

Multifunctional Nanoplatfom Based on pH-responsive Micelle Coated with Discontinuous Gold Shell for Cancer Photothermo-chemotherapy and Photoacoustic Tomography

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Electronic Supplementary Information

Abstract Photothermo-chemotherapy, as a new strategy for cancer treatment, incorporates the complementary advantages of photothermal therapy and chemotherapy. In this study, a pH-sensitive diblock copolymer poly(aspartic acid-butanediamine)-poly(2-(diisopropylamino)ethyl methacrylate) (PAsp(DAB)-PDPA) was synthesized and self-assembled into doxorubicin-loaded micelle, which was further used as a template to form a gold nanoshell. After further modification with poly(ethylene glycol), the resulting nanoplatfom provided good biocompatibility and desirable photo-thermal conversion efficiency to facilitate photothermal therapy. Meanwhile the nanoparticle also exhibited pH sensitivity, which prevented drug loss while circulating in the blood but enabled rapid drug release after endocytosis. An improved effect was achieved with the combination of photothermal therapy and chemotherapy. In addition, systemic delivery of the nanoplatfom could be monitored by photoacoustic tomography. Thereby, this multifunctional nanoplatfom would be highly potential for the diagnosis and therapy of cancer.

Keywords Gold nanoshell; Photoacoustic tomography; Photothermo-chemotherapy; pH-sensitive micelle

Citation: Huang, Y.; Li, X. X.; Zhang, L.; Chen, X. Y.; Liu, C. B.; Chen, J. Q.; Wang, Y.; Shuai, X. T. Multifunctional Nanoplatfom Based on pH-responsive Micelle Coated with Discontinuous Gold Shell for Cancer Photothermo-chemotherapy and Photoacoustic Tomography. Chinese J. Polym. Sci. 2018, 36(10), 1139–1149.

INTRODUCTION

Various strategies have been developed for treatments of cancer, such as chemotherapy, gene therapy, hyperthermia and *etc.*, but unfortunately none of them are adequate to confront cancer perfectly^[1]. For instance, chemotherapy can cause severe adverse effects^[2, 3] and is often rendered ineffective by multidrug resistance^[4]. Conversely, photothermal therapy (PTT) has the advantage of selectively damaging the tumor tissue, but tumor cells at the periphery of the irradiated area may remain unaffected, leading to recurrence of the disease^[5]. Therefore, attempts have been made to combine different strategies to improve the therapeutic effect, and one of the successful cases is photothermal-chemotherapy, *i.e.*, combination of PTT and chemotherapy^[6, 7]. Incorporating photothermal agents and anti-cancer drugs into one nanoplatfom proves to be beneficial in the combined therapy, because it guarantees the

simultaneous delivery of both therapeutic agents, and the delivery efficiency can also be improved due to the enhanced extravasation into tumor at elevated temperatures^[8]. During the past few years, a number of nanoplatfoms have been designed to facilitate photothermal-chemotherapy, such as polymeric nanoparticles encapsulating both photosensitizers and drugs^[9, 10], inorganic nanoparticles as PTT agents carrying chemotherapeutic agents^[11–13] and *etc.*

Gold-based nanoparticles are chief materials used in photothermal therapy^[14] as they have tunable optical properties^[15], low toxicity^[16], stability against photo-bleaching^[17] as well as facility for synthesis and modification^[18]. Another advantage of Au nanoparticles is that they can be used as contrast agents for photoacoustic tomography (PAT)^[19]. Detecting the acoustic energy converted from absorbed optical energy, PAT features much deeper penetration compared with optical imaging, as biological tissue is more transparent to sound than to light. Besides, PAT also provides scalable spatial resolutions and thereby well-suited for both microscopic and macroscopic imaging^[20, 21]. Thus, Au nanoparticles serve to be ideal multifunctional nanoplatfoms for the diagnosis and therapy

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Received February 27, 2018; Accepted April 2, 2018; Published online May 10, 2018

of cancer.

Representative Au nanoparticles include gold nanospheres^[22], nanorods^[23], nanocages^[24], hollow nanoshells^[25], and *etc.* Apart from PTT agents, they can also function as delivery systems by either conjugating drugs to the surface or encapsulating them in the core^[18]. Nevertheless, the former strategy is limited to the chemotherapeutic agents containing active functional groups for conjugation and partly impairs the bioactivity of the drugs^[26]. Conversely, drug-encapsulating nanoparticles coated with compact gold nanoshell can be applied to nearly all kinds of drugs, yet drug release might be hindered without enough NIR irradiation^[27].

Herein, a novel nanoplatform based on pH-responsive polymeric micelle coated with a discontinuous gold nanoshell has been developed to address the problems mentioned above. As outlined in Scheme 1, the diblock copolymer poly(aspartic acid-butanedi-amine)-poly(2-(diisopropylamino) ethyl methacrylate) (PAsp(DAB)-PDPA) was synthesized to prepare doxorubicin (DOX)-loaded polymeric micelles. As the hydrophobic block PDPA turned hydrophilic after protonation, the micelle would decompose in response to the acidic conditions. With the pH-sensitive micelles as templates, negatively-charged gold nanoseed (GS) was absorbed to the micellar surface through electrostatic interaction. Au(III) was subsequently reduced to form a discontinuous gold coating with GS as the crystal nucleus. Poly(ethylene glycol) was eventually conjugated to the gold nanoshell for better stability and prolonged circulation time. The resulting multifunctional nanoplatform GNS@PDPM exhibited the maximum absorption in the first NIR window as well as provided desirable photo-thermal conversion efficiency and site-specific drug release in response to the acidic environment of lysosomes, and meanwhile biodistribution of the nanoparticles could be monitored with photoacoustic imaging. In the present study,

both *in vitro* and *in vivo* experiments were conducted to evaluate the potential of GNS@PDPM for photothermal-chemotherapy and photoacoustic imaging.

EXPERIMENTAL

General Considerations

Poly(β -benzyl L-aspartate) (PBLA) was synthesized through ring-opening polymerization of ϵ -benzyloxycarbonyl L-aspartic acid (BLA-NCA) as previously reported^[28]. *S*-1-dodecyl-*S'*-(α,α' -dimethyl- α'' -acetic acid)trithiocarbonate (DDAT) was synthesized according to the literature^[29]. 2-(Diisopropyl amino)ethyl methacrylate (DPA, Sigma-Aldrich) was removed of the stabilizer with aluminium oxide. 1-(3-Dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC·HCl, J&K), *N*-hydroxysuccinimide (NHS, J&K), 1,4-butanedi-amine (DAB, J&K), chloroauric acid (J&K) and sodium borohydride (J&K) were used as purchased. 2,2'-Azobis(2-methylpropionitrile) (AIBN, J&K) was recrystallized twice in methanol.

