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Charge-Conversional PEG-Polypeptide Polyionic Complex Nanoparticles from Simple Blending of a Pair of Oppositely Charged Block Copolymers as an Intelligent Vehicle for Efficient Antitumor Drug Delivery

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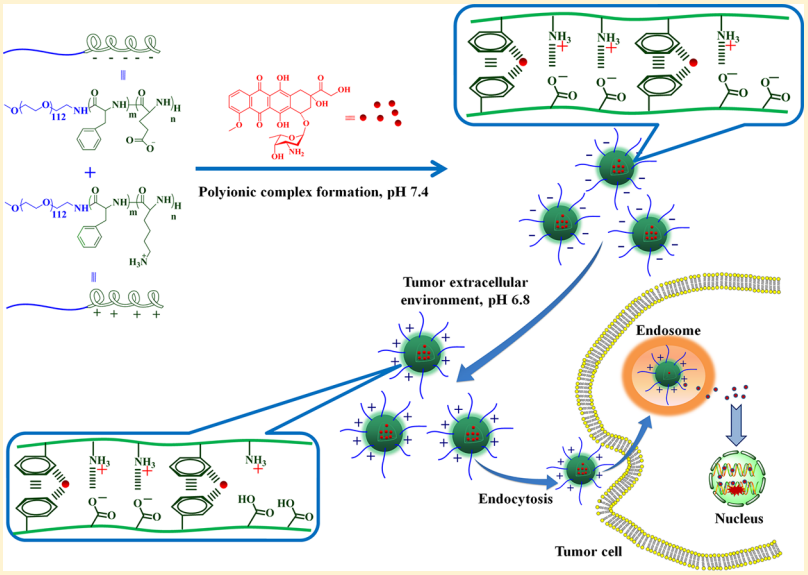

ABSTRACT: A tumor-acidity-activated charge-conversional polyionic complex nanoparticle system was developed by simply mixing a pair of oppositely charged block copolymers: anionic methoxy poly(ethylene glycol)-*b*-poly(L-glutamic acid-*co*-L-phenylalanine) (mPEG-*b*-P(Glu-*co*-Phe)) and cationic methoxy poly(ethy1ene glycol)-*b*-poly(L-lysine-*co*-L-phenylalanine) (mPEG-*b*-P(Lys-*co*-Phe)). The nanoparticles could stay negatively charged under normal physiological pH value and reverse the surface charge to positive at the tumor extracellular environment. Doxorubicin (DOX) was encapsulated into the nanoparticles fabricated by a self-assembly process, and the DOX-loaded polyionic complex nanoparticles (DOX-NPs) retained the charge-conversional property. *In vitro* DOX release study demonstrated that DOX release was promoted by the significantly increased acidity in endosomes and lysosomes (pH ≈ 5−6). Cellular uptake studies confirmed that the DOX-NPs could be more effectively internalized by cells at the tumor extracellular pH value. *In vitro* cytotoxicity assays demonstrated that the polyionic complex nanoparticles had good biocompatibility, and DOX-NPs showed efficient cell proliferation inhibition to HeLa and A549 tumor cells. Maximum tolerated dose (MTD) studies revealed that DOX-NPs had a significantly higher MTD (more than 25 mg of DOX/kg) in mice compared to that for free DOX (5 mg of DOX/kg). Furthermore, DOX-NPs showed superior antitumor activity and reduced side toxicity compared to free DOX in A549 tumor bearing nude mice.

KEYWORDS: *charge-conversion, polypeptide, doxorubicin, delivery, polyionic complex*

INTRODUCTION

In recent years, abundant efforts have been made to develop nanoscaled drug delivery systems for cancer therapy. However, the efficient delivery of antitumor drugs to tumor cells is still a

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major barrier due to poor cellular uptake and unsatisfactory intracellular drug release. Nanosized drug delivery systems using amphiphilic copolymers have been attracting great attention for improving the treatment efficacy in cancer therapy.[1,2] Most anticancer drugs with potent pharmacological effects are small molecules and have great limitation in their clinical applications due to nonspecific drug distribution in the body and serious side toxicity.[3] By altering pharmacokinetics and biodistribution profiles of these drugs through the enhanced permeability and retention (EPR) effect, nanoscaled drug delivery systems have resulted in successful efficacy for cancer therapy with decreased toxicity and enhanced efficacy.[4,5] However, the EPR effect just increases the accumulation of nanomedicines in solid tumors, and the low cellular uptake level and unsatisfactory intracellular drug release are still particularly serious problems which greatly influence the final therapeutic effect for these drug delivery systems. To overcome these limitations, stimulus-responsive delivery systems, which have well preprogrammed structures to change their properties during the drug delivery course, have drawn much more attention to improve drug bioavailability.[6] Among all the biological stimuli utilized for drug delivery (e.g., temperature, pH, glucose, glutathione, magnetic field, bioactive ligands, etc.), pH-sensitivity is one of the most frequently used due to significant differences of pH values between different tissues and cellular compartments.[7−10] For example, the pH value of the tumor extracellular environment is slightly lower (pH ≈ 6.8) than that of the normal tissues (pH ≈ 7.4), and the pH values of the intracellular organelles such as lysosome and endosome are even lower (pH ≈ 5−6).[11−14] Accordingly, a number of nanocarriers have been designed for pH-responsive drug delivery based on pH-induced structural change. However, most of those delivery systems only utilize the pH-triggered drug release acceleration at the acidic tumor tissues or tumor cells, while the enhancement of the cell internalization is rarely involved, which reduces the drug delivery efficacy and related anticancer efficiency.[8,15−18] The development of dual pH-responsive nanocarriers which have not only pH-sensitive drug release but also pH-induced cell internalization enhancement is still an urgent requirement.

In order to meet the requirement, pH-dependent charge conversion has been proved to be a very efficient strategy for improved drug delivery.[19] It has been reported that the surface charge of nanoparticles exerts a great impact on their *in vitro* and *in vivo* fate. Nanoparticles with positive surface charge have higher affinity with negatively charged cell membranes, which is conducive to cell internalization.[20,21] However, positively charged nanoparticles always have serious aggregation and rapid clearance after injection due to their strong interaction with blood components.[22] In contrast, negatively charged nanoparticles demonstrate the excellent blood compatibility and the prolonged blood circulation for the reduced clearance by the reticuloendothelial system (RES).[23] Therefore, the design of a pH-dependent charge conversional drug delivery system that stays negatively charged under neutral conditions and positively charged at the tumor extracellular environment is a meaningful approach for enhanced tumor cell internalization. For example, Yoon et al. utilized poly(amino acid) derivatives as a charge-conversional drug carrier in response to the tumor environment.[24] Wang's group developed a tailor-made dual pH-sensitive polymer−drug conjugate with the charge-conversional property at the tumor extracellular pH value and acid-triggered intracellular drug release.[25] These nanocarriers acquired charge conversion features mainly by employing a reversible conjugation of modifying the amino groups on the polymers with 2,3-dimethylmaleic anhydride or *cis*-aconitic anhydride. However, the unstable chemical linkage is still cleavable under normal physiological conditions, which may hamper the final application of these vehicles.

To implement the charge-conversional concept through a simple method, we designed a pH-sensitive polyionic complex as a nanoparticulate drug delivery system by simply mixing two ionic polymers in aqueous solution. For the material foundation, an anionic methoxy poly(ethylene glycol)-*b*-poly(L-glutamic acid-*co*-L-phenylalanine) (mPEG-*b*-P(Glu-*co*-Phe)) copolymer and a cationic methoxy poly(ethy1ene glycol)-*b*-poly(L-lysine-*co*-L-phenylalanine) (mPEG-*b*-P(Lys-*co*-Phe)) copolymer were utilized in this study. The two copolymers applied were based on poly(ethylene glycol)-poly(amino acid) (PEG-PAA) copolymers which have been widely utilized as nanocarriers in preclinical and clinical trials due to their outstanding biocompatibility, excellent biodegradability, and versatile functions.[26] The two copolymers are expected to form the polyanion−polycation complex nanoparticles resulting in the PEG shell and the PAA inner core through electrostatic and hydrophobic/aromatic interactions. The PEG shell of the nanoparticles reduces nonspecific interactions with blood components, and this enables prolonged blood circulation for facilitated drug accumulation at the tumor site through the EPR effect.[27] The poly(L-glutamic acid) and poly(L-lysine) segments in two block copolymers provide the pH-dependent charge-conversional feature to the nanoparticles. It has been demonstrated that the dissociation degree of carboxyl groups and amino groups in polymers is significantly influenced by environmental acidity, therefore, the surface charge of the polyionic complex nanoparticles greatly depends on the environmental acidity.[28] By adjusting the feed ratio of mPEG-*b*-P(Glu-*co*-Phe) and mPEG-*b*-P(Lys-*co*-Phe), the surface charge of the nanoparticles may vary from negative to positive at the tumor extracellular environment. The poly(L-phenylalanine) component of the copolymer serves as a reservoir of hydrophobic drugs. In addition, the aromatic interactions of phenylalanine units within the nanoparticle core can further enhance construct stability toward dissociation and consequently possibly reduce the drug-release rate. Doxorubicin (DOX) is a very effective anthracycline antitumor drug used as the first line treatment for a variety of cancers.[3] However, the dose-dependent side effects of free DOX such as cardiotoxicity, myelosuppression, and nephrotoxