ROS-triggered self-accelerating drug release nanosystem with charge conversion for enhanced cancer therapy

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Abstract

**Background:** The enhancement tumor retention and of cellular uptake of drugs are important factors in maximizing anticancer therapy and minimizing side effects of encapsulated drugs. Herein, a delivery nanoplatform with a pH-triggered charge-reversal capability and self-amplifiable reactive oxygen species (ROS) level inducing drug release pattern was constructed by encapsulating doxorubicin (DOX) in pH/ROS-responsive polymeric micelle.

**Results:** The surface charge of this system can be converted from negative to positive for enhanced tumor cell uptake in response to the weakly acidic tumor tissue. In addition, methionine-based system was dissociated in a ROS-rich intracellular environment, resulting in a phase transition and the release of DOX. Then, the exposed α-tocopheryl succinate (α-TOS) segments can be capable of producing ROS, which further induced the self-amplifiable disassembly of the micelles and drug release.

**Conclusions:** We confirmed efficient DOX delivery into cancer cells, upregulation of tumoral ROS level and induction of the apoptotic capability in vitro. The system exhibited outstanding tumor inhibition capability in vivo, indicating that dual stimuli nanosystem would be great potential as an anticancer drug delivery platform.
Keywords: Doxorubicin, Reactive oxygen species, Charge-reversal, Lung cancer

Background

Plenty of smart drug delivery systems have been proposed to improve the therapeutic efficacy and reduce undesirable side effects through achieving “on demand” drug release at tumor site under unique internal or external stimuli including pH gradient [1-6], intracellular reductive agents [7,8], peculiar enzymes [9,10], ROS [11,12], and so on. However, the stimuli levels of the tumor extracellular microenvironments are particularly subtle, such as relatively low ROS concentration even under pathological conditions, which accordingly imposes strict demand for the sensitivity of the materials responsive to the tumor microenvironments [13-15]. Besides, the poor tumor cells' uptake and incomplete drug release are another two critical challenges hindering the clinical translation of polymeric drug [6,15]. Tremendous efforts have been devoted to the development of stimuli-responsive tumor-targeted drug delivery systems to deal with aforementioned dilemma. On one hand, pH-dependent charge conversion strategy was utilized for the construction of polymeric drug, which would maintain their stealth features during circulation and then undergo a charge reversal process for achieving enhanced tumor cells’ internalization once exposed to tumor relative acidic environment [15-18]. On the other hand, polymeric drug with ROS generation capability would be an effective tactic to cope with insufficient ROS concentration for activating the complete drug release [13-15,19].

Inspired by above implement, we designed a self-amplifiable drug release system with charge reversal ability by loading doxorubicin in a polymeric micelle methoxyl poly(ethylene glycol)-block-poly(L-lysine)-graft-α-tocopheryl succinate and methionine modified with dimethylmaleic anhydride (denoted as PPT/D(DMA)@DOX).

Firstly, a moderate amount of dimethylmaleic anhydride (DMA) was conjugated to the backbone of the polymer, endowing the pH-triggered charge reversal property. The pH-dependent charge reversal delivery systems could remain negatively charged under physiological environment (pH 7.4) to reduce nonspecific interactions with
serum components and avoid clearance by reticuloendothelial system (RES), while they could be converted to positive in to enhance targeted tumor uptake [6,15]. Secondly, α-TOS, an analogue of vitamin E, could rapidly generate ROS in cells after interacting with mitochondrial respiratory complex II and interfering the electron transportation chain in mitochondria [13]. Thioether groups in poly-methionine segments could be changed to hydrophilic sulfoxide groups in cancer cells due to an inundation of ROS [11,12,20]. We hypothesized that ROS concentration under pathological conditions can induce the less decomposition of the polymeric drug by the phase transitions and then exposed α-TOS segment interacted with mitochondria in tumor cells to generate additional ROS. In other words, the intracellular ROS would be self-regenerated and amplified. Finally, α-TOS was less toxic to normal cells and shows specific anticancer activities to various cancers [21]. DOX could be encapsulated by hydrophobic interaction with α-TOS and methionine and electrostatic attraction with DMA. Thus, we hypothesized that PPT/D(DMA)@DOX system could effectively enhance tumor therapeutic efficacy with self-amplifiable drug release. In this study, 1H-NMR was used to confirm the successful synthesis of polymers. Several in vitro and in vivo characteristics were performed and the indexes indicated a great pH/ROS-sensitive antitumor efficacy of micelle, which would be potential nanocarrier for lung cancer therapy.
Scheme 1 Schematic illustration of the successive behaviors of the multifunctional micelle of the charge reversal PPT/D(DMA)@DOX system with self-amplifiable drug release for tumor therapy in vivo
Scheme 2 Synthesis routes of PPT/D(DMA)

Materials and methods

Materials

Methoxy poly(ethylene glycol) amine (mPEG-NH₂, \( M_n=5000 \)), doxorubicin hydrochloride (DOX) and \( \alpha \)-tocopheryl succinate (\( \alpha \)-TOS) were purchased from
Macklin Company, China. N(ε)-benzyloxycarbonyl-L-lysine and L-methionine was purchased from Energy Chemical, China. Dimethylmaleic anhydride (DMA) was purchased from Bidepharm, China. L-lysine-N-carboxyhydrde (Lys-NCA) were synthesized by the Fuchs-Farthing method using bis(trichloromethyl) carbonate (triphosgene) as reported earlier [6,22]. Propidium iodide and 4′, 6-diamidino-2-phenylindole dihydrochloride (DAPI) were purchased from Sigma Company. Annexin V-FITC Apoptosis Detection Kit was purchased from Kengen. All other reagents and solvents were purchased from Sinopharm Chemical Reagent Co., Ltd., China.

