributed in the whole cell, while the PDA-RGDC/DOX was selectively concentrated in the nucleus (Figure. 3B, Figure. S11). Such phenomenon suggested that the targeting RGDC motif was beneficial for nanoparticles to locate in the perinuclear region [43, 48]. As we know, location in the nucleus is of vital importance for DNA intercalation drugs to efficiently kill tumor, such as DOX and cisplatin derivatives [50]. Therefore, the improved uptake and perinuclear localization of DOX demonstrated the effectiveness of PDA-RGDC as drug carrier to recognize and enter pathological cells.

Next, we wondered whether the NIR light would trigger drug release from PDA-RGDC/DOX inside cells. After being incubated with PDA-RGDC/DOX at 37 °C for 4 h, HeLa cells were washed with PBS to remove free nanoparticles and then irradiated by the 808 nm laser at the power intensity of 2 W/cm² for 5 min. As revealed by confocal

fluorescence images, the DOX fluorescence inside cells enhanced dramatically after laser irradiation relative to the control experiments (Figure. 4A).

In order to investigate the therapeutic efficacy of the PDA-RGDC/DOX *in vitro*, different treated HeLa cells were stained with calcein acetoxymethyl ester (Calcein AM) and imaged by confocal microscopy. As shown in Figure 4B, the cells treated with PDA or PDA-RGDC alone showed negligible cell death. In contrast, the PDA-RGDC/DOX with NIR irradiation exhibited the highest rate of cell death compared to non-targeted PDA/DOX (chemo-photothermal therapy), single treatment of PDA-RGDC/DOX without NIR irradiation (chemotherapy), and PDA-RGDC with NIR irradiation (photothermal therapy). It was worthy to be noted that the nanoparticles could not only target the cancer cells, but also improved the therapy efficiency compared with individual photothermal or chemotherapy. We further quantitatively measured their cytotoxicity on HeLa cells using an MTT assay (Figure. S12A). The individual photothermal treatments based on PDA (0.84) or PDA-RGDC (0.72) and chemo-treatments based on DOX (0.55) or PDA-RGDC/DOX (0.48) showed slight damages to cancer cells. In comparison, for the PDA-RGDC/DOX group with NIR laser irradiation, the cell viability reduced to 0.22. The therapy combined chemotherapy and PTT led to a significant reduces of the cell viability relative to the use of each method alone. The prominent synergistic effect may result from hyperthermia effect on physiological function of cells and photothermal enhanced chemotherapeutic efficacy of DOX at elevated temperatures [48]. In addition, we investigated the effects of the laser power intensity and irradiation duration time on the efficiency of chemo-photothermal therapy by the MTT assay (Figure. S12B). The experimental results suggested that 5 min of irradiation at 2 W/cm² and 10 min of

irradiation at 1 W/cm² were sufficient for the cancer therapy. However, cells may possess a powerful resistance mechanism (thermotolerance) under long time hyperthermia (42–47 °C), which was mediated by the induction of heat shock proteins [49] and markedly increase their survival rate. Thus, we chose a higher power density for in vivo study to decrease irradiation duration time.

3.4 The biocompatibility of PDA-RGDC *in vivo*

Considering that the degradation of the nanoparticles might induce subsequent damage to the organs, the *in vivo* long-term toxicity of the nanoparticles was further examined. After intravenous injection of a single dose of the nanoparticles, the mice remained healthy over one-month period with slightly increased body weight after intravenous injection of a single dose of the nanoparticles (Figure. S13), displaying no abnormalities in eating, drinking, grooming, activity, exploratory behavior, urination, or neurological status. One month later, the mice were then euthanized and several main organs were stained with hematoxylin and eosin (H&E) for histology analysis (Figure. S14). In contrast with the control group, there was nearly no distinct tissue damage, inflammation, or lesion post injection. Moreover, the five liver function markers including alkaline phosphatase (ALP), alanine transaminase (ALT), aspartate transaminase (AST), total protein (TP) and albumin (ALB), were tested, and the liver did not exhibit noticeable lesions compared with those of the control group (Figure. S15A). Clinical routine complete blood tests such as white blood cells (WBC), red blood cells (RBC), hemoglobin (HGB), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), platelet (PLT), and so on were measured to be normal compared

with the control group, revealing no obvious interference with the physiological regulation of haem or immune response (Figure. S15B), highlighting that PDA-RGDC had no toxic side effects to normal tissues. Furthermore, the PDA-RGDC could be oxidized to degrade by the hydrogen peroxide existed *in vivo* [34]. Thus, PDA-RGDC was proved to be an excellent biocompatible and biodegradable material for applications in biological medicine.

