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Co-delivery of Doxorubicin and Afatinib with pH-responsive Polymeric Nanovesicle for Enhanced Lung Cancer Therapy

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Abstract Drug-resistance and drastic side effects are two major issues of traditional chemotherapy which may result in trial failure even death. Nanoparticle-mediated multidrug combination treatment has been proven to be a feasible strategy to overcome these challenges. In the present study, amphipathic block polymer of methoxyl poly(ethylene glycol)-poly(aspartyl(dibutylethylenediamine)-co-phenylalanine) (mPEG-P(Asp(DBA)-co-Phe)) was synthesized and self-assembled into pH-responsive polymeric vesicle. The vesicle was utilized to co-deliver cancer-associated epidermal growth factor (EGFR) inhibitor of afatinib and DNA-damaging chemotherapeutic doxorubicin hydrochloride (DOX) for enhanced non-small-cell lung cancer (NSCLC) therapy. As evaluated *in vitro*, the pH-responsive design of nanovesicle resulted in a rapid release of encapsulated drugs into tumor cells and caused enhanced cell apoptosis. In addition, *in vivo* therapeutic studies were conducted and the results evidenced that the co-delivery of DOX and afatinib using pH-sensitive nanovector was a promising strategy for NSCLC treatment.

Keywords Nanovesicle; Polymeric vector; Combination therapy; pH-responsive

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INTRODUCTION

To date, lung cancer has become the most life-threatening malignant tumor by causing more than 1.5 million deaths each year.^[1] Even worse, the incidence and mortality of lung cancer increase rapidly in recent years due to the prevalence of cigarette smoking.^[2,3] Approximately 85% of lung cancers are diagnosed as non-small-cell lung cancer (NSCLC).^[4] As one of the standard treatments for NSCLC, chemotherapy is facing tremendous challenges including drug-resistance and the severe side effects to normal tissues, both of which will lead to trial failure and even death.^[5,6] In this case, there is an urgent clinical demand to develop new strategies for enhanced NSCLC treatment.

Combined pharmacotherapy is one of the effective methods. The multiple drugs affect survival pathways and result in complementary and synergistic effects in killing tumor cells.^[7–9] In addition, combination therapy reduces the dosage of drugs compared to monotherapy, leading to a decreased possibility of side effects and drug resistance.^[10,11] For example, it has been validated that NSCLC cells can be significantly sensitized to the DNA-damaging agents like doxorubicin (DOX) when the cancer-associated epidermal growth factor receptor (EGFR) signaling pathway is suppressed

by EGFR tyrosine kinase inhibitors (EGFR-TKIs).^[12] Specifically, EGFR-TKIs such as gefitinib, erlotinib, and afatinib can block the signaling pathway *via* binding covalently and irreversibly to the intracellular tyrosine kinase domain, then induce the rewiring of signaling networks with persistent EGFR inhibition, and finally lead to a more effective DNA damaging efficacy of DOX.^[13,14] However, the poor water solubility of TKIs^[15] as well as the distinct pharmacokinetics and biodistributions of different drugs limit the clinical success of combination therapy.^[16,17]

In recent years, nanovector-mediated drug delivery has been extensively developed due to its promising properties.^[18,19] Specifically, nanoparticles can improve the water solubility of hydrophobic drugs, prolong the half-life of small molecule drugs, increase the tumor accumulation *via* an enhanced permeation and retention (EPR) effect,^[20–22] and reduce the toxic side effects by the achievement of controlled drug release.^[23,24] For example, in our previous study, an interlayer-crosslinked micelle with reduction and pH dual sensitivity was used for DOX delivery. With the reduced drug leakage in bloodstream and increased DOX release in tumor cells, the therapeutic effect was significantly enhanced.^[25] Most importantly, it is entirely possible that nanoparticles encapsulate different drugs into one whole to endow them with the same biodistribution and pharmacokinetics,^[26] which conduces to an efficient and manageable drug administration. Among the nanosized delivery systems, poly-

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meric vesicles are frequently used for the delivery of multiple drugs owing to their potential to simultaneously encapsulate hydrophilic agents and hydrophobic ones into the aqueous core and membrane, respectively.^[27,28]

Herein, we developed a pH-responsive polymeric vesicle to co-deliver DOX and afatinib for the combination therapy of NSCLC. The polymeric vesicles were prepared from the self-assembly of amphiphilic polymer methoxyl poly(ethylene glycol)-poly(aspartyl(dibutylethylenediamine)-co-phenylalanine), *i.e.*, mPEG-P(Asp(DBA)-co-Phe). The PEG block served as hydrophilic corona to increase the stability and biocompatibility of vesicles. Additionally, the hydrophobic membrane of the vesicles consisted of P(Asp(DBA)-co-Phe) blocks, in which the PAsp(DBA) block could be either hydrophobic in normal physiological environment or hydrophilic after protonation in the acidic lysosome of tumor cells (Scheme 1). In this case, the vesicles were expected to keep stable in bloodstream and release the encapsulated drug into tumor cells rapidly in response to the pH change. With the highly efficient co-delivery of DOX and afatinib, the drug-loaded vesicles could exert an enhanced antitumor effect both *in vivo* and *in vitro*.

EXPERIMENTAL

Materials

Methoxy- ϵ -hydroxy-poly(ethylene glycol) (mPEG-OH, $M_n = 2$ kDa) and dibutylethylenediamine (DBA) were purchased from TCI (Shanghai, China). β -Benzyl-L-aspartate (BLA, Adamas-beta, China), L-phenylalanine (Phe, J&K, China), fluorescein diacetate (FDA, J&K, China), and doxorubicin hydrochloride (DOX·HCl, Aladdin, China) were used as received. Dialysis bags (MWCO: 3.5 and 14 kDa) were purchased from Green Bird Technology Development Co., Ltd. (Shanghai, China). Anhydrous dimethyl sulfoxide (DMSO) and anhydrous *N,N*-dimethylformamide (DMF) were purchased from Sigma Aldrich (St. Louis, MO, USA). Menthol, ethanol, diethyl ether, and chloroform (CHCl₃) were purchased from Guangzhou Chemical Reagent Factory (Guangzhou, China). CHCl₃ was dried over CaH₂ and then distilled under ambient pressure. Other reagents were used without further purification. Methoxy- ϵ -amino poly(ethylene glycol) (mPEG-NH₂) was synthesized from mPEG-OH according to the literature.^[29] *N*-carboxyanhydride of β -benzyl L-aspartate (BLA-NCA) and *N*-carboxyanhydride of L-phenylalanine (Phe-NCA) were synthesized from BLA and Phe separately as reported procedure.^[30]