Synthesis of the Polymers

Synthesis of poly(2-(diisopropyl amino)ethyl methacrylate) (PDPA)

AIBN (0.006 g, 3.6×10^{-5} mol), DPA (2.0 g, 3.6×10^{-3} mol) and DDAT (0.136 g, 3.6×10^{-4} mol) were dissolved in 30 mL of dioxane. The mixture was frozen with liquid nitrogen and vacuumed for three times to remove oxygen. Then the reaction was allowed to proceed for 12 h at 80 °C. The solution was quickly cooled to room temperature, dialyzed (MWCO: 1000 Da) against dioxane for 72 h and against deionized water for 48 h subsequently and freeze-dried to obtain 1.1 g of yellow powder. Subsequently, 0.017 g of AIBN and 0.6 g of PDPA-DDAT were dissolved with 30 mL of dioxane. After the same operation as mentioned above, 0.4 g of white powder was obtained.

Synthesis of poly(β -benzyl L-aspartate)-poly(2-(diisopropylamino)ethyl methacrylate) (PBLA-PDPA)

PDPA (0.30 g), EDC·HCl (0.046 g, 0.002 mol) and NHS (0.026 g, 0.002 mol) were dissolved in 20 mL of chloroform and placed into a 50 mL flask. After the solution was stirred for 4 h at room temperature, PBLA (0.112 g) dissolved in 10 mL of chloroform was added. The solution was stirred at room temperature for 48 h and then precipitated into cool diethyl ether. The product was recovered by filtration and vacuum-dried to get 0.20 g of PBLA-PDPA.

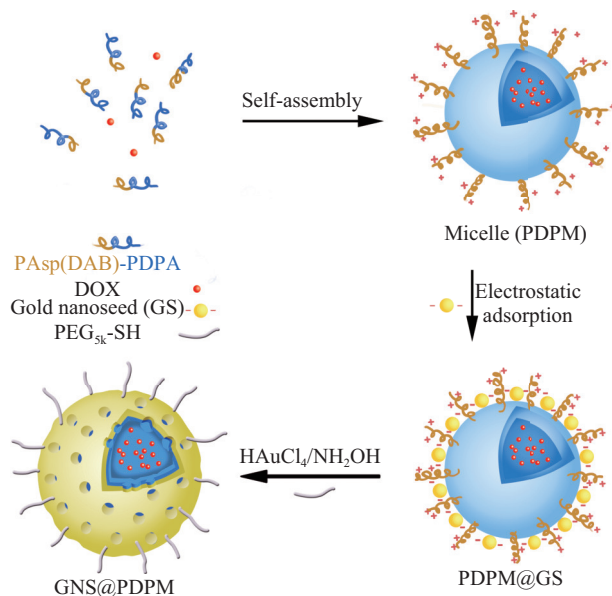
Aminolysis of PBLA-PDPA

DAB (0.44 g) and PBLA-PDPA (0.30 g) were dissolved in 20 mL of DMSO and then stirred for 4 h at room temperature. Subsequently the solution was dialyzed (MWCO: 3500 Da) against methanol for 72 h, concentrated by rotary evaporation and finally vacuum-dried to obtain 0.15 g of final product PAsp(DAB)-PDPA.

Synthesis of the Nanoparticles

Preparation of DOX-loaded cationic micelles (D-PDPM)

PAsp(DAB)-PDPA (10 mg) was dissolved in 4 mL of methanol and mixed with 2 mL of DOX·HCl solution (0.5 mg/mL in methanol). The mixture was added slowly into 20 mL of deionized water under ultrasonic oscillation. The resulting mixture was dialyzed (MWCO: 14 kDa) for



Scheme 1 Schematic illustration of GNS@PDPM preparation (The online version is colorful.)

12 h against deionized water.

Preparation of gold nanoseed (GS)

Sodium citrate (250 μ L, 25 mmol/L) and chloroauric acid (250 μ L, 25 mmol/L) were mixed and diluted to 20 mL. Sodium borohydride (30 μ L, 1 mol/L) was added to the solution mentioned above and stirred for 10 min. Subsequently the mixture was filtered with 220 nm microfiltration membrane and kept in a 50 mL centrifuge tube.

Preparation of GNS@PDPM

GS solution (300 μ L) was added to the polymeric micelle solution (40 mL) and stirred for 2 h. Chloroauric acid solutions (300, 500 and 800 μ L, 20 mmol/L, pH = 8) were subsequently added to the mixture and stirred for 10 min, respectively. Then hydroxylamine (5%, 400 μ L) was added and further stirred for 10 min. The mixture was centrifuged at 10000 r/min for 15 min. The residue at the lower layer was collected and dissolved in deionized water. 10 mg of PEG5k-SH was added to the resulting solution and stirred in vortex for 12 h. The mixture was dialyzed (MWCO: 14 kDa) against deionized water for 12 h, filtered with 450 nm microfiltration membrane and concentrated.

Characterization of the Polymers and Nanoparticles

^{1} H-NMR spectrum was recorded by a Bruker 400 MHz spectrometer. UV-Vis spectra of PDPA-DDAT and PDPA were recorded by a UV-Vis spectrometer (PerkinElmer UV75003040425) at a concentration of 1 g/mL. GPC chromatogram was measured with a Waters gel permeation chromatography (GPC) system (Waters Corp., Milford, Massachusetts, USA). Specifically, PBLA and PBLA-PDPA were measured in DMF containing LiBr (1.0 g/L) at a flow rate of 1.0 mL/min, while PAsp(DAB)-PDPA was measured in phosphate buffer solution (pH 5.0) at a flow rate of 0.6 mL/min. The size distribution and zeta potential of nanoparticles were measured by 90Plus/BI-MAS equipment (Brookhaven Instruments Corporation, USA) at 25 °C with a detection angle of 15° and 90°, respectively. The samples were dropped on copper grids, dried and observed under transmission electron microscopy (Hitachi model JEM-1400, Japan).

Evaluation of pH Sensitivity

DOX-loaded micelle solution (4 mL) was divided into two groups and the pH was adjusted to 7.4 and 5.0, respectively. The same process was conducted with 4 mL of GNS@PDPM. The aforementioned solutions were further divided into two groups and one of them was irradiated by 808 nm laser for 5 min at 1.5 W/cm², while the other group was not irradiated. The samples were eventually dialyzed against water for 4 h, and the solutions outside the dialysis bag were subjected to fluorometric analysis. Fluorescence from 500 nm to 750 nm was determined with an excitation wavelength of 485 nm on a RF5301PC spectrophotometer (Shimadzu, Japan) at a speed of 50 nm/min. The slit width was 5 nm for both excitation and emission.

Determination of Drug Loading Content

10% HCl was added to 200 μ L of D-GNS@PDPM in order that pH was adjusted to 1.0. After 10 min sonication, the

solution was dialyzed against HCl solution at pH 1.0 for 24 h. The absorption of the resulting sample at 485 nm was measured and the concentration of DOX was calculated according to the standard curve.

Determination of the Gold Content

Gold content of the nanoparticles was determined by thermogravimetric analysis (TGA) from 40 °C to 800 °C at a heating rate of 10 °C/min on a Perkin-Elmer TGA system under oxygen.

Determination of Photo-thermal Conversion Efficiency

GNS@PDPM or PBS (1 mL) was irradiated with 808 nm laser for 5 min at 1.5 W/cm². The temperature of the solutions was recorded every 30 s.