**Synthesis of methoxyl poly(ethylene glycol)-block-poly(L-lysine) (mPEG-b-PLL)**

Firstly, poly(ethylene glycol)-block-poly(N(ε)-benzyloxycarbonyl-L-lysine) (mPEG-b-PLL(Z)) was synthesized by ring open polymerization (ROP) of Lys(Z)-NCA in DMF using mPEG-NH₂ as initiator and the deprotection of benzyl groups as described earlier [22,23]. Typically, dried mPEG-NH₂ (1.0 g, 0.2 mmol) and Lys-NCA (1.8 g, 6.0 mmol) were added in a 50 mL dried glass reactor with 30 mL of DMF. After stirring at 25 °C for 3 days, the reaction solution was precipitated into 150.0 mL of ice ether three times to obtain pure mPEG-b-PLL(Z) polymers (Yield: 88.6%). The degree of polymerization (DP) of mPEG-b-PLL(Z) was determined to be 30 by ¹H NMR spectra (Figure S1). The number-average molecular weights (Mₙ) of mPEG-b-PBLG polymer caculated by ¹H-NMR was 12870 (mPEG₅₀₀₀₋b-PBLG(Z)₇₈₇₀). Subsequently, 1.6 g of mPEG-b-PLL(Z) was added into 10.0 mL of trifluoroacetic acid (TFA) and HBr/HOAc (0.8 mL) to remove Z groups. After stirring for 1 h under ice bath, the mixture was precipitated into 150.0 mL of ice diethyl ether, following dialyzed (molecular weight cut-off (MWCO) = 3500 Da) against distilled water, and then freeze-dried to obtain mPEG-b-PLL product and the yield was 75.6 %. The purified product was dried under vacuum at room temperature.

**Synthesis of methoxyl poly(ethylene glycol)-block-poly(L-lysine)-graft-α-tocopheryl succinate and methionine modified with dimethylmaleic**
anhydride (PPT/M(DMA))

The N-(tert-Butoxycarbonyl)-L-methionine (D(Boc)) was firstly prepared as described previously \textsuperscript{[24]}. Dried mPEG-\textit{b}-PLL (1.0 g, 0.11 mmol), D(Boc) (0.74 g, 3.30 mmol), NHS (1.71 g, 14.85 mmol) and DCC (3.06 g, 14.85 mmol) was dissolved in 30.0 mL of DMSO under nitrogen gas. After stirring at 25°C for 24 h, the reaction solution was firstly filtered and dialyzed against DMSO and then dialyzed against distilled water, following freeze-dried to obtain methoxyl poly(ethylene glycol)-\textit{block}-poly(L-lysine)-\textit{graft}-methionine (Boc) (PPT/D(Boc)) product (PPD(Boc)). The number of grafted D(Boc) was 24 from \textsuperscript{1}HNMR (Figure S2). PPD(Boc) (1.15 g, 0.08 mmol), \(\alpha\)-TOS (0.35 g, 0.66 mmol), N-hydroxysuccinimide (NHS, 0.15 g, 1.32 mmol) and \(N,N^\prime\)-dicyclohexylcarbodiimide (DCC, 0.27 g, 1.32 mmol) was dissolved in 30.0 mL of DMSO under nitrogen gas. After stirring at 25°C for 48 h, the reaction solution was firstly filtered dialyzed against DMSO and then dialyzed against distilled water, following freeze-dried to obtain methoxyl poly(ethylene glycol)-\textit{block}-poly(L-lysine)-\textit{graft}-\(\alpha\)-tocopheryl succinate and methionine (Boc) (PPT/D(Boc)) product (Figure S3). Subsequently, 0.8 g of PPT/D(Boc) was dissolved in 5.0 mL of DCM and 5.0 mL of TFA was added dropwise under ice bath. After stirring for 1.0 h, the mixture was filtrated and precipitated into 100.0 mL of ice diethyl ether, following dialyzed against distilled water, and then freeze-dried to obtain poly(ethylene glycol)-\textit{block}-poly(L-lysine)-\textit{graft}-\(\alpha\)-tocopheryl succinate and methionine (PPT/D) product. The shell was prepared by the reaction between PPT/D and DMA \textsuperscript{[25-26]}. Briefly, PPT/D and two times amount of DMA were dissolved in DMSO, then triethylamine (TEA) and pyridine was added followed by stirring under nitrogen protection at the room temperature overnight. The mixture was purified by dialysis (MWCO 3500 Da) against DMSO for 24 h, followed dialyzed in dialysis bag (MWCO 10,000 Da) for 24 h to remove DMSO, and lyophilized. The succinic anhydride (SA)-modified shell (Shell-SA) was prepared in the similar route and denoted as PPT/D(SA). PPD was obtained with deprotection of PPD(Boc) and modified with DMA without \(\alpha\)-TOS and denoted as PPD(DMA) (Figure S3).
Micelle preparation and characterization