3.5 The photoacoustic imaging of tumor *in vivo*

The strong absorption of PDA-RGDC in the NIR region (Figure. 5A, 5C) motivated us to investigate its potential application as a contrast agent for PA imaging (PAI). To demonstrate the capacity of PDA-RGDC as a new PAI contrast agent, we first evaluated the photoacoustic signals from aqueous solutions containing different concentrations of PDA-RGDC. As shown in Figure 5B and 5D, a higher PA signals was detected with increased PDA-RGDC concentration ($R^2 = 0.987$). Notably, the signal of PDA-RGDC could be clearly detected even with a concentration as low as $15 \mu\text{g mL}^{-1}$. As a proof-of-concept experiment, PDA-RGDC was further utilized as a contrast agent for *in vivo* PA imaging. Mice bearing subcutaneous integrin $\alpha_v\beta_3$ -positive HeLa tumor [45] were intravenously injected with PDA and PDA-RGDC, respectively, followed by PAI at appointed time points (0 (before injection), 4, 8, 24, 48 and 72 h). The PA images revealed that PDA-RGDC was an excellent PAI agent, which could significantly illuminate the tumor site and contribute to delineate the margins of the tumor clearly (Figure. 5E-5F). We calculated the photoacoustic signal by drawing a region of interest (ROI) around the tumor, and the photoacoustic signal variation was quantified as a

function of time (Figure. S16). We found that the plain PDA only led to a temporary increase in the photoacoustic signal of the tumor, arising from circulating nanoparticles that would be eventually cleared from the bloodstream. Conversely, PDA-RGDC led to a consistently higher photoacoustic signal, due to the bind between PDA-RGDC and the tumor cell or vasculature [51]. Notably, the PA signals reached maximum accumulation at 24 h and 8 h for PDA and PDA-RGDC (Figure. S16), respectively, which was of importance to choose optimal time for the 808 nm irradiation and guaranteed a maximum accumulation of nanoparticles at the tumor site and the best PTT efficacy.

We further validated the suitability of the PDA-RGDC toward other type tumor. Balb/c mice bearing 4T1 xenograft tumor were treated with PDA and PDA-RGDC similarly as HeLa tumor bearing mice. The PA signal from the tumors was 3 times higher in mice injected with PDA-RGDC than that in mice injected with plain PDA (Figure. S17). All results suggested that the PDA-RGDC dramatically boosted target-to-background ratios *in vivo* for PA imaging and could be potentially used as the molecular nanoprobe for tumor theranostic.

3.6 Combined chemo-phototherapy synergistic effect of PDA-RGDC/DOX *in vivo*

Encouraged by the above results, we then proceeded to estimate the synergistic antitumor efficiency of PDA-RGDC/DOX on tumor-bearing mice. When the tumor reached about 3-4 mm in diameter, Balb/c nude mice bearing HeLa tumors were divided into six groups with 6 mice per group, PBS (Group1), free DOX (Group 2), PDA+808 nm laser (Group 3), PDA-RGDC+808 nm laser (Group 4), PDA-RGDC/DOX (Group 5) and PDA-RGDC/DOX+808 nm laser (Group 6). Following PA imaging results as

described above, laser treatment was conducted at 8 h post injection for group 4 and 6, and at 24 h post injection for group 3, at which tumor uptake of nanoparticles has plateaued. An IR thermal camera was used to monitor the tumor temperature (Figure. 6A-B). As expected, the temperature of tumor site treated with PDA-RGDC and PDA-RGDC/DOX rapidly increased and maintained at ~52 °C during laser irradiation. This temperature is sufficient to cause irreversible damage to cancer cells [52-53] and the temperature is lower than most reported work *in vivo* (Table S1). In marked contrast, the photothermal effect of the mice treated with PDA showed relatively smaller temperature range than PDA-RGDC. The tumor sizes and body weights were then measured every the other day for two weeks (Figure.6 C-D). The real photographs of tumor-bearing nude mice were recorded in different groups, respectively (Figure. S18). At day 14, three mice were sacrificed and tumors were excised and the remains were observed for 50 days. Compared to treatment with free DOX, PDA-RGDC/DOX without laser irradiation showed improved therapeutic effect, probably owing to the enhanced tumor uptake by the active targeting and the enhanced permeability retention (EPR) effect. Although enhanced inhibition of tumor growth was observed with injection of only PDA-RGDC/DOX or PDA-RGDC with NIR laser irradiation, the tumor growth was only partially delayed, indicating the poor efficacy in inhibiting tumor growth. In marked contrast, the tumor in the combination therapy group (PDA-RGDC/DOX +laser irradiation) was completely eradicated without regrowth in 50 days (Figure. S19). In addition, hematoxylin and eosin (H&E) staining of tumor slices confirmed that whole cells in control groups of tumors largely retained their normal morphology with distinctive nuclear structures and membrane, but there almost no tumor cells in the group

receiving PDA-RGDC/DOX injection and NIR laser irradiation (Figure. 6F). The *in vivo* anticancer results strongly suggested PDA-RGDC/DOX under laser irradiation could provide remarkable chemo-photothermal synergistic effect for cancer therapy. Thus, the combination of photothermal and chemotherapy therapy has been proved to be more effective in cancer treatments. Besides the use of heat to directly destroy the tumor cells, the photothermal effect could also be utilized to stimulate drug release enhancement and improve chemotherapy efficacy, which offered a synergistically enhanced therapeutic efficacy superior to that obtained by mono-therapy.