Human esophageal cancer A549 cell lines and BALB/C nude mice (male, 6–8 weeks) were purchased from the Animal Experiment Centre of the Sun Yat-sen University. Cell culture medium, phosphate buffer saline (PBS), fetal bovine serum (FBS), 1% penicillin-streptomycin double antibody, and Trypsin-EDTA (0.25%) solution were purchased from Gibco (Carlsbad, CA.). DAPI was purchased from Beyotime Biotech (Shanghai, China). Paraformaldehyde (4%) was purchased from KeyGEN BioTECH Corp., Ltd. (Nanjing, China). Hematoxylin and eosin (H&E) staining kit was purchased from Huntz Enterprises Inc. (Shanghai, China). MTT cell cytotoxicity assay kit was purchased from Roche Diagnostics Ltd-(Shanghai, China). Fragel™ DNA fragmentation detection kit was purchased from Merck (Darmstadt, Germany).

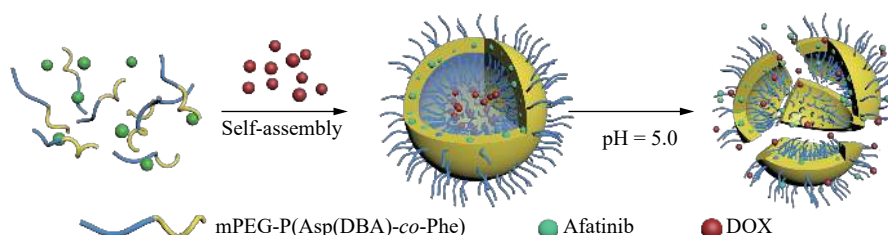
Synthesis of mPEG-P(Asp(DBA)-co-Phe)

Methoxy poly(ethylene glycol)-poly(β -benzyl L-aspartate-co-L-phenylalanine), *i.e.* mPEG-P(BLA-co-Phe), was synthesized by ring-opening polymerization with mPEG-NH₂ as the macroinitiator. Firstly, 0.33 g of PEG-NH₂ was vacuum-dried at 70 °C for 4 h in a 50 mL flask under nitrogen protection and then dissolved in 30 mL of anhydrous CHCl₃ after cooling to room temperature. Subsequently, 1.00 g of BLA-NCA and 2.61 g of Phe-NCA dissolved in 3 mL of anhydrous DMF were added into the PEG solution. The reaction was kept stirring for 48 h at 35 °C. After reaction, the solution was precipitated into excessive cool diethyl ether. The precipitate was centrifuged, washed with diethyl ether, and vacuum-dried to obtain the product as white solid (mPEG-P(BLA-co-Phe), $M_n = 22.7$ kDa as calculated from ¹H-NMR spectrum, yield: 96.8%).

mPEG-P(Asp(DBA)-co-Phe) was obtained by the aminolysis reaction of mPEG-P(BLA-co-Phe) with DBA. Briefly, 1.50 g of mPEG-P(BLA-co-Phe) and 2.02 mL of DBA were dissolved in 25 mL of anhydrous DMSO under nitrogen protection. Then the solution was kept stirring for 48 h at 35 °C. The solution was dialyzed (MWCO: 3.5 kDa) against methanol for 48 h after the reaction, and the solvent was removed by rotary evaporation to get the crude product. The crude product was redissolved in 20 mL CHCl₃, precipitated into diethyl ether, centrifuged, washed with diethyl ether, and finally dried under vacuum to obtain mPEG-P(Asp(DBA)-co-Phe) as light yellow solid ($M_n = 27.4$ kDa as calculated from ¹H-NMR spectrum, yield: 88.5%).

Preparation of the Nanovesicles

The dual-drug-loaded (A/D-PADP) vesicle was prepared by the double emulsion method as reported before.^[31] Briefly,



Scheme 1 Schematic illustration of the dual-drug-loaded vesicle preparation and the pH-triggered release of DOX and afatinib

20 mg of mPEG-P(Asp(DBA)-co-Phe) and 0.5 mg of afatinib were dissolved in 3 mL of CHCl_3 , then mixed with 0.3 mL of purified water containing 2 mg of DOX·HCl under sonication using a probe sonicator (VCX130, Sonics, 20 kHz, 40% power level). The obtained emulsion was emulsified again into 20 mL of purified water by sonication. Then CHCl_3 in the final emulsion was removed by a rotary evaporator. The vesicle solution was concentrated and washed using the centrifugal filter tube (MILLIPORE, MWCO: 100 kDa) to remove free DOX, then filtered through a 0.45 μm filter membrane to eliminate free afatinib and big aggregation. The blank (B-PADP) vesicle without drug encapsulation, the single DOX-loaded (D-PADP) vesicle, and the single afatinib-loaded (A-PADP) vesicle were prepared similarly without the addition of corresponding drug(s). Besides, the vesicle containing DOX and FDA (F/D-PADP) was prepared by replacing afatinib with FDA.

Characterization of the Polymers and Nanoparticles

^1H -NMR spectra of the polymers were acquired on an AVANCE III 400 MHz spectrometer using DMSO-d_6 as the solvent. Fourier transform infrared (FTIR) spectral measurements were conducted using a FTIR spectrometer (Nicolet NXR 9650, USA) with a resolution of 2 cm^{-1} and the powder samples were compressed into KBr pellets. The molecular weight distributions of the polymers were tested by gel permeation chromatography (GPC) system (Waters Breeze, USA) equipped with a Waters 1515 pump, an Ultrahydrogel™ 500 column, an Ultrahydrogel™ 250 column, and a Waters 2417 differential refractive index detector. The GPC measurements were carried out using DMF as the eluent at a flow rate of 1.0 mL/min. Methoxyl poly(ethylene glycol) (PEG) was used as a pre-established calibration standard. After the vesicle solution was obtained, the morphology of the A/D-PADP vesicle was analyzed using the transmission. The grid was dried overnight and then stained for electron microscopy (Hitachi JEM-1400, Japan) at 120 kV. The TEM samples were prepared by dropping the vesicle solution (10 μL , 1 $\mu\text{g}/\mu\text{L}$) on a copper grid coated with amorphous carbon. The grid was dried overnight, then stained with uranyl acetate solution (2 wt% in water) for 1 min. The sample was dried overnight at 40 °C before observation. The size distribution of the vesicle was determined using a dynamic light scattering (DLS) measurement device (Brookhaven 90 Plus/BI-MAS, USA). The data were collected on an autocorrelator with a detection angle of scattered light at 90°. Each sample was measured five times, and the collected data were averaged to obtain the results.