The solvent exchange method was used to prepare micelles in this study. DOX·HCl (5.0 mg) was dissolved in DMF (1 mL) with TEA (2.6 mg) to remove the HCl of the DOX·HCl and stirred for 2 h. A amount of PPT/D(DMA) were dissolved in DMF (1 ml), followed added to the above solution and then stirred in dark for another 2 h. Then the solution was added to 5 mL PBS (pH 9.0) by using infusion pump at a constant rate of 2 mL/h and stirred for another 3 h. After that the solution was loaded into a MWCO 3500 dialysis bag and dialyzed against pH 8.0-9.0 water for 24 h. The obtained solution was mildly centrifuged and the supernatant was stored in 0 °C which would be used directly. The similar process was for other micelles. The DOX loading content was determined by lyophilizing 1.0 mL of the above solution and dissolved the obtained powder in DMSO. The concentration of the DOX-loaded micelles was measured at excitation and emission wavelengths of 485 nm and 550 nm. The drug-loading content (DLC) and drug-loading efficiency (DLE) of DOX micelles were calculated by the following equations.

\[
\text{DLC\%} = \frac{\text{weight of drug in nanoparticles}}{\text{weight of drug loaded nanoparticles}} \times 100\% \quad (1)
\]

\[
\text{DLE\%} = \frac{\text{experimental drug loading}}{\text{theoretical drug loading}} \times 100\% \quad (2)
\]

For the characterizations of the empty micelle or DOX-loaded micelles, the particle sizes, size distributions and zeta-potentials were measured by using a Zetasizer (Malvern 3000HSA). The morphologies of the micelles were identified by transmission electron microscopy (TEM) images obtained using a JEM-2000EXII transmission electron microscope with an accelerating voltage of 200 kV. The morphology changes of micelles were also evaluated when micelles were incubated in different pH solutions or H\textsubscript{2}O\textsubscript{2} solutions.

pH-sensitive property of PPT/D(DMA)@DOX

In order to evaluate pH-sensitive property of PPT/D(DMA)@DOX, the micelle
was tested compared with PPT/D(SA)@DOX. Two above micelles (500 μg/mL) were incubated in PBS at pH 7.4 and 6.8 for 200 min, respectively. At predetermined intervals, the mean diameter and zeta potentials of the micelles were measured by DLS.

**Drug release behavior**

To study the ROS responsibility of PPT/D(DMA)@DOX, 3.0 mg of micelles suspended respectively in 1.0 mL of PBS (pH=7.4) containing various concentrations of H₂O₂ (0, 0.1, and 10.0 mM) were sealed in a dialysis bag (MWCO 3.5 kDa). The dialysis tubes were subsequently immersed into glass tube containing 30.0 mL of PBS (pH=7.4) with same concentrations of H₂O₂. The fluorescence intensity of the supernatant of incubation solution was measured at given time intervals by using a Lambda Bio40 UV/Vis spectrometer. Likewise, Briefly, the same concentration of PPT/D(DMA)@DOX and PPT/D(SA)@DOX was immersed in PBS with various pH values (pH 7.4 and 6.8) at 37 °C under shaking at 100 rpm. The release media were taken out at predetermined times and an equal volume of fresh PBS was added. After that, the amounts of released DOX were detected.

**Cell culture**

Human lung adenoma cell lines A549 were purchased from the American Type Culture Collection (ATCC, Rockville). The cells were cultured in complete Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 U/mL streptomycin and grown in a 37°C humidified environment containing 5% CO₂.

**Evaluation of the ROS-responsiveness in cells**

ROS level changes in cells were determined with 2',7-dichlorofluoresceindiacetate (DCFA-DA) dye. A549 cells were seeded onto plates at a density of 1.0 × 10⁵ cells per well. After an incubation of 24 h, the cells were treated with empty micelles (PPT/D(DMA) or PPD(DMA)) for different
incubation times. And the cells without any treatment were used as control. After co-incubation for predetermined time, the cells were washed with PBS for three times and the media were replaced with DCFH-DA (10 μM) at 37 °C for 30 min. Finally, all the cells were observed by fluorescence microscopy and the green fluorescence intensity was measured with a microplate reader (ELIASA of Perkin Elmer) at 490 nm.

**Cell apoptosis assay**

To investigate the apoptotic effect of free DOX and DOX-loaded micelles, a flow cytometry (FCM) assay was performed. The extent of apoptosis in A549 was evaluated by FCM (ESP Elite, Beckman-Coulter, Miami, FL) analysis using FITC-conjugated AnnexinV/propidium iodide (PI, BD PharMingen) staining, following the manufacturer's instructions. Both early apoptotic (Annexin V-positive, PI-negative) and late apoptotic (Annexin V-positive and PI-positive) cells were included in cell death determinations.