In Vitro DOX Release

GNS@PDPM was concentrated to 4 mg/mL and divided into 12 groups with 2 mL of solution in each group. Six of them were irradiated by 808 nm laser for 5 min at 1.5 W/cm² and the others were not irradiated. The samples were then transferred to dialysis bags (MWCO: 14 kDa), which were placed in 50 mL centrifugal tubes and dialyzed against 30 mL of PBS (pH 7.4 or 5.0) at 37 °C in an incubator shaker. 4 mL of the solution outside the dialysis bag was taken and replaced with 4 mL of fresh buffer solution at different time points. Absorption of the samples at 485 nm was detected by a UV-Vis spectrometer and the cumulative amount of released DOX was calculated accordingly.

MTT Assay

The C6 cells were seeded at a density of 5×10^3 cells per well in 96-well plates and cultured in 5% CO₂ at 37 °C for 24 h. The samples were added to each well at different concentrations and cultured for 8 h. Subsequently the cells were exposed to the NIR laser of 808 nm at an intensity of 1.5 W for 6 min and cultured in the humidified atmosphere with 5% CO₂ for another 16 h. Then 10 μ L of 1% MTT was added to each well and the cells were incubated for another 4 h. After the medium was carefully removed, 100 μ L of DMSO was added to each well. The absorbance of each well at the wavelength of 562 nm was recorded on a microplate reader.

Measurement of Cellular Uptake

The C6 cells were seeded in petri dishes at a density of 5×10^3 cells per dish and incubated for 24 h in 5% CO₂. The cells were then incubated with GNS@PDPM for 2, 4, 6, 8, 10 and 12 h, respectively. Then the petri dishes were washed with PBS, fixed by 4% paraformaldehyde and stained with DAPI for 15 min. The samples were observed on a LSM 710 confocal microscope (German). The fluorescence emission of DOX and DAPI was excited at 485 and 359 nm, respectively, and collected at 595 and 461 nm, respectively.

Photoacoustic Imaging

All surgical interventions and post-operative animal care were approved by the Institutional Animal Care and Use Committee of the Sun Yat-sen University. Each nu/nu mouse was subcutaneously injected with 1×10^6 C6 cells in the right hind leg and GNS@PDPM was injected through tail vein at a dose of 40 mg/kg after the tumors reached 50–

100 mm³. Photoacoustic imaging was carried out with a photoacoustic imaging system (Vevo LAZR, FUJIFILM, VisualSonics Inc., Toronto, Ontario, Canada) as previously reported^[27].

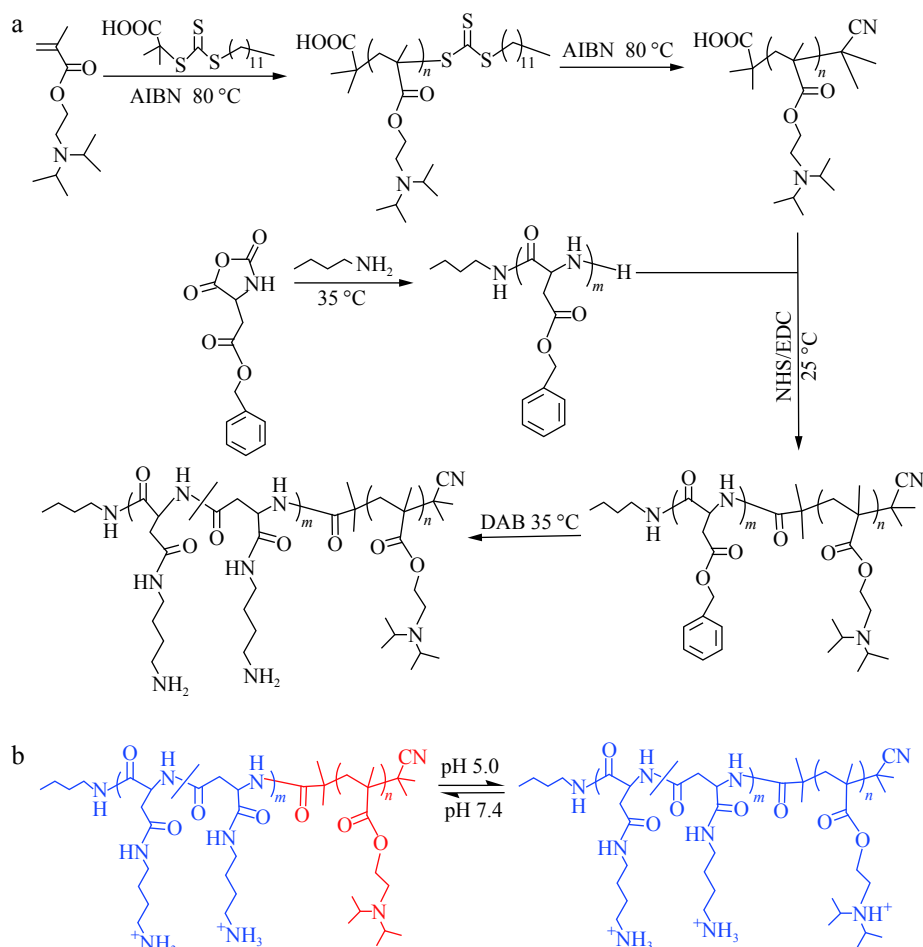
RESULTS AND DISCUSSION

Synthesis and Characterization of the Diblock Copolymer

The pH-sensitive diblock copolymer PAsp(DAB)-PDPA was synthesized through multistep reactions as outlined in Scheme 2(a) and the successful synthesis of the polymer was verified by ¹H-NMR, gel permeation chromatography (GPC) as well as UV-Vis measurements. The RAFT agent DDAT was synthesized according to the literature and verified by well-matched chemical shifts of protons in the ¹H-NMR spectrum (Fig. 1A1: 0.88 ppm, $-\text{CH}_3$; 1.25–1.47 ppm, $-(\text{CH}_2)_9$; 1.62–1.81 ppm, $-\text{C}(\text{CH}_3)_2$ ^[29]. Subsequently, PDPA-DDAT was synthesized by RAFT polymerization and the characteristic resonance signals of DPA units (Fig. 1A2, 1.01 ppm $(\text{CH}_3)_2\text{CH}-$; 3.00 ppm, $(\text{CH}_3)_2\text{CH}-$; 2.63 ppm, $-\text{OCH}_2\text{CH}_2-$; 3.85 ppm, $-\text{OCH}_2\text{CH}_2-$; 1.82 ppm, $-\text{CH}_2\text{C}(\text{CH}_3)(\text{CO})-$; 1.01 ppm, $-\text{CH}_2\text{C}(\text{CH}_3)(\text{CO})-$) appeared in the ¹H-NMR spectrum, consistent with previous

report^[30]. The degree of polymerization for PDPA was 24 according to the integral values of the characteristic peaks at 1.82 ppm ($-\text{CH}_2\text{C}(\text{CH}_3)(\text{CO})-$ of PDPA) and 3.40 ppm ($-\text{SCH}_2\text{CH}_2-$ of DDAT) (Fig. 1A). As the trithiocarbonate group is highly toxic^[31], it is removed with excess AIBN for better biocompatibility of the polymer. As shown in Figs. 1(A) and 1(B), the characteristic resonance signals of trithio-carbonate groups in the ¹H-NMR spectrum disappeared and the UV-Vis absorption at 315 nm^[32] was no longer detected, demonstrating the successful removal of trithiocarbonate groups.