Loading Content of DOX and Afatinib

To determine the drug loading content of DOX, the absorbance of the DOX-loaded vesicle was measured by a UV-Vis spectrophotometer (Thermo AVATER 330FT-IR, USA). Briefly, the freeze-dried vesicle was weighed, then redissolved in 3 mL of $\text{CHCl}_3/\text{DMSO}$ (1/3, *V/V*) mixed solution. The DOX concentration in the solution of A/D-PADP was quantified by measuring the absorbance intensity of DOX at 485 nm according to a pre-established calibration curve. To determine the loading content of afatinib, high performance

liquid chromatography (HPLC) was carried out on an Agilent 1260 chromatography device. Similarly, the freeze-dried A/D-PADP vesicle was weighed, then redissolved in 3 mL of $\text{CHCl}_3/\text{methanol}$ (1/3, *V/V*) solution for HPLC test. The chromatography was performed on a Beckman C8 column (Thermo, USA). The effluents were monitored at the excitation wavelength of 254 nm at 30 °C. The mobile phase consisted of purified water and acetonitrile (1/3, *V/V*) with a flow rate of 1 mL/min. The concentration of afatinib was determined by calculating the corresponding peak area in HPLC spectrum according to a pre-established calibration curve.

In Vitro DOX and Afatinib Release from Vesicle

To study the pH-responsive drug release behavior of DOX and afatinib, tests were performed at pH 7.4 and pH 5.0, respectively. Briefly, 1 mL of A/D-PADP vesicle solution was dispersed in 1 mL of PBS at pH 7.4 and 5.0, respectively, and then dialyzed (MWCO: 14 kDa) against 20 mL of the same PBS at 37 °C in an incubator shaker. At pre-determined time points, 2 mL of solution outside the dialysis bag was replaced by fresh PBS of the same pH and collected for HPLC test to determine the concentration of DOX and afatinib. The drug release data were measured in triplicate for each pH value.

Confocal Laser Scanning Microscopy (CLSM)

The cellular uptake of vesicle and the intracellular distribution of vesicle in A549 cells were analyzed using a confocal laser scanning microscope (TCS SP8, Leica). The FDA-containing F/D-PADP vesicle was used instead of A/D-PADP vesicle because of the non-fluorescent afatinib. A549 cells were incubated overnight in a confocal dish at 37 °C. After incubation with F/D-PADP vesicle for different times, the cells were washed with PBS twice and permeabilized with 0.1% Triton X-100. Then the cells were washed with PBS twice and the nuclei were stained with DAPI solution (1 $\mu\text{g}/\text{mL}$ in PBS) for 5 min. The A549 cells were observed under CLSM. DAPI, FDA and DOX were excited at 504, 488, and 514 nm, respectively. The emission wavelengths of DAPI, FDA, and DOX were 461, 520, and 595 nm, respectively.

Cell Viability Assay

The cell viability was measured using MTT assay. Briefly, A549 cells were seeded into 96-well plates at a density of 5×10^4 cells per well and incubated overnight at 37 °C in 5% CO_2 atmosphere. Afterwards, the cells were separately incubated with 4 kinds of vesicles (B-PADP, A-PADP, D-PADP, and A/D-PADP) at various concentrations at 37 °C in 5% CO_2 atmosphere for 24 h. After co-incubation, the used medium in each well was replaced with 100 μL of fresh medium containing 10 μL of MTT solutions (5 mg/mL in PBS), and the cells were incubated for another 4 h. DMSO (100 μL) was added into each well. After vibration for 10 min, a multimode plate reader (Infinite F200, Tecan) was used to measure the absorbance of each well at 570 nm. The cell viability for each well was calculated by the following equation: Cell viability = $(A_{\text{co}} - A_{\text{blank}})/(A_{\text{control}} - A_{\text{blank}}) \times 100\%$ where A_{co} , A_{blank} , and A_{control} denote the absorbance of

the cells treated with vesicle, the blank well, and the vesicle-free cells, respectively. All experiments were carried out in triplicate.

Flow Cytometry Assay

A549 cells were seeded into 6-well plates at a density of 1×10^4 cells per well, incubated overnight at 37 °C in 5% CO₂ atmosphere, then treated with various vesicles (B-PADP, A-PADP, D-PADP, and A/D-PADP) separately, and co-incubated for 24 h. Subsequently, the used medium was discarded and the cells were washed with PBS twice. Then, the A549 cells were treated with 0.25% trypsin without EDTA for digestion, which was terminated using the serum-containing medium. The cells were collected, washed with PBS twice, and re-suspended in 500 μ L of binding buffer. After stained with 5 μ L of Annexin V-FITC and 5 μ L of DAPI for 15 min, the A549 cells were detected using a Gallios flow cytometer (Attune NxT Acoustic Focusing Cytometer, Life). The excitation wavelengths of Annexin V-FITC and DAPI were 488 and 358 nm, respectively, and the emission fluorescence of Annexin V-FITC and DAPI was collected at 530 and 455 nm, respectively. The A549 cells without vesicle co-incubation but cultured in the same condition were used as control for background calibration. The collected data were analyzed using Attune™ NxT Software.