**Cell uptake**

A549 cells were seeded in the glass bottom dishes at a density of 1.0×10^5 cells per well for 24 h. Then all the cells were incubated with PPT/D(SA)@DOX or PPT/M(DMA)@DOX (both containing 2.5 μg/mL DOX) for 2 h and 4 h at pH 7.4 or 6.8 respectively. Thereafter, the media were removed and the cells were washed with PBS to remove the extracellular micelles. The cell nuclei were stained by DAPI according to the standard protocol provided by the supplier. At last, the cellular uptake of samples was visualized under fluorescence microscopy.

**Cell viability study**

The cytotoxicity of free DOX, PPT/D(SA)@DOX and PPT/D(DMA)@DOX were evaluated by the MTT assay. A549 cells were seeded in 96-well plates at a density of 5000 cells per well in 100 μL DMEM containing 10% FBS and cultured for 24 h at 37°C. Then the cells were treated with 100 μL culture medium of pH 7.4 or
6.8 containing fixed amount of micelles for 48 h. After that the medium was replaced with 200 μL of fresh DMEM and 20 μL MTT (5 mg/mL in PBS) and incubated for another 4 h. Then the medium was removed and 200 μL DMSO was added. The optical absorbance was measured at 450 nm of each well using a microplate reader. The cell viability (%) was determined by comparing the absorbance at 450 nm with control wells containing only cell culture medium. All the cytotoxicity tests were conducted in triplicate.

$$\text{Cell viability (\%)} = \frac{\text{OD}_{\text{sample}} - \text{OD}_{\text{blank}}}{\text{OD}_{\text{control}} - \text{OD}_{\text{blank}}} \times 100\%$$  \hspace{1cm} (3)

**In vivo antitumor study and histochemistry analysis**

Nude mice (5-6 weeks old) were purchased from Beijing Institution for Drug Control, China. A549 cell tumor-bearing mice model was established by subcutaneous injection of A549 cells (2×10⁶) into the right groin side of each mouse. In vivo/ex vivo imaging and biodistribution experiments were performed around about day 10 after tumor cell injection, by which time tumors had grown to 0.8 cm in diameter. At that time, A549 cell tumor-bearing nude mice were injected intravenously with PBS, free DOX, PPT/D(SA)@DOX and PPT/D(DMA)@DOX micelles at a dose of 0.5 mg/kg DOX. The mice were sacrificed after 24 h post-injection. At 24 h post-injection, the mice were sacrificed, and the hearts, livers, spleens, lungs, kidneys, and tumors were excised to directly observe the fluorescence distribution. The emission fluorescence was collected from 450 to 700 nm, with the 455 nm excitation filter used.

Moreover, when the tumor volume reached 50 mm³, A549 tumor-bearing mice were divided into 4 groups randomly (n=4) and treated with PBS, free DOX, PPT/D(SA)@DOX and PPT/D(DMA)@DOX (fixed concentration of DOX at 2 mg/kg) through intravenously injection at day 0, 3, 6 and 9 respectively. And tumor volumes and body weights of all the mice were recorded every two days. Tumor volumes (V) and body weights were measured to evaluate the antitumor activity and systemic toxicity. Tumor volume (V) was calculated using the following formulas:
\[ V = \frac{a \times b^2}{2} \]  

where \( a \) and \( b \) were major and minor axes of the tumors measured by a caliper, respectively and all the mice were sacrificed at day 16. Tumors were dissected, washed and used for histology analysis. Cell state in tumor tissue was analyzed by hematoxylin-eosin (H&E) staining. Simultaneously, to evaluate the levels of apoptosis in tumor areas, tumor tissues were stained by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (Tunel) according to the manufacturer's protocol (Roche, Penzberg, Germany). Ki-67-stained sections were observed under a microscope (X51 Olympus; Olympus Corp. Tokyo, Japan).

**Statistical analysis**

The results were presented as mean ± standard deviation (SD). Statistical significance was analyzed using Student’s t-test.

**Results and discussion**

**Synthesis and characterization of micelles**

In this study, we have synthesized a biocompatible pH/ROS-responsive micelle via ROP polymerization and stepwise chemical grafting reactions as illustrated in Scheme 2. Successful synthesis of PPT/D(DMA) was confirmed using \(^1\)H-NMR as shown in the Figure 1A. PPT/D(DMA) \(^1\)H NMR; 500 MHz, DMSO-d₆, ppm): 0.82 (CH₃- from α-TOS); 1.00-1.70 (-CH₂CH₂CH₂CH₂- from mPEG and CH₃- from α-TOS); 1.88 broad (-CH₂- from chromanol ring and CH₃- from DMA); 1.9-2.1 (CH₃- from chromanol ring and CH₃-S- from methionine); 2.74 (g, -CH₂- from α-TOS); 2.80-2.90 (f, -CH₂- from α-TOS); 3.51 (-CH₂CH₂-O- from mPEG). The repeating units in PPT/D(DMA) were 1:30:6:24 for mPEG, PLL, α-TOS and D, respectively, as calculated from the \(^1\)H-NMR results. \( M_n \) of PPT/D(DMA) calculated by \(^1\)H-NMR was 22.1 kDa, similar with the data of 23000 g/mol measured by GPC with polydispersity of 1.25. The empty micelle and DOX-loaded micelles (denoted as PPT/D(DMA) and PPT/D(DMA)@DOX) were prepared with the dialysis method. The PPT/D(DMA) block polymer was dissolved in DMSO and then the solvent was gradually removed.
for self-assembly though solvent exchange processes via dialysis. Fluorescence study indicated that the resulting PPT/D(DMA) micelle had a relatively low critical micelle concentration (CMC) of 1.74 μg/mL (Figure 1B). Average size of blank micelle and DOX-loaded micelle was measured as shown in Table 1 and Figure 1C, D. The mean diameter of empty micelle was found to be 98.1 ± 4.5 nm. When DOX was encapsulated in the nanocarriers in proportion of 10%, the mean size of micelles was decreased to 84.3 ± 3.6 nm with a particle size distribution of PDI = 0.108. The DLC and DLE were calculated to be 9.64% and 96.4%, respectively.