On the other hand, PBLA was prepared by the ring-opening polymerization of BLA-NCA with *n*-butylamine as initiator. The degree of polymerization for PBLA was 9 according to the integral values of the characteristic resonances at 0.78 ppm ($\text{CH}_3(\text{CH}_2)_3\text{NH}-$) and 7.28 ppm ($\text{C}_6\text{H}_5\text{CH}_2-$) (Fig. 2A). Other characteristic resonance signals were matched with the protons well (Fig. 2B, 1.18 ppm, CH_3CH_2- ; 1.31 ppm, $\text{CH}_3\text{CH}_2\text{CH}_2-$; 4.62 ppm, $-(\text{CO})\text{C}(\text{NH}_2)\text{CH}_2-$; 2.61 ppm, 2.80 ppm, $-\text{CH}_2\text{COO}-$; 5.01 ppm, PhCH_2-). After coupled with PDPA by amidation reaction, the PBLA block was aminolyzed by 1,4-butanediamine (DAB), resulting in the elimination of benzyl group of PBLA and the appearance of DAB group



Scheme 2 (a) Synthetic approach of PAsp(DAB)-PDPA; (b) Schematic illustration of hydrophilic-hydrophobic interconversion of pH-sensitive polymer PAsp(DAB)-PDPA at pH 5.0 and pH 7.4

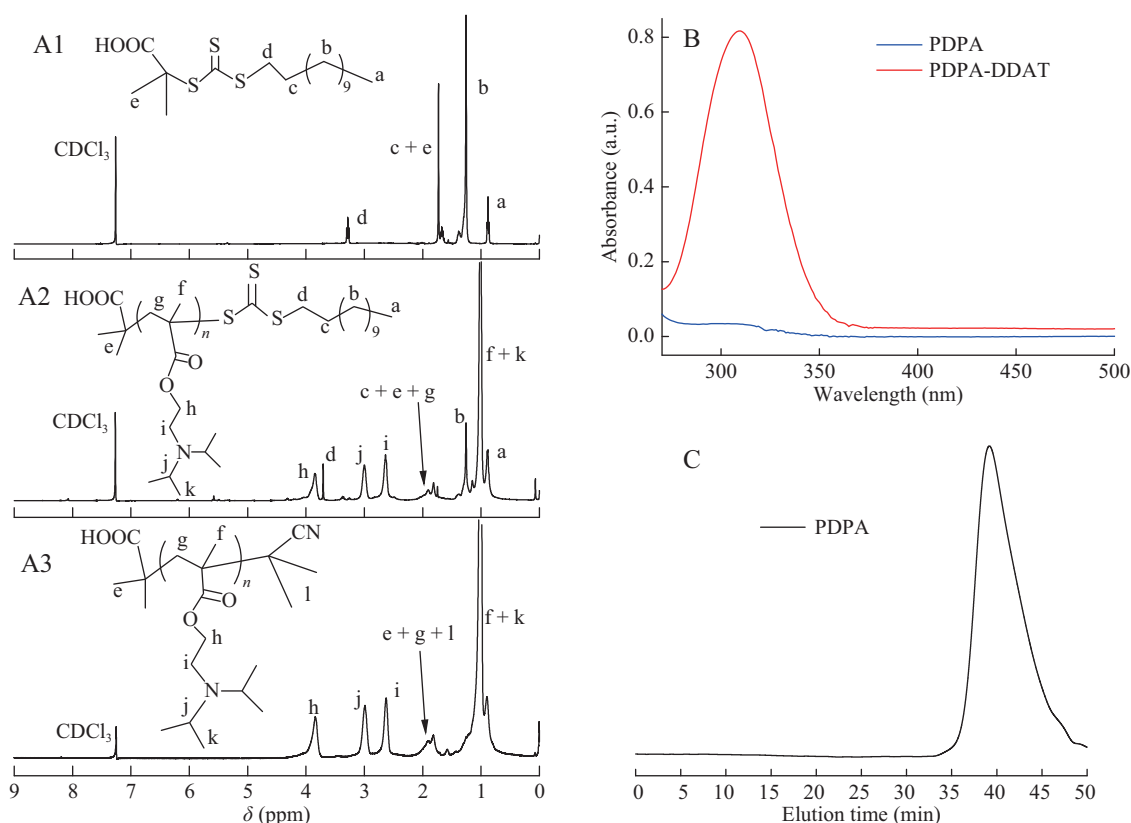


Fig. 1 (A) ¹H-NMR spectra of DDAT (A1), PDPA-DDAT (A2) and PDPA (A3) in CDCl₃; (B) UV-Vis spectra of PDPA-DDAT and PDPA in DMF; (C) GPC chromatogram of PDPA in phosphate buffer solution (pH 5.0) at a flow rate of 0.6 mL/min

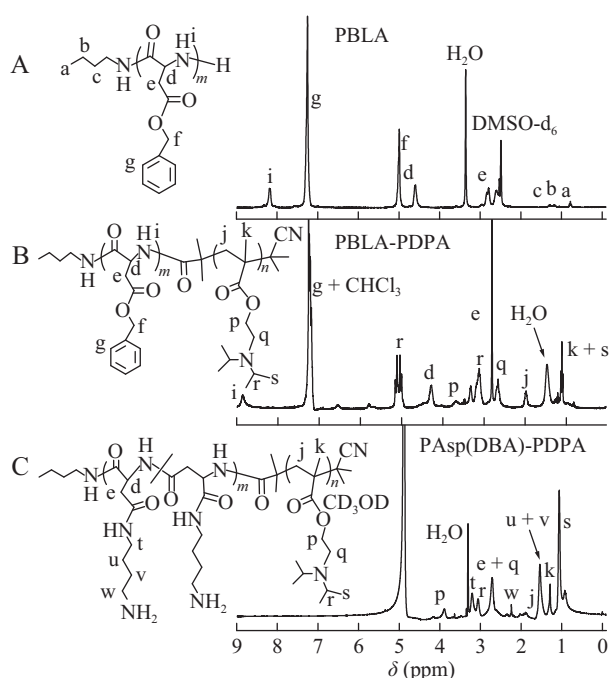


Fig. 2 (A) ¹H-NMR spectrum of PBLA in DMSO-d₆; (B) ¹H-NMR spectrum of PDPA-PBLA in CDCl₃; (C) ¹H-NMR spectrum of PAsp(DAB)-PDPA in CD₃OD

signal in the ¹H-NMR spectrum (Fig. 2C). In addition, GPC chromatograms showed all the polymer and pre-polymers were monodisperse with the narrow polydispersities

(Figs. 1C and 3). After the coupling reaction, the molecular weight of PBLA-PDPA was obviously increased compared with those of PDPA and PBLA, demonstrating the successful coupling of PDPA and PBLA (Table 1). All these results indicated the successful synthesis of PAsp(DAB)-PDPA. In order to investigate the pH sensitivity of the polymer, ¹H-NMR analysis in D₂O with different pH values was conducted. As shown in Fig. 4(a), characteristic peaks of PDPA at pH 5.0 were much stronger than those at pH 7.4, which indicated that the PDPA block turned from hydrophobic to hydrophilic as pH decreased from 7.4 to 5.0 (Scheme 2b)^[33]. The transition was due to the protonation of tertiary amines in PDPA^[30] and suggested the promising prospects of PAsp(DAB)-PDPA to serve as a pH-responsive drug carrier.