4. Conclusions

In summary, the PDA-RGDC/DOX was developed as NIR-adsorbed theranostic agents to realize the combination of photoacoustic imaging and chemo-photothermal therapy of tumor *in vitro* and *in vivo*. The PDA-RGDC/DOX nanoparticles showed strong NIR absorbance, high PA imaging efficiency, good photothermal performance, targeting accumulation at tumor sites, as well as excellent biocompatibility and biodegradability. The loaded DOX could be triggered by external NIR light and lower intracellular pH value. The *in vitro* and *in vivo* results demonstrated that PDA-RGDC/DOX had the ability to achieve synergistic therapeutic effect under the accurate guide of PA imaging, effectively eradicating tumor without regrowth. Our results offer a way to harness PDA-based theranostic agents to achieve PA imaging-guided cancer therapy.

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Figure legends

Figure.1. The characteristic and biocompatibility of PDA-RGDC. A) Schematic showing the preparation of PDA-RGDC. B) The MALDI-TOF-MS of PEG and PEG-RGDC: molecular weight from 7420 g mol⁻¹ (PEG) to 7910 g mol⁻¹ (PEG-RGDC). C) FTIR spectra of PDA before and after modification PEG-RGDC on their surface. D-E) TEM images of PDA and PDA-RGDC, respectively. F) DLS of PDA and PDA-RGDC. G) The UV-vis-NIR absorbance spectrum of PDA-RGDC. H) The photothermal heating curves of pure water and PDA-RGDC with different concentrations under 808-nm laser irradiation at the power density of 2 W/cm². Inset: IR images of water and PDA-RGDC solutions after laser irradiation for 5 min.

Figure.2. The drug loading and releasing ability of PDA-RGDC. A) UV-vis-NIR absorbance spectra of PDA (A) PDA-RGDC (B) PDA-RGDC/DOX (C) and DOX (D) in water. B) DOX loading capacity with different concentrations of DOX. C) DOX release from PDA-RGDC/DOX overtime in buffers at the different pH values (5.0 and 7.4) and the NIR irradiation triggered release of DOX from PDA-RGDC/DOX at pH values of 5.0. D) The samples at different pH values were irradiated with an NIR laser (2 W/cm²) for 5 min at different time points as indicated by the arrows. E) Relative viability of HeLa cells after incubation at different concentration of PDA-RGDC. F) Relative viability of HeLa cells after treated with different loaded PDA-RGDC/DOX (808 nm, 2 W/cm², and 5 min).

Figure.3. Fluorescence images of HeLa cells after incubation with PDA-RGDC/DOX and PDA/DOX for various times indicated (A). Confocal laser scan microscopy images of HeLa cells incubated with PDA-RGDC/DOX and PDA/DOX, Images are taken from DAPI, DOX, respectively (B).

Figure.4. The chemo-phototherapy synergistic therapeutic effect *in vitro*. A) Confocal images of HeLa cells incubated with PDA-RGDC/DOX with or without laser irradiation (808 nm, 2 W/cm², and 5 min). Blue and red colors represent DAPI stained nuclear and DOX fluorescence, respectively. B) Confocal laser scan microscopy images of HeLa cells incubated with (b1) control + 808 nm (b2) PDA + 808 nm NIR light, (b3) PDA-RGDC+808 nm NIR light, (b4) DOX, (b5) PDA-RGDC/DOX, (b6) PDA-RGDC/DOX+808 nm NIR light. Viable cells were stained green with calcein AM.

Figure.5. The PA imaging ability of PDA-RGDC. A) UV-vis-NIR absorbance spectra of aqueous solutions containing PDA-RGDC at varied concentration and C) the corresponding linear plot of absorbance of PDA-RGDC concentration at 808 nm. PA values (B) and PA images (D) of PDA-RGDC solutions with different concentrations. PA images of HeLa tumors (region pointed by yellow circle and arrows) before and after tail vein injection of PDA (E) and PDA-RGDC (F) (20 mg kg⁻¹) in living mice. The red arrows pointed the background interference after injection of PDA.

Figure.6. The chemo-phototherapy synergistic therapeutic effect in vivo. A) IR thermal images of HeLa tumor-bearing mice recorded by an IR camera. B) Temperature changes of tumors monitored by the IR thermal camera in different groups during laser irradiation. C) Relative body weight of mice after various treatments indicated. D) The growth of HeLa tumors in different groups of mice after various treatments indicated. The relative tumor volumes were normalized to their initial sizes. E) Photographs of tumors collected from mice at the end of treatments (day 14). F) The H&E-stained tumor slices collected from mice post various treatments.

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