To investigate the sensitivity of micelles to pH and ROS, TEM and DLS were used to study the morphology and size change of PPT/D(DMA)@DOX micelle in response to pH and ROS stimuli. The micelle was characterized in terms of size and surface morphology at pH 6.8 after 24 h incubation and with H2O2 for 6 h as shown in Figure 1E, F. At pH 7.4, the average size of the PPT/D(DMA)@DOX was 84.3 ± 3.6 nm, which increased to 116.2 ± 6.3 nm at pH 6.8. This might result from the swell of exposed amine from methionine segments with positive charge after the cleavage of DMA groups from the micelle at pH 6.8 [27]. Similarly, the TEM image also revealed increase in the size at pH 6.8. Moreover, PPT/D(DMA)@DOX exhibited a disintegrated morphology in the existence of H2O2. The destruction of the micelle structures was also determined with DLS and two peaks containing a large aggregated form were observed, indicating the high sensitivity of the micellar system to ROS. The underlying mechanism was that thioether groups in methionine segments could be changed to hydrophilic sulfoxide groups by ROS and then induced the disassembly of micelle [20, 28].
Fig. 1  

a $^1$H-NMR spectra of PPT/D(DMA) in DMSO-d$_6$.  
b Dependence of excitation fluorescence intensity ratio of pyrene (I$_{338}$/I$_{333}$) on the logarithmic concentration of PPT/D(DMA).  
c DLS and TEM images of PPT/D(DMA) blank micelles in PBS (pH 7.4).  
d DLS and TEM images of PPT/D(DMA)@DOX micelles in PBS (pH 7.4).  
e DLS and TEM images of PPT/D(DMA)@DOX micelles at pH 6.8 after 24 h incubation.  
f DLS and TEM images of PPT/D(DMA)@DOX micelles at pH 7.4 with H$_2$O$_2$ for 6 h

Tab. 1 Investigation in DLC and DLE of DOX micelles

| Number | DOX:Polymer ratio (w/w) | Designed DLC(%) | Measured DLC(%) | DLE(%) | Size (nm) (PDI)$^{a}$ |
|--------|-------------------------|-----------------|-----------------|--------|-------------------|
| 1      | 0:100                   | 0               | -               | -      | 98.1(0.165)       |
| 2      | 10:90                   | 10              | 9.64            | 96.4   | 84.3(0.108)       |

$^{a}$Average diameter and PDI of the nanoparticles measured by DLS.
**pH and ROS-responsive property and drug release**

In this work, the preparation and drug release behavior of PPT/D(DMA)@DOX was used illustrated in Figure 2A. In order to assess the charge-reversal property and stability of PPT/D(DMA)@DOX in pH 7.4 and 6.8, we investigated the changes of zeta potential and size in PBS (pH 7.4 and 6.8) in 200 min. When the microenvironment changed from physiological pH (pH 7.4) to slightly acidic conditions (pH 6.8), the negatively charged PPT/D(DMA)@DOX can turn to positive charge owing to the conversion reaction between carboxyl groups and amino groups (Figure 2B). By contrast, the zeta potentials of PPT/D(SA)@DOX were negatively charged at both pH 7.4 and 6.8. This result suggested that PPT/D(DMA)@DOX had a charge-reversal property at pH 6.8, which was attributed to the cleavable amide linkages formed between methionine segments and DMA. To further evaluate the stability in physiological and tumor microenvironment, we measured the size changes of PPT/D(DMA)@DOX and PPT/D(SA)@DOX. From Figure 2C, the average sizes of them remained their initial sizes and exhibited a good stability at pH 7.4 within 200 min. Nevertheless, at pH 6.8, the average size of PPT/D(DMA)@DOX decreased from 84.3 ± 3.6 nm to 72.2 ± 4.4 nm in 100 min, whereas that of PPT/D(SA)@DOX was slightly increasing in 200 min. The above results demonstrated that PPT/D(DMA)@DOX was negatively charged during the blood circulation (pH 7.4), which could reduce the interaction with negatively charged proteins in the blood. When it reached the tumor microenvironment (pH 6.8), the surface of micelle exhibited a charge reversal and turned into positive charge, which would be taken up easily by tumor cells.