Preparation and Characterization of DOX-loaded Micelle

Considering that PDPA is hydrophobic while PAsp(DAB) is hydrophilic under neutral conditions, the amphiphilic diblock polymer could self-assemble into micelle due to the hydrophobic interaction. The blank micelle of PAsp(DAB)-PDPA (denoted as B-PDPM) and DOX-loaded micelle (denoted as D-PDPM) were prepared with sonication and characterized by transmission electron microscopy (TEM) (Fig. 4b) and dynamic light scattering (DLS). Both B-PDPM and D-PDPM were spherically shaped with a diameter of (21.0 ± 7.5) nm (Fig. 4c) and (24.4 ± 6.2) nm (Fig. S1, in electronic supplementary information, ESI), respectively. Meanwhile, both of the micelles were positively-charged

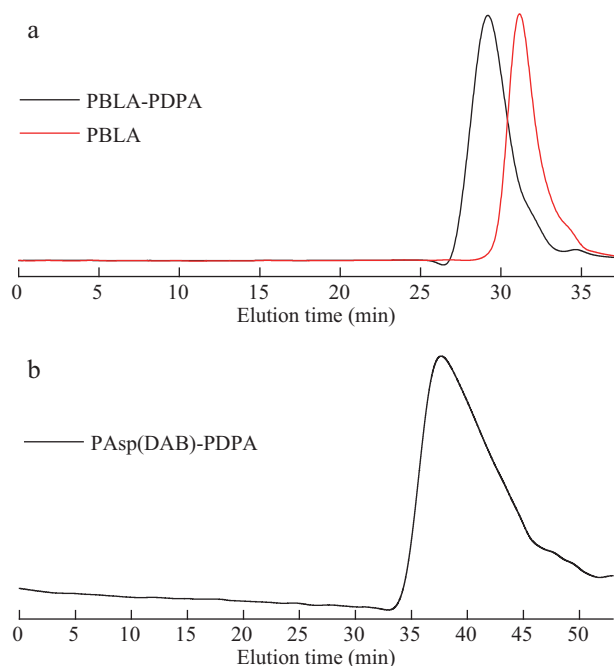


Fig. 3 GPC curves of (a) PBLA and PBLA-PDPA in DMF with LiBr (1.0 g/L) at a flow rate of 1.0 mL/min, and (b) PAsp(DAB)-PDPA in phosphate buffer solution (pH 5.0) at 0.6 mL/min

with a zeta potential nearly 18 mV owing to the protonated amines in PAsp(DAB), which were ideal to facilitate further complexation with negatively-charged gold nanoseed (GS) and subsequent formation of the gold shell. The pH

Table 1 Characteristics of the synthesized polymers

Sample	M_n^a	M_n^b	M_w^b	PDI
PBLA	1900	2000	2200	1.09
PDPA	5100	5400	5500	1.03
PBLA-PDPA	7000	7100	7800	1.10
PAsp(DAB)-PDPA	6900	6700	7600	1.12

^a Calculated from $^1\text{H-NMR}$ spectra; ^b Estimated by GPC measurements

sensitivity of D-PDPM was further evaluated with fluorescence measurements at different pH (Fig. 4d). According to literatures, the fluorescence of DOX would be decreased while encapsulated in the micelle due to the fluorescence quenching effect^[34], therefore the dramatically increased fluorescence intensity at pH 5.0 compared to that at pH 7.4 indicated that the micelle was decomposed and the drug was released under acidic condition.

Preparation and Characterization of Gold Nanoshell-coated Micelle

Negatively-charged gold nanoseeds (GS) were prepared as reported^[35] and characterized by TEM, DLS and UV-Vis-NIR spectroscopy. As shown in Fig. 5, the GS were spherically shaped with a diameter around 3 nm and the maximum absorption appeared at 510 nm. With the polymeric micelle as a template, GS were absorbed on the surface of the nanoparticles through electrostatic interaction to form a complex (denoted as PDPM@GS). As the optical properties of the gold nanoparticles vary with their structural dimensions^[14], different amounts of chloroauric acid were added and reduced by hydroxylamine^[36] to form gold shell

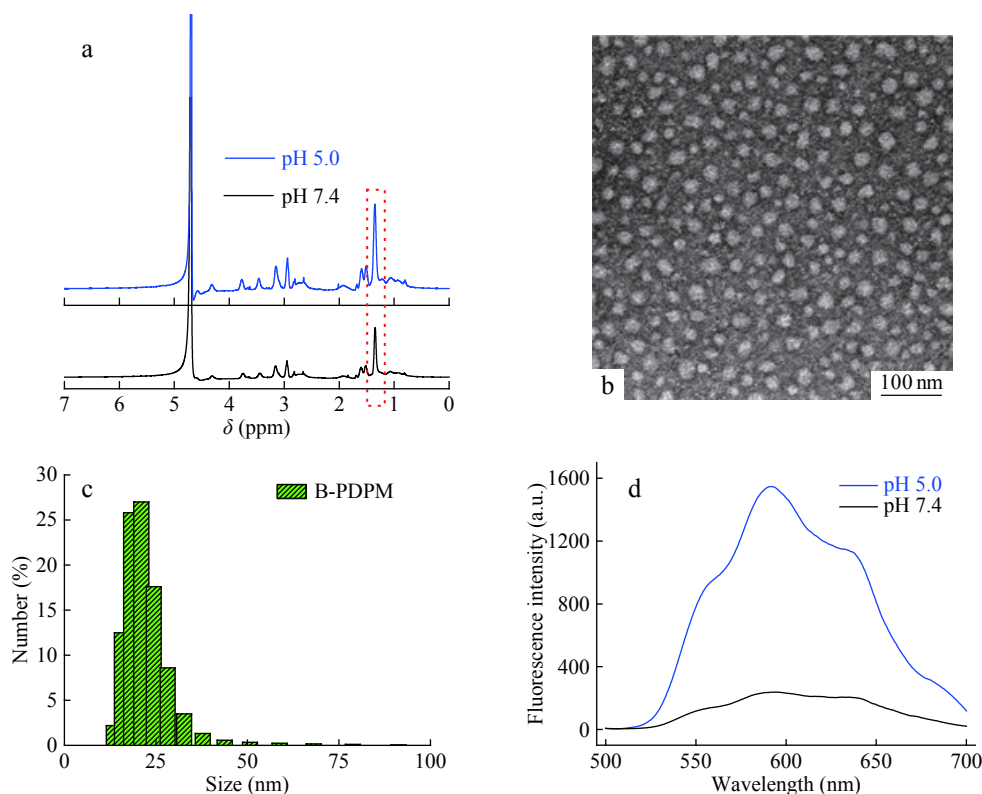


Fig. 4 (a) $^1\text{H-NMR}$ spectra of PAsp(DAB)-PDPA at pH 5 and pH 7.4; (b) TEM image of DOX-loaded micelles at pH 7.4; (c) The hydrodynamic size of B-PDPM; (d) Fluorescence intensity of D-PDPM at pH 7.4 and pH 5.0