Animal Model and Treatment Protocol

All the *in vivo* experiments and animal care were approved by the Institutional Animal Care and Use Committee of the Sun Yat-sen University, and in strict accordance with the “Guide for the Care and Use of Laboratory Animals”. Nude mice (male, 6–8 weeks) bearing subcutaneous xenograft tumor were used for *in vivo* test. The mice were anesthetized, and then A549 cells (1×10^6) suspended in 50 μ L of PBS were subcutaneously injected in the right back of the mice. After the tumor volume reached approximately 50 mm³, the mice were randomly divided into five groups ($n = 6$) and injected with the following samples *via* tail vein: B-PADP, A-PADP, D-PADP, A/D-PADP, and PBS. The treatment was conducted every 3 days. The length (L) and width (W) of

the tumor was recorded using Vernier calipers. Then the tumor volume was calculated by the following formula: $\text{Volume} = 0.5 \times L \times W^2$. The tumor volume and the weight data were measured every 3 days, and the survival rate of the mice was recorded every day.

Histology Study

For the histological evaluation, the tumor tissues were excised from mice and then fixed in 4% paraformaldehyde for 48 h. After dehydration with a series of ethanol solutions with increasing concentration, the tumor tissues were paraffin embedded for 5 h at 60 °C. Subsequently, the tumor tissues were sectioned into 4- μ m-thick slides, then deparaffinized and rehydrated for later use. Further, hematoxylin and eosin (H&E) staining and terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling (TUNEL) assays were performed following the manufacturer's operation procedures. The resulting tumor sections were observed and recorded using an optical microscope.

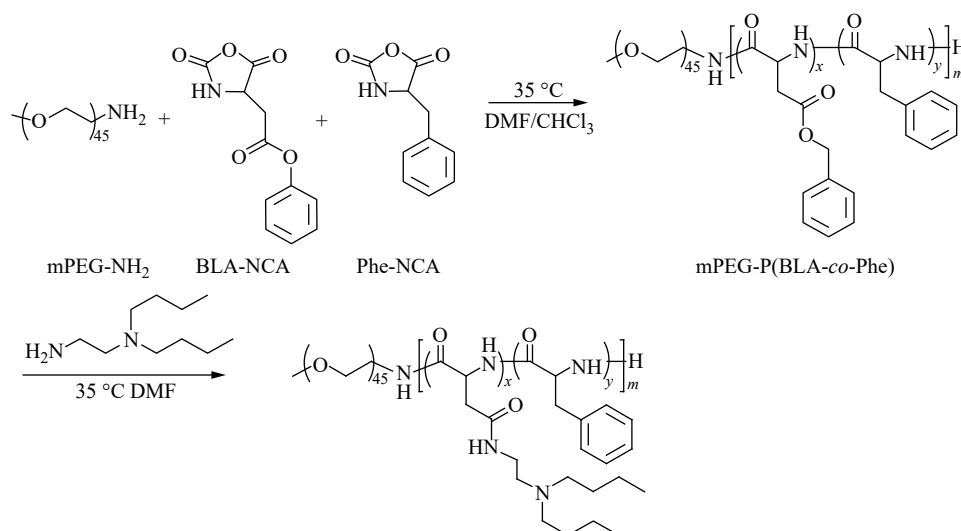
Statistical Analysis

All the results are shown as the mean $\pm$ standard deviation (SD). Data were statistically analyzed using one-factor analysis of variance (SPSS software, version 18.0, SPSS Inc.), and $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Polymer Synthesis and Vesicle Preparation

The pH-sensitive block copolymer mPEG-P(Asp(DBA)-*co*-Phe) was synthesized following the route in Scheme 2. As described in the experimental section, the polymer was prepared through ring-opening polymerization of BLA-NCA monomer and Phe-NCA monomer with mPEG-NH₂ as the initiator. Sequentially, ammonolysis reaction was conducted with DBA. The successful synthesis was confirmed using ¹H-NMR, FTIR, and GPC analyses. The ¹H-NMR spectra of mPEG-P(BLA-*co*-Phe) and mPEG-P(Asp(DBA)-*co*-Phe) are shown in Fig. 1(a). The expected structure of mPEG-P(BLA-*co*-Phe) matched the characteristic resonance signals well.



Scheme 2 The synthesis approach of copolymer mPEG-P(Asp(DBA)-*co*-Phe)