Subsequently, the quantitative analysis was employed to investigate the pH and ROS-responsive drug release behavior of micelles. As shown in Figure 2D, the DOX release profiles of the micelles (PPT/D(SA)@DOX and PPT/D(DMA)@DOX) were monitored at different pH values (pH 7.4 and 6.8) using the dialysis method. The amounts of DOX released from the micelles were all less than 30% within 60 h at pH 7.4, which validated that these systems were relatively stable and the release rate of DOX was rather slow under physiological conditions. At pH 6.8, the released DOX...
drugs from PPT/D(SA)@DOX and PPT/D(DMA)@DOX were increased to 36.6% and 46.2% respectively, which were markedly higher than that of pH 7.4. These micelles all exhibited significant pH-dependent release behavior. This can be ascribed to the fact the protonation of carboxylic groups of glutamic acid, weakening the electrostatic interaction of DOX with carboxylic groups [31]. Moreover, the surface of PPT/D(DMA)@DOX was turned to positive charge at pH 6.8, which could absorb water to swell, further increasing of the DOX release rate [32]. As shown in Figure 2E, around 62% DOX released from PPT/D(DMA)@DOX micelle upon treatment with $0.1 \times 10^{-3}$ mol/L H$_2$O$_2$ for 60 h. Furthermore, when the H$_2$O$_2$ concentration increased to $10 \times 10^{-3}$ mol/L, the release percentage reached 74%. It again indicated the triggering effect of H$_2$O$_2$ concentration on drug release. These results consistently demonstrated the stability of the system under physiological condition and their ROS responses for controllable drug release.

Fig. 2 a Illustration of the preparation procedure and pH/ROS-dual sensitivity of PPT/D(DMA)@DOX. The micelle took out DMA at pH 6.8 to expose the positive
charges, and the release of DOX was triggered quickly by the ROS below pH 6.8. The zeta potential PPT/D(DMA)@DOX and PPT/D(SA)@DOX after incubation at pH 7.4 or 6.8 for 200 min. Average size of PPT/D(DMA)@DOX and PPT/D(SA)@DOX after incubation at pH 7.4 or 6.8 for 200 min. Cumulative release of DOX from PPT/D(DMA)@DOX and PPT/D(SA)@DOX micelles at pH 7.4 or 6.8. Cumulative release of DOX from PPT/D(DMA)@DOX and PPT/D(SA)@DOX micelles at pH 7.4 after treatment with various concentrations of H2O2

**Analysis of the ROS regenerating ability of PPT/D(DMA) in vitro**

In order to better explore the feasibility of positive feedback strategy to overcome the obstacles in ROS-responsive PPT/D(DMA)@DOX, the prevailing intracellular ROS sensitive probe 20,7-dichlorofluoresceindiacetate (DCFH-DA) was utilized to confirm the ROS generation [33]. Cell permeable nonfluorescent DCFH-DA could be rapidly oxidized to dichlorofluorescein (DCF) with green fluorescence by the intracellular ROS. As shown in Figure 3A and B, the conspicuous green fluorescence of DCF in A549 cells clearly proved the inherent existence of intracellular ROS. When the A549 cells were incubated with PPT/D(DMA) micelle, the green fluorescent signal was noticeably stronger than that without PPT/D(DMA) micelles, owing to the ROS generating capability of α-TOS segments, which could restrain the bioactivity of mitochondrial respiratory complex II, resulting in the electron transfer to produce ROS from oxygen [13]. In addition, after prolonging the incubation time, the green fluorescence in A549 cells became stronger. Further, compared to the A549 cells as the control for the same time, the fluorescence signal in A549 cells with the treatment of PPT/D(DMA) micelles for 10 h incubated with DCFH-DA increased approximately 8 folds measured by microplate reader (Figure 3B). The results above confirmed the intracellular ROS producing ability of α-TOS segments and also revealed the possibility of PPT/D(DMA) for positive feedback strategy.
Apoptosis assay

In vitro antitumor activities of the PPT/D(DMA)@DOX micelles were analyzed by apoptosis assay using FITC Annexin V and Propidium Iodide (PI). As shown in Fig. 3C-D, in the control cells, negligible apoptotic cells were confirmed in the A549 cells. After 24 h, the apoptosis percentage of free DOX and PPT/D(SA)@DOX micelles treated cells was 14.4% and 25.6%; however, the percentage of PPT/D(DMA)@DOX micelles treated cells exhibiting apoptosis was 34.3%. PPT/D(DMA)@DOX micelles had relative high percentages of apoptotic cells, confirming the enhanced antitumor efficacy of PPT/D(DMA)@DOX system [34].

In vitro cytotoxicity

To investigate the cytotoxicity of free DOX, PPT/D(SA)@DOX and PPT/D(DMA)@DOX, the MTT assay in A549 cells was performed at pH 7.4 and 6.8. From Figure 5E, all the groups presented a DOX concentration-dependent growth inhibition effect on A549 cells. When the DOX concentration increased to 2 μg/mL, all micelles groups exhibited a lower cell viability than the free DOX group, indicating that the micelles improved the cytotoxicity of DOX. As for the free DOX group, there was no significant difference in cytotoxicity at pH 7.4 and 6.8. In contrast, the cells incubated with PPT/D(SA)@DOX and PPT/D(DMA)@DOX exhibited a higher cytotoxicity at pH 6.8 than pH 7.4. Moreover, half-maximal inhibitory concentration (IC50) of PPT/D(SA)@DOX and PPT/D(DMA)@DOX micelles for 48 h was 1.08 μg/mL and 0.75 μg/mL respectively at pH 6.8 meaning that the pH-sensitive micelles with charge reversal promoted the phagocytosis effect of tumor cells and enhanced the DOX accumulation in tumor cells [35].
Fig. 3  

**a** Evaluation of the ROS regenerating ability of PPT/D(DMA) *in vitro*. Fluorescence images of A549 cells treated with 50 μM PPT/D(DMA) for different time. PPD(DMA) Green channel: DCF fluorescence illustrated intracellular ROS. Scale bar: 20 μm.  

**b** Quantitative analysis the ROS of corresponding A549 cells by microplate reader.  

**c** Apoptosis analysis of A549 cells induced by PBS (control), DOX (2.0 μg/mL), PPT/D(SA)@DOX and PPT/D(DMA)@DOX (20.0 μg/mL, equivalent of 2.0 μg/mL DOX).  

**d** Quantification of apoptosis result.  

**e** Cell viability of A549 cells treated with free DOX, PPT/D(SA)@DOX and PPT/D(DMA)@DOX at various concentrations of DOX at pH 7.4 or 6.8 (n = 4). All error bars present as mean ± SD.
*p < 0.05, **p < 0.01

**Cellular uptake study**

To explore the mechanisms of exerted cytotoxicity and apoptosis effect of PPT/D(SA)@DOX and PPT/D(DMA)@DOX, we used the cellular uptake of PPT/D(SA)@DOX and PPT/D(DMA)@DOX on A549 cells at pH 7.4 or 6.8 by fluorescence microscopy. As demonstrated in Figure S5 and Figure 4, upon culturing with PPT/D(SA)@DOX and PPT/D(DMA)@DOX at pH 6.8, cells exhibited much stronger DOX fluorescence compared to those at pH 7.4, especially after 4 h incubation. Figure 4 showed the images of cells treated with free DOX and DOX-NPs for 2 h and 4 h, respectively. In the PPT/D(SA)@DOX group, slight red fluorescence was observed at 2 h after incubation and fluorescence intensity was increased after 4 h incubation. Meanwhile, the more fluorescence in cytosol and nucleus was observed. Moreover, PPT/D(DMA)@DOX group could rapidly accumulate in the cytosol after 2 h incubation, which was revealed by the slight red fluorescence. After 4 h, the more fluorescence nucleus was observed. By contrast, PPT/D(DMA)@DOX showed higher fluorescence intensity than PPT/D(SA)@DOX, indicating that PPT/D(DMA)@DOX had the strongest cellular uptake effect at pH 6.8, which was in accordance with the MTT results. These data demonstrated that the charge-reversal could obviously enhance cellular uptake in the tumor microenvironment [36].
**Fig. 4** Fluorescence images of A549 cells incubated with PPT/D(SA)@DOX and PPT/D(DMA)@DOX with the same concentration of 2.0 μg/mL for 2 h or 4 h at pH 6.8. The scale bar is 100 μm. Blue: DAPI; red: DOX

**Ex vivo imaging**

To verify the biodistribution of DOX during systemic circulation, the mice after injection with saline, free DOX, PPT/D(SA)@DOX and PPT/D(DMA)@DOX for 24 h were sacrificed, and various organs and tumors were isolated to image *ex vivo* along with intensity detected. As showed in Figure 5A and B, for free DOX group, the liver, spleen, kidney and tumor showed the DOX fluorescence at 24 h post-injection and the fluorescence intensity of liver was strongest. The results suggest that they were captured mainly in the organs such as liver and kidney leading to severe organ damage and low drug efficacy. By contrast, weaker fluorescence in the liver and stronger fluorescence in the tumor was observed in PPT/D(SA)@DOX and
PPT/D(DMA)@DOX micelles group, indicating that DOX-loaded micelles could significantly improve the tumor targeting and accumulation, which was on account of the EPR effect. Furthermore, the tumor fluorescence intensity of PPT/D(DMA)@DOX group was distinctly was much stronger than those of other groups, which further demonstrated the potency of charge-reversal in enhancing the cellular uptake to improve the accumulation and prolong the retention of DOX at the tumor site \[37\].

**In vivo tumor therapy test**

A xenograft model was constructed by implanting BALB/c nude mice with $2.0 \times 10^6$ A549 cells. Tumor volumes and body weights were measured every two days and shown in Figure 5D and E. Different treatments induced various extents of tumor growth suppression compared to control (saline). In comparison, the tumor growth inhibition of PPT/D(SA)@DOX and PPT/D(DMA)@DOX groups was higher than free DOX, indicating the enhanced antitumor efficiency of the micelle system. The body weight loss is another important indicator for evaluating drug-induced toxicity. Treatment with free DOX resulted in the greatest body weight loss (8.4%) compared with DOX-loaded micelles (~5.2%), which revealed that free drug had significant treatment-related toxicities. More importantly, PPT/D(DMA)@DOX induced the greatest tumor suppression among all groups without body weight loss, and the tumor size was significantly reduced after treatment, indicating the superior antitumor effects. The reasons could be elucidated as follows. Firstly, the long-circulating PEG layer and negative charged surface of PPT/D(DMA)@DOX system could prolong the circulation time and increase nanocarrier accumulation at tumor sites through EPR effect \[38\]. Secondly, tumor acidity-activating charge conversion could effectively improve cell uptake of PPT/D(DMA)@DOX. Moreover, after internalization, the endogenous ROS would induce micelle disassembly and drug release, and the exposed $\alpha$-TOS segments could further produce ROS for amplifying micelle disassembly and drug release \[39, 40\]. Above reasons contributed to the superior therapeutic efficacy of PPT/D(DMA)@DOX system.
H&E and Ki67 with immunofluorescence staining were performed to further confirm the enhanced antitumor activity of the PPT/D(DMA)@DOX system based on proliferation activity. As shown in Figure 5F, PPT/D(SA)@DOX and PPT/D(DMA)@DOX groups inhibited proliferation markedly than free DOX, as revealed by the distinct cell shrinkage and chromatin condensation in H&E observation and fewer magenta dots co-located with nuclei in Ki67 images. Meanwhile, the proliferation of mice treated with PPT/D(DMA)@DOX was lowest, which was consistent with Tunel assay for tumor tissues. PPT/D(DMA)@DOX group displayed the highest level of Tunel expression among all groups, meaning the severe cell apoptosis. The results demonstrate that PPT/D(DMA)@DOX could effectively deliver DOX to tumor and induce tumor cells apoptosis with high efficiency in vivo.
**Fig. 5 a** *Ex vivo* representative fluorescence imaging of various organs and tumor tissue from mice treated with Control, DOX, PPT/D(SA)@DOX, and
PPT/D(DMA)@DOX. The mice were sacrificed at 24 h post-injection. 

b Quantitative analysis of organ distribution of DOX in tumor-bearing mice 24 h after intravenous injection. 

c Photographs of the tumors extracted from A549 tumor cell-bearing mice after treatments with Control, DOX, PPT/D(SA)@DOX and PPT/D(DMA)@DOX (equivalent of 2 mg/kg DOX) for 16 days. 

d The volume growth curves of tumors at different time points post-treatment in 4 groups (n = 4 per group). 

e Effect of different treatments on body weight. 

f Images of H&E, Tunel and Ki67 of tumor sections, respectively. Scale bar: 100 μm. Error bars present as mean ± SD (n = 4). *p < 0.05, **p < 0.01

Conclusions

In summary, we developed a pH/ROS-responsive micelle drug delivery system with charge reversal and self-amplifiable drug release for tumor therapy. The micelle with negatively charged surface in blood had a great ability of prolonging circulation time; the charge reversal occurred followed by the exposure of positively charged amine of methionine due to pH responsiveness in tumor tissue, resulting in excellent cell membrane penetrating. It was found that owing to the ROS-responsive thioether groups, this designed nano-system could disassemble and deliver the drug to tumor cells and produce the cell toxicity. Moreover, exposed α-TOS segments would lead to the augmented concentration of intracellular ROS and accelerate release of DOX. Due to its unique advantages such as efficient cellular uptake and triggering targeted drug release, this designed system with charge reversal will exhibit great potential for achieving better therapeutic effects in cancer treatment.

Abbreviations

DMA: dimethylmaleic anhydride; PPT/D(DMA): methoxyl poly(ethylene glycol)-block-poly(L-lysine)-graft-α-tocopheryl succinate and methionine modified with dimethylmaleic anhydride; DOX: doxorubicin; α-TOS: α-tocopheryl succinate; PBS: Phosphate buffer saline; TEM: Transmission electron microscopy; ROS: reactive oxygen species; CMC: critical micelle concentration.
Authors’ contributions
Xinyi Zhang drafted the manuscript, performed the experiment and analyzed the data. Tiantian Zhu performed the experiment, analyzed the data and modified the manuscript. Yaxin Miao performed the experiment, analyzed the data and purchased reagent. Lu Zhou performed the experiment and analyzed the data. Weifang Zhang firstly conceived, designed the study and thereafter supported experimental guidance and modification of manuscript.

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Acknowledgements
Not applicable.

Competing interests
The authors declare no competing financial interest.

Availability of data and materials
All data generated or analyzed during this study are included in the article and additional file. The additional file is available.

Consent for publication
All authors have provided consent for the manuscript to be published.

Ethical approval
All experiments in the research were conducted under a protocol approved by the Institutional Animal Care and Use Committee at Southeast University, China.

**Funding**
The authors are grateful for the financial support provided by the National Natural Science Foundation of China (Nos. 81960015 and 81800051), the Youth Science Foundation from the Science and Technology Department of Jiangxi province (Nos.20171BAB215002 and 20171BAB215040), Project of Jiangxi Provincial Health and Family Planning Commission (No.20165188); Project of Jiangxi Provincial Department of Education (No.170107) and the Youth Science Foundation from the Second Affiliated Hospital of Nanchang University (No. 2016YNQN12038).

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