with different thicknesses. The thus prepared nanoparticles were further coated with thiolated PEG *via* S–Au bond for better biocompatibility and enhanced stability in aqueous solutions^[37]. The maximum SPR absorption peaks of the resulting nanoparticles, denoted as GNS@PDPM-1/2/3, moved to 630, 680 and 750 nm, respectively (Fig. 5e). Considering that only light in the first (650–850 nm) or second (950–1350 nm) NIR window can deeply penetrate the healthy tissues^[14], GNS@PDPM-3 was selected for further experiments, and GNS@PDPM refers to GNS@PDPM-3 unless otherwise indicated. TEM observation (Fig. 5c) indicated that the resulting nanoparticles had an irregular structure with a diameter of approximately 45 nm while the weight percentage of the gold layer was 14% as determined by thermogravimetric analysis (Fig. 5f).

The biodistribution and toxicity of nanoparticles depend largely on the particle size as well as surface properties^[38]. As shown in Fig. 5(d) and Table 2, the diameter of GNS@PDPM was (43.8 ± 11.3) nm, which was desirable for systemic delivery as nanoparticles ranging from 20 nm to 100 nm are reported to accumulate at tumors due to the enhanced permeability and retention (EPR) effect^[39]. In addition, the zeta potential of GNS@PDPM was (0.5 ± 1.2) mV, which was also advantageous because a slightly positive zeta potential is favorable for cellular uptake and meanwhile the toxicity will not be as high as those with high positive charge^[40]. In order to evaluate the stability of the multifunctional nanoplatform under physical conditions, GNS@PDPM was dispersed in PBS and PBS containing 10% FBS, respectively. As shown in Fig. S2 (in ESI), no

Sample	Size (nm)	PDI	Zeta potential (mV)
B-PDPM (pH 7.4)	21.0 ± 7.5	0.198	18.5 ± 7.4
D-PDPM (pH 7.4)	24.4 ± 6.2	0.203	17.3 ± 7.9
Gold nanoseed	3.1 ± 2.1	0.210	-15.6 ± 7.0
GNS@PDPM	43.8 ± 11.3	0.225	0.5 ± 1.2

obvious changes in particle size were observed within 48 h, indicating the adequate stability of the nanoparticles.

Photo-thermal Conversion Efficiency

To assess the photo-thermal conversion efficiency of GNS@PDPM, different concentrations of GNS@PDPM were exposed to 808 nm NIR laser at a power density of 1.5 W/cm^2 , which is a moderate power commonly used in photothermal therapy (Fig. 6a). With 0.5 mg/mL GNS@PDPM, the temperature of the solution increased by 30.2°C after 5 min irradiation. The thermal effect was slightly weaker with 0.1 mg/mL GNS@PDPM (24.8°C), but still remarkable compared with PBS solution (6.2°C), which indicated the promising potential of GNS@PDPM for photothermal therapy. The photo-thermal conversion was induced by the melting of the gold shell after receiving laser irradiation, resulting in an obvious deformation of nanoparticle as demonstrated by TEM observation (Fig. 6b). In addition, the caused temperature increase would generate acoustical signal which could be used for imaging. As demonstrated by the various photoacoustic signals depending on the sample concentrations, the GNS@PDPM showed considerable potential for photoacoustic tomography in tumor treatment (Figs. 6c and 6d).

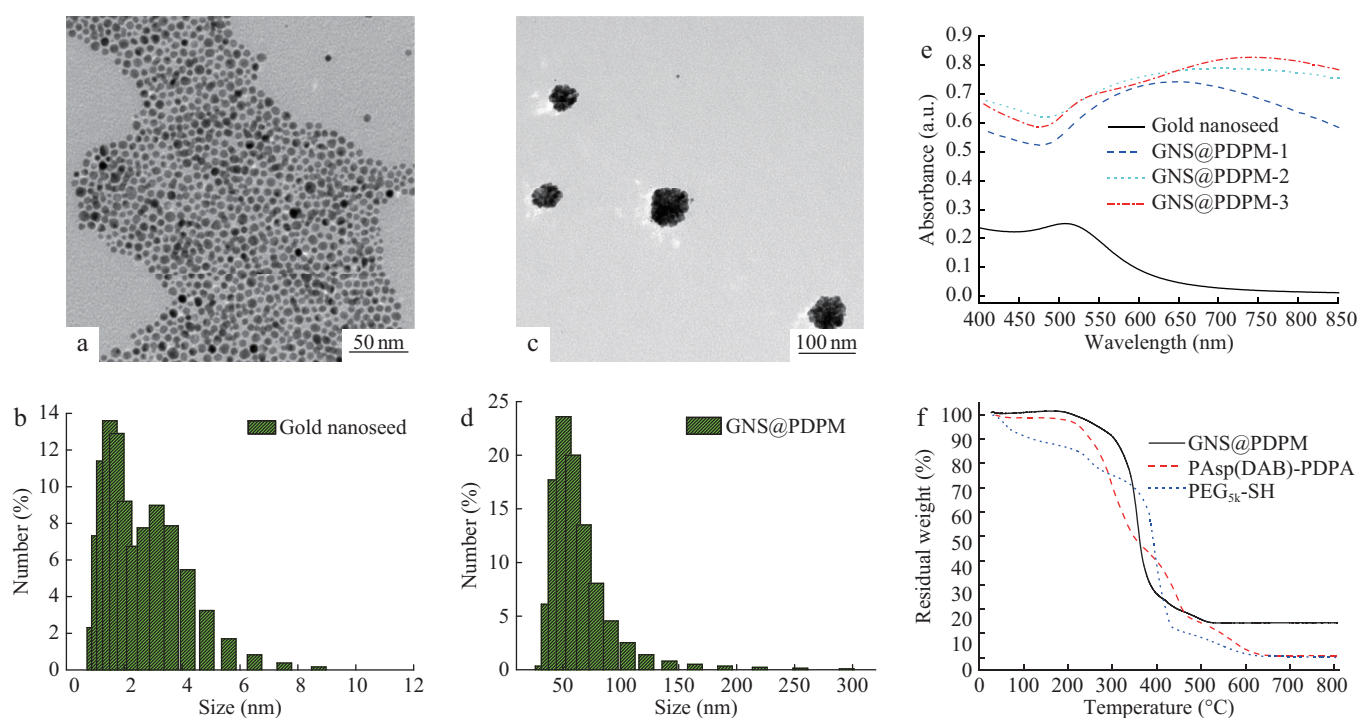


Fig. 5 (a) TEM image of GS; (b) The hydrodynamic size of GS; (c) TEM image of GNS@PDPM; (d) The hydrodynamic sizes of GNS@PDPM; (e) UV-Vis-NIR absorption spectra of gold nanoseed, GNS@PDPM-1, GNS@PDPM-2 and GNS@PDPM-3; (f) Thermogravimetric (TG) curves of GNS@PDPM, PAsp(DAB)-PDPA and PEG_{5k}-SH