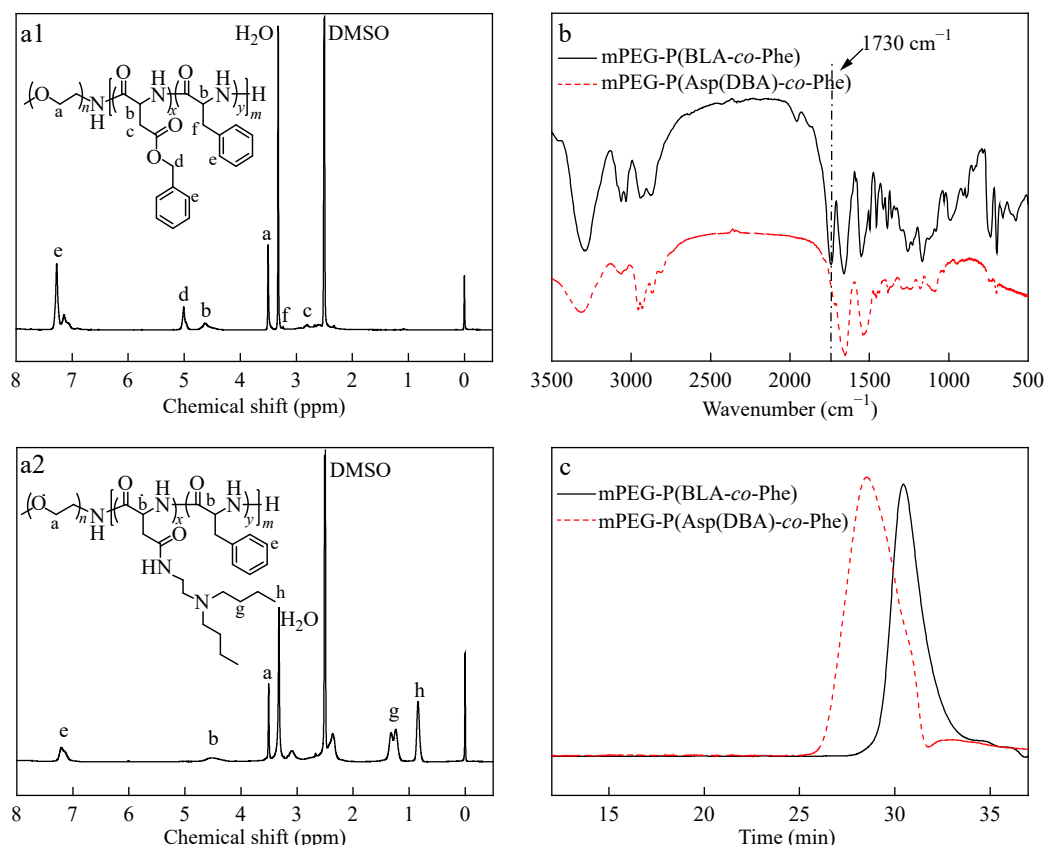


Fig. 1 ¹H-NMR spectra of (a1) mPEG-P(BLA-co-Phe) and (a2) mPEG-P(BLA(DBA)-co-Phe) in DMSO-d₆; (b) FTIR spectra and (c) GPC traces recorded for mPEG-P(BLA-co-Phe) and mPEG-P(BLA(DBA)-co-Phe)

The characteristic proton signals in the ¹H-NMR spectrum of mPEG-P(BLA-co-Phe) (Fig. 1a1) are (in ppm): 3.5 (a, $-\text{OCH}_2\text{CH}_2-$), 4.6 (b, $-\text{CHCONH}-$), 2.6 (c, $-\text{CH}_2\text{COOCH}_2-$), 5.0 (d, $-\text{CH}_2\text{C}_6\text{H}_5$), 7.1–7.3 (e, $-\text{C}_6\text{H}_5$), 3.2 (f, $-\text{CH}_2\text{C}_6\text{H}_5$). According to the integral values of characteristic signals areas ($-\text{OCH}_2\text{CH}_2-$, $-\text{C}_6\text{H}_5$, and $-\text{CH}_2\text{C}_6\text{H}_5$), the polymerization degree of BLA moiety and Phe moiety was determined to be 74 and 38, respectively. After the ammonolysis reaction, the disappearance of the characteristic signals of benzyl protons ($-\text{COOCH}_2\text{C}_6\text{H}_5$) at 5.0 ppm and the appearance of the characteristic signals of butyl protons in DBA at 0.82 ppm ($-\text{CH}_2\text{CH}_2\text{CH}_3$) and 1.28 ppm ($-\text{CH}_2\text{CH}_2\text{CH}_3$) indicated the complete reaction (Fig. 1a2). In addition, the successful synthesis of mPEG-P(Asp(DBA)-co-Phe) was also validated by FTIR spectra (Fig. 1b) in which the characteristic band of ester bond (1730 cm^{-1}) in mPEG-P(BLA-co-Phe) disappeared after the ammonolysis reaction. The molecular weight of mPEG-P(Asp(DBA)-co-Phe) and mPEG-P(BLA-co-Phe) was 28 kDa (PDI: 1.27) and 25 kDa (PDI: 1.13), respectively, as determined by GPC analysis (Fig. 1c). mPEG-P(Asp(DBA)-co-Phe) showed a narrow polydispersity in GPC trace, and the molecular weight was in line with the result calculated from ¹H-NMR spectra.

After the vesicle was prepared by double-emulsion method, the morphology and size distribution of the vesicle were analyzed by TEM and DLS, respectively. As shown in Fig. 2(a), the TEM image demonstrates that the drug-loaded

system possessed a spherical and vesicle structure with a uniform nanosize around 130 nm, which was in line with the size data obtained from DLS detection ($(130 \pm 10)\text{ nm}$, Fig. 2b). The suitable particle size of polymeric vesicle might facilitate the accumulation in tumor site *via* EPR effect.^[32,33] As measured by UV-Vis absorption and HPLC, the drug loading content in A/D-PADP vesicle was determined to be 3.72% and 1.73% for DOX and afatinib, respectively. The results evidenced that the polymeric nanovesicle exhibited great potential in co-encapsulation of both hydrophilic and hydrophobic therapeutic agents.

In Vitro Release of DOX and Afatinib

The release of DOX and afatinib from A/D-PADP vesicle was conducted using the dialysis method under different conditions (pH 7.4 and 5.0). The drug concentration outside the dialysis bag was measured at selected time points to determine the cumulative drug release. As shown in Fig. 3, both DOX and afatinib were released very little at the condition of pH 7.4 in 24 h, indicating the high stability of nanovesicle during circulation. Conversely, when the pH was adjusted to 5.0, both of them were released much more rapidly, and the cumulative drug release reached 90% in 24 h. In addition, the release of afatinib was faster than that of DOX, which might be due to the fact that the medium encapsulated in the superficial membrane was easier to be released from vesicle. Overall, the *in vitro* release profiles of DOX and afatinib indicated that the vesicle could prevent

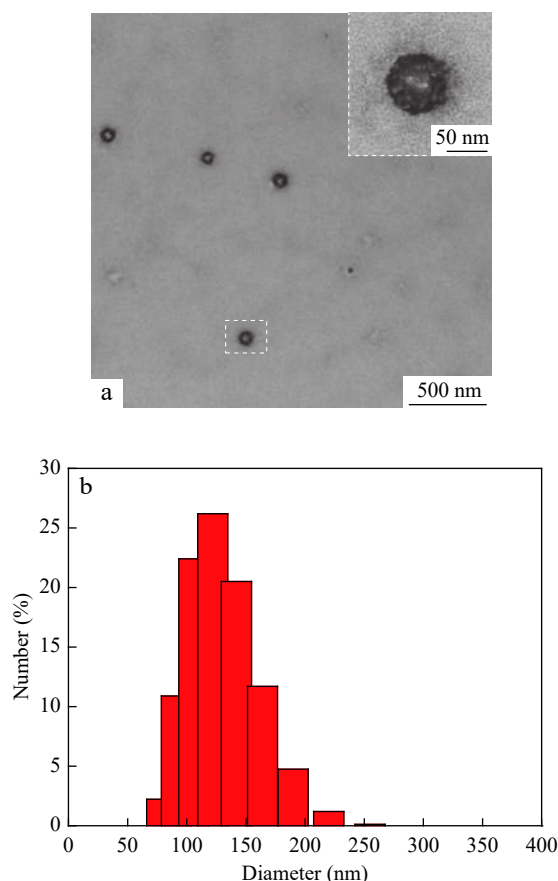


Fig. 2 (a) TEM image and (b) particle size distribution of the A/D-PADP vesicle

drug leakage in physiological condition (pH $\sim$ 7.4) but achieve in-site rapid drug release in tumor cells (pH $\sim$ 5.0) due to the pH-responsive feature of the polymer, which may improve the chemotherapy efficiency *in vivo* by increasing the local drug concentration.