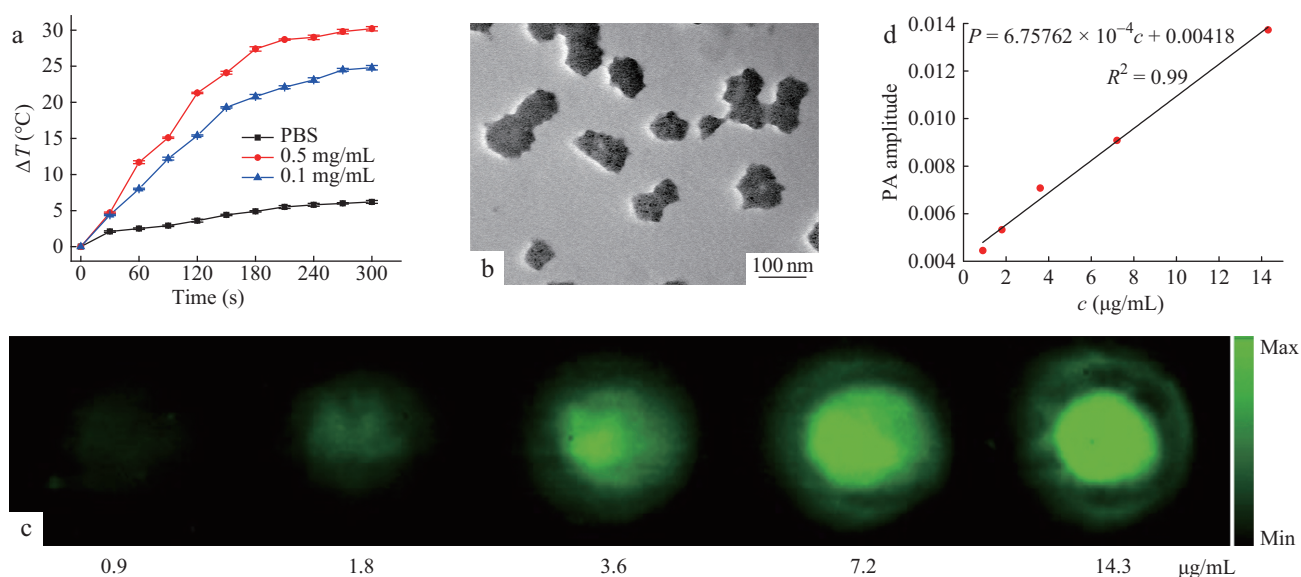


Fig. 6 (a) Temperature increase as a function of time with NIR irradiation (808 nm at 1.5 W/cm^2); (b) TEM image of GNS@PDPM after 5 min irradiation (1.5 W/cm^2); (c) The maximum amplitude projection of photoacoustic tomography with GNS@PDPM concentration range from $0.9 \text{ }\mu\text{g/mL}$ to $14.4 \text{ }\mu\text{g/mL}$; (d) Photoacoustic signal amplitude as a function of the concentration of GNS@PDPM

In Vitro Drug Release Triggered by pH and NIR Laser

A primary challenge for most drug delivery systems is to simultaneously prevent drug loss in the blood while release the drug rapidly at the pathologic sites. To address this problem, a variety of stimuli-responsive drug carriers have been designed and developed. Such stimulation can be either external (such as light, heat and magnetism) or internal (including pH, redox, enzymes and *etc.*)^[41]. In the present study, GNS@PDPM are exposed to not only the externally applied NIR laser but also the acidic environment of lysosomes (pH 4.5–5.5) as nanoparticles are normally internalized by the cells *via* endocytosis^[42]. Therefore, both the influences of light and pH on drug release were evaluated. Qualitative assessment was carried out with fluorescence measurements. As shown in Fig. 7(a), the fluorescence intensity of GNS@PDPM at pH 5.0 was always stronger than that at pH 7.4, regardless of the irradiation, which indicated the pH-responsive drug release. On the other hand, the fluorescence intensity at pH 5.0 was stronger with the presence of NIR laser than that without laser, which may be attributed to deformation of the discontinuous gold shell upon irradiation. Quantitative assessment was further carried out and the accumulative release curves are shown in Fig. 7(b). At pH 7.4, no more than 10% DOX was released within 24 h, whether there was laser radiation or not. In contrast, GNS@PDPM released drug swiftly at pH 5.0, with 65.9% released in 24 h without irradiation and 71.0% released in 24 h after 5 min irradiation. The results suggested a site-specific drug release in response to the acidic microenvironment, especially after laser irradiation. Thereby most of the drug release would be confined to tumors and consequently better therapeutic effect might be achieved.

Cellular Internalization of Drug

Cellular uptake of drug was investigated with confocal laser scanning microscopy (CLSM). As shown in Fig. 8(A), the

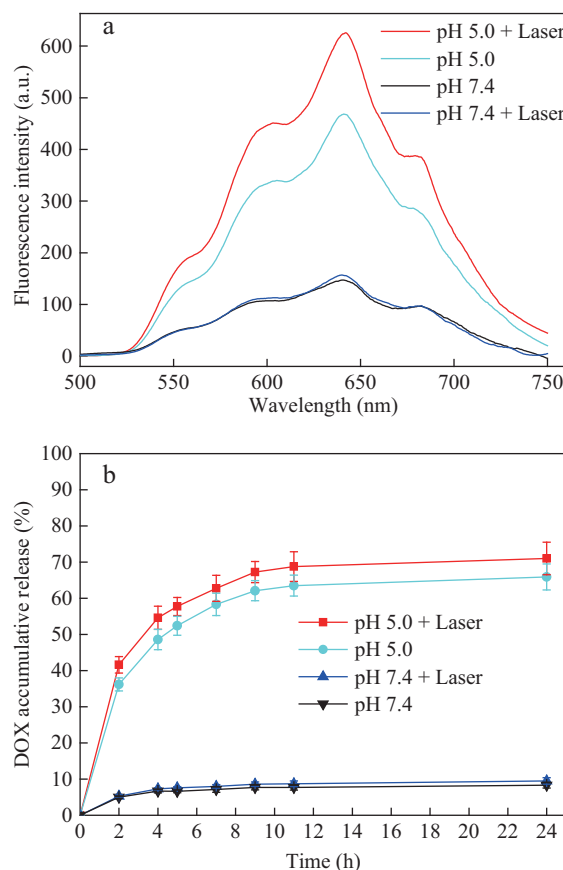


Fig. 7 (a) Fluorescence spectra of D-GNS@PDPM at 2 various conditions and (b) *in vitro* DOX release from D-GNS@PDPM

red fluorescence of DOX was observed in the cytoplasm within 1 h, indicating a rapid cellular uptake was obtained. After 6 h, DOX was found to enter the nuclei and the intensity of red fluorescence became much stronger, which suggested that DOX had been released from the