Cell Uptake and Intracellular Distribution

The cell uptake and intracellular distribution of the vesicles were analyzed using CLSM. A549 cells were incubated with FDA-containing F/D-PADP vesicles, in which fluorescent FDA was used instead of the non-fluorescent afatinib. As shown in Fig. 4, the red fluorescence (DOX) and green fluorescence (FDA) merged and reproduced yellow fluorescence in cell cytoplasm in the first 2 h, indicating both agents were co-delivered into the cells by vesicles *via* endocytosis quickly. After 4 h, the vesicles collapsed in lysosomes because of their rapid degradation in the acidic environment, releasing the drugs into cytoplasm. DOX started to migrate into nuclei gradually and FDA kept staying in cytoplasm. In addition, the vesicles kept collapsing in a time-dependent manner, resulting in an increasingly stronger fluorescence of DOX and FDA due to the fluorescence dequenching effect. When the incubation time reached 8 h, most of DOX accumulated in nuclei, leading to a pink fluorescence merged by DOX and DAPI. These results indicated that the nanovesicle could effectively deliver the co-encapsulated drugs into A549 cells and release them rapidly in response to

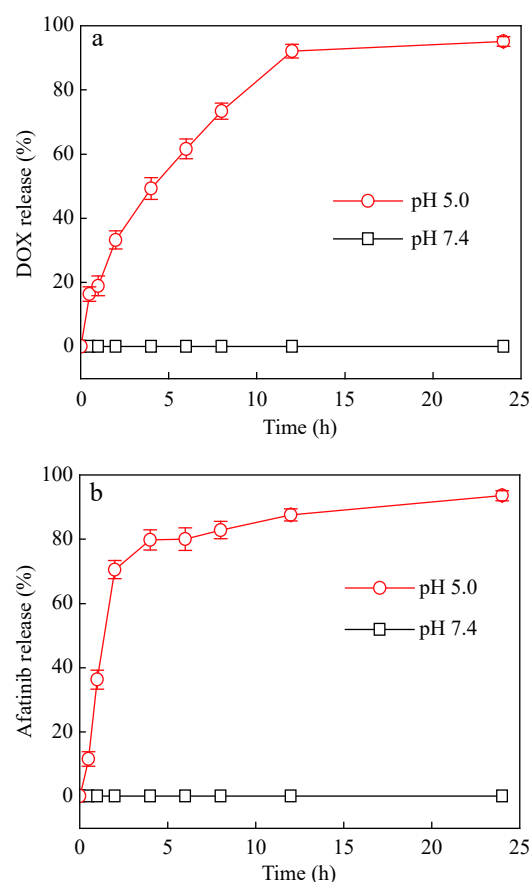


Fig. 3 *In vitro* (a) DOX and (b) afatinib release from the A/D-PADP vesicle at different conditions (pH 7.4 and 5.0). The release study was performed at 37 °C ($n = 3$).

the intracellular acidic pH of tumor cells.

Cell Viability and Cell Apoptosis

The cytotoxicity of B-PADP vesicle, A-PADP vesicle, D-PADP vesicle, and A/D-PADP vesicle in A549 cells was evaluated by MTT assay. The results showed that the blank vesicle (B-PADP) was nontoxic as more than 80% of A549 cells remained viable when the polymer rose to a high concentration of 300 μ g/mL (Fig. 5a1). The nontoxicity was attributed to the good biocompatibility in chemical structure of the polymer. After treated by single-drug-loaded vesicles (A-PADP or D-PADP), the viability of A549 cells decreased with the rising drug concentrations (Fig. 5a). To verify the enhanced anti-cancer effect of DOX in combination with afatinib, the viability of A549 cells incubated with the dual-drug-loaded vesicle A/D-PADP was analyzed. As shown in Fig. 5(a3), the A/D-PADP vesicle was much more effective in inducing cell death than D-PADP vesicle and A-PADP vesicle did. For example, at the DOX concentration of 8 μ g/mL, the cell viability decreased from 39.79% for D-PADP and 93.07% for A-PADP to 20.37% for A/D-PADP vesicle (afatinib concentration: 2.4 μ g/mL).

Additionally, the apoptosis of A549 cells incubated with various vesicles was determined using flow cytometry assay. As shown in Fig. 5(b), the B-PADP group exhibited a low apoptosis rate (7.3%). In the meantime, the A/D-PADP group

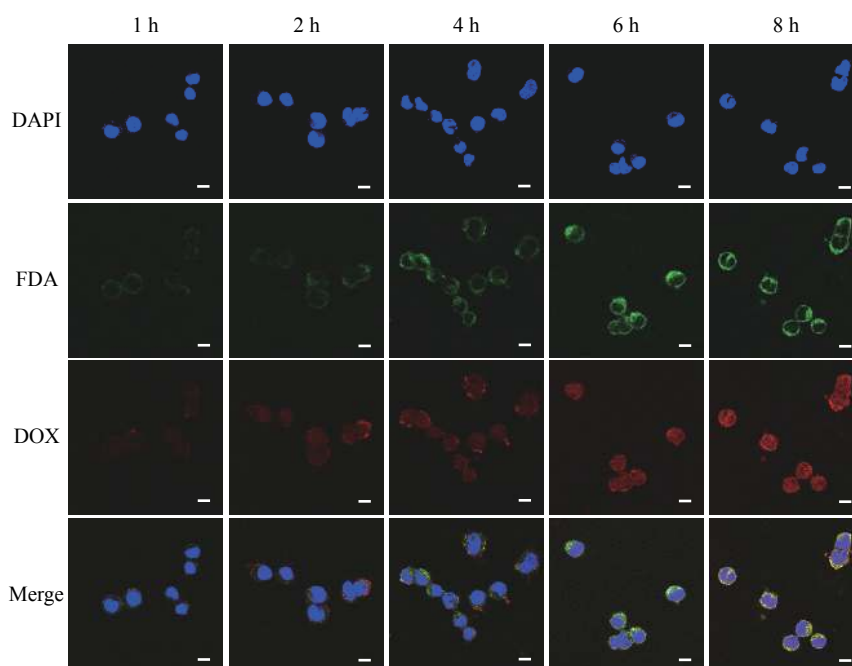


Fig. 4 Confocal laser scanning microscopy (CLSM) images of A549 cells after incubated with F/D-PADP vesicle for different time. Red fluorescence: DOX; green fluorescence: FDA; blue fluorescence: nuclei stained with DAPI. The scale bars are 10 μm .

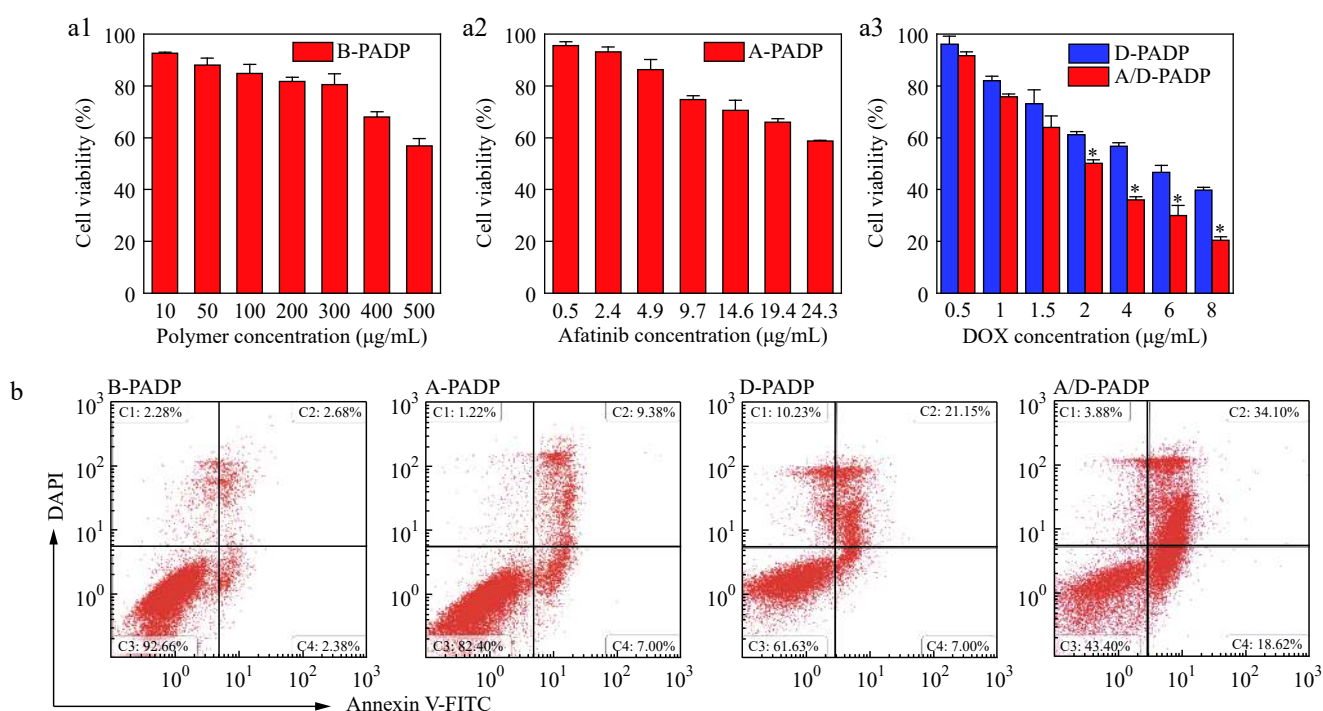


Fig. 5 Cell viability of A549 cells after treated with different vesicles: (a1) B-PADP vesicle with various polymer concentrations; (a2) A-PADP vesicle with various afatinib concentrations; (a3) D-PADP vesicle and A/D-PADP vesicle at various DOX concentrations. Data were detected by MTT assay ($n = 3$). The afatinib concentration of A/D-PADP vesicle was 2.4 $\mu\text{g/mL}$. * $P < 0.05$ versus D-PADP group with the same DOX concentration; (b) The apoptotic ratios of A549 cells after incubated with different vesicles. DOX concentration if applied: 4 $\mu\text{g/mL}$; afatinib concentration if applied: 2.4 $\mu\text{g/mL}$; C1: necrotic cells; C2: late stage apoptotic cells; C3: normal viable cells; C4: early stage apoptotic cells. The sum of C1, C2, and C4 indicated the apoptosis rate.

showed a much higher level of cell apoptosis (56.60%) compared to the single drug-loaded groups, *i.e.* 17.60% for A-PADP and 38.37% for D-PADP. These results indicated that A/D-PADP group had a high efficacy of inducing cell apop-

tos, attributed to the combined activity of DOX and afatinib. Thus, a significant enhanced effect of DOX and afatinib in A/D-PADP vesicle was achieved in inducing cell apoptosis, which was in consistent with the cell viability results.