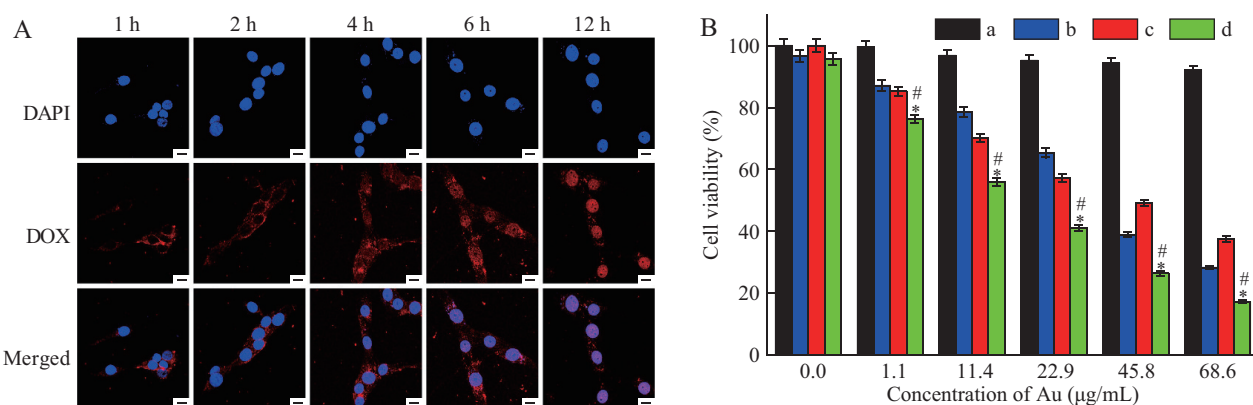


Fig. 8 (A) Confocal laser microscopic images of C6 cells incubated with D-GNS@PDPM at different time points. Red fluorescence: DOX; blue fluorescence: nuclei stained with DAPI. Bar: 10 μ m; (B) Cytotoxicity of B-GNS@PDPM without irradiation (a), B-GNS@PDPM with irradiation (b), D-GNS@PDPM without irradiation (c) and D-GNS@PDPM with irradiation (d) evaluated by MTT assay. * $p < 0.05$ and # $p < 0.05$ compared with B-GNS@PDPM with irradiation and D-GNS@PDPM without irradiation group, respectively.

nanoparticles^[43]. The swift drug release was most likely due to the decomposition of polymeric micelles in response to the acidic microenvironment of lysosomal compartments.

The Cytotoxicity of GNS@PDPM

The cytotoxicity of blank nanoplateform (denoted as B-GNS@PDPM) and DOX-loaded nanoplateform (denoted as D-GNS@PDPM) was evaluated in C6 cells by MTT assay. As shown in Fig. 8(B), B-GNS@PDPM exhibited low toxicity in the absence of irradiation with 92.1% cell survival rate in 480 μ g/mL B-GNS@PDPM. Nevertheless, the cell survival rate decreased significantly as the concentration of B-GNS@PDPM increased when the NIR laser irradiation was applied. For the highest concentration (480 μ g/mL), the survival rate was reduced to 28.3% owing to the remarkable photo-thermal conversion effect of the gold nanoshell. For cells treated with DOX-loaded nanoparticles, the survival rate was in negative correlation with the concentration of D-GNS@PDPM, and the IC_{50} value was 8.1 μ g/mL DOX without irradiation and 2.7 μ g/mL DOX in the presence of irradiation. The result was encouraging as it indicated that combined with photothermal therapy, the systemic side effect of chemotherapy could be dramatically reduced by lowering the drug dose while maintaining the same therapeutic effect.

In Vivo Photoacoustic Tomography Effect

Photoacoustic tomography (PAT), as a hybrid imaging modality that detects acoustic signals converted from optical absorption, incorporates both desirable imaging depth and scalable spatial resolutions^[21]. Due to the surface plasmon resonance effect, Au nanoparticles generally provide high sensitivity at depth and consequently serve as contrast agents in PAT^[19]. As shown in Figs. 6(c) and 6(d), the photoacoustic signal increased linearly with the concentration of GNS@PDPM within the range used in the measurement (≤ 0.1 mg/mL), which indicated its potential to be monitored by PAT. Therefore, in this study, the intratumor distribution of intravenously administered GNS@PDPM was detected by PAT. As shown in Fig. 9, the photoacoustic signal was dramatically intensified at 24 h after the injection, which suggested the accumulation of

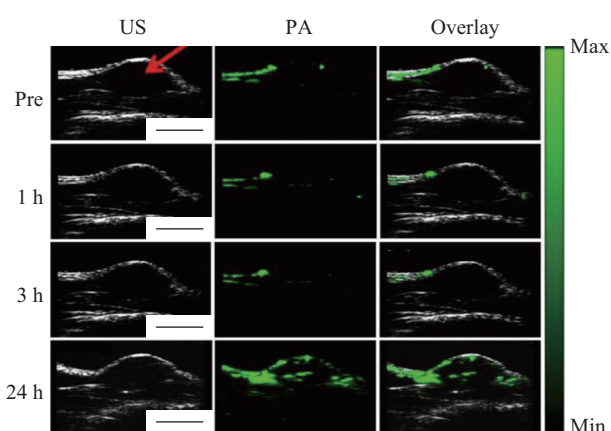


Fig. 9 Ultrasound (US), photoacoustic (PA), and their overlay images of the tumor region at different time points after the injection of GNS@PDPM. Scale bars represent 3 mm. The red arrow points out the tumor.

GNS@PDPM at the tumor through EPR effect^[39]. The result indicated that systemic delivery of GNS@PDPM would be practical in cancer therapy as the nanoparticles could automatically accumulate at the pathologic sites and meanwhile the delivery system could also be used as a contrast agents in PAT for enhanced detection sensitivity.

CONCLUSIONS

A pH-sensitive micelle coated with gold nanoshell was designed to facilitate photothermal-chemotherapy of cancer, in which the micelle was self-assembled by amphiphilic diblock copolymer PAsp(DAB)-PDPA and encapsulated anti-cancer drug DOX in its core. The nanoparticle (GNS@PDPM) exhibited high stability in serum-containing aqueous solutions, high photo-thermal conversion efficiency and rapid drug release in response to the acidic environment of lysosomes. The combined photothermal-chemotherapy provided enhanced effects to kill C6 cells as demonstrated by cytotoxicity assay. In addition, the biodistribution of intravenously-injected nanoparticles was facilely detected

with photoacoustic tomography. Therefore, GNS@PDPM would serve as a highly prospective multifunctional nanoplatfrom in cancer theranostics.

Electronic Supplementary Information

Electronic supplementary information (ESI) is available free of charge in the online version of this article at <http://dx.doi.org/10.1007/s10118-018-2141-8>.

ACKNOWLEDGMENTS

This work was financially supported by the National Natural Science Foundation of China (No. U1401242), National Basic Research Program of China (No. 2015CB755500), Natural Science Foundation of the Guangdong Province (No. 2014A030312018) and the Fundamental Research Funds for the Central Universities (Nos. 17lgjc01 and 17lgpy08).

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