In Vivo Therapeutic Effect

The cell assays above indicated that the A/D-PADP vesicle could realize quick release of DOX and afatinib in A549 cells attributed to the pH-sensitive feature and induced an enhanced tumor cell apoptosis *via* the combined effect. Furthermore, animal studies were performed to test whether the *in vivo* combined therapeutic effect could be achieved as expected. As shown in Fig. 6(a), the tumor volumes of the mice in PBS group and B-PADP group rose to (1205 ± 115) and (1164 ± 102) mm³ at the end of the treatment, respectively, indicating that B-PADP vesicle had no significant effect on tumor growth inhibition. On the contrary, the single-drug-loaded groups of A-PADP and D-PADP inhibited tumor growth to some extent as their tumor volumes $((731 \pm 127)$ and (615 ± 132) mm³, respectively) were much smaller than those in PBS and B-PADP group. Furthermore, the A/D-PADP group exhibited the highest antitumor effect with a final tumor volume of (216 ± 78) mm³, which is only 18% of the value in PBS control group. The effective antitumor effect of dual-drug-loaded vesicle resulted from the in-site released drug delivery as well as the enhanced cytotoxic effect of DOX and afatinib. The cumulative survival rates of each group confirmed the same conclusions. In Fig. 6(b), only one mouse died in the A/D-PADP group after 40-day treatment. Contrarily, no mouse survived more than 35 days when receiving the treatment of PBS and B-PADP vesicle. Additionally, the survival rates for D-PADP group and A-PADP group were both no more than 50%. Thus, the dual-

drug-loaded vesicle could inhibit the tumor growth and prolong the mice survival effectively. On the other hand, the drug-loaded vesicle decreased the side effects of free drugs efficiently by obtaining a similar body weight with the control groups after treatment (Fig. 6c).^[34,35] After treatment, the histological study of tumor tissues using H&E staining and TUNEL analysis were conducted. As illustrated in Fig. 6(d), the A/D-PADP group showed the lowest cancer cell density and the most cell apoptosis in tumor sections, which was in line with the result of tumor growth inhibition test. Consequently, the *in vivo* treatment studies and histological assays demonstrated that DOX and afatinib could act jointly in suppressing tumor cells *via* co-encapsulation in our pH-responsive vesicle and generate the best anti-tumor effect.

CONCLUSIONS

In conclusion, a pH-sensitive copolymer mPEG-P(Asp-(DBA)-*co*-Phe) was successfully synthesized to self-assemble into nanosized vesicle with hydrophilic DNA-damaging agent DOX and hydrophobic EGFR TKI afatinib co-encapsulated in the aqueous core and membrane, respectively. The *in vitro* tests indicated that the disintegration of the vesicle was triggered by the acidic tumor lysosomal environment, which resulted in an in-site drug release that enhanced the tumor killing effect of nanomedicine.

Additionally, the potent antitumor capabilities in suppressing tumor growth and enhancing survival rate were verified by *in vivo* studies, confirming again the combined effect of

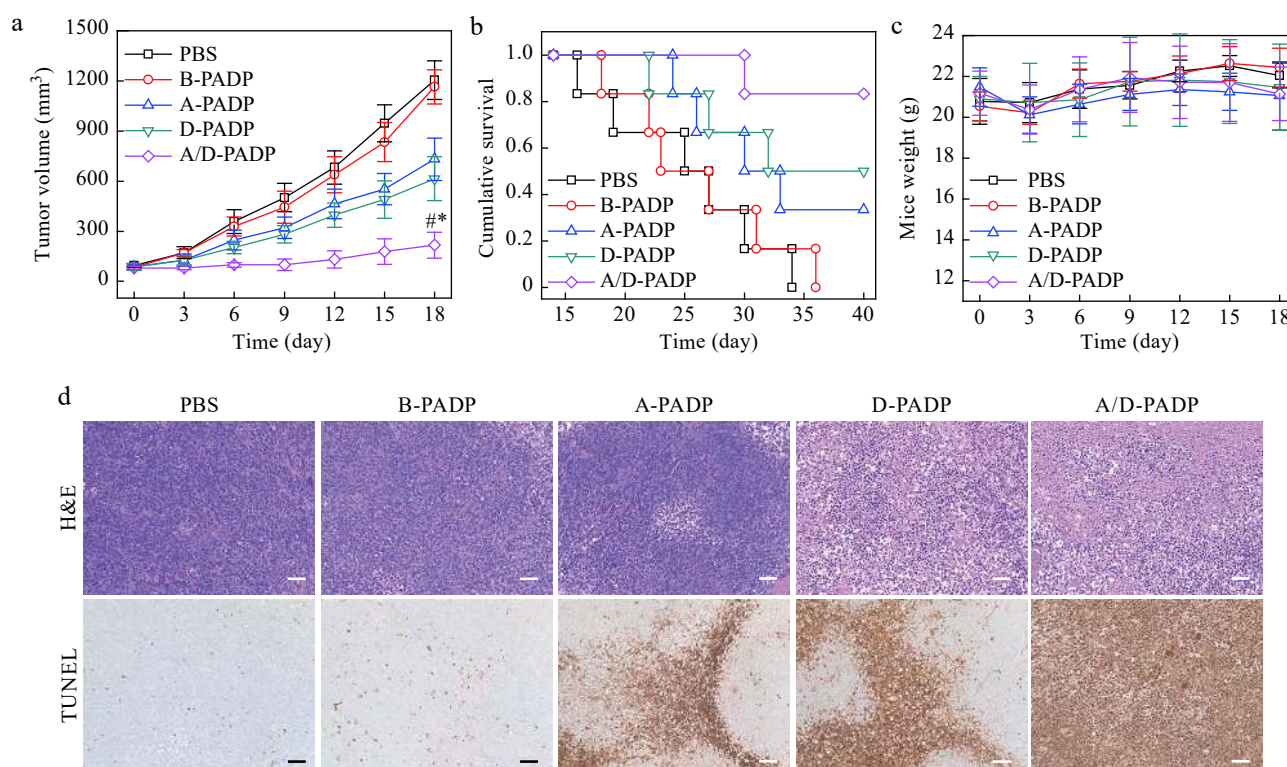


Fig. 6 (a) *In vivo* tumor growth inhibition, (b) survival rate, and (c) body weight changes of mice bearing A549 lung cancer xenografts after being intravenously injected with PBS, B-PADP, A-PADP, D-PADP, and A/D-PADP, respectively ($n = 6$). $^{*}P < 0.001$ versus D-PADP group; $^{*}P < 0.001$ versus PBS group; (d) Histopathology of tumor tissue sections from mice after different treatment with H&E and TUNEL staining. DOX dosage if applied: 2.5 mg/kg body weight; afatinib dosage if applied: 1.2 mg/kg body weight. The scale bars are 50 μ m.

DOX and afatinib. Conclusively, the promising antitumor effect of this co-delivery system may provide a potential method for the development of combination cancer treatment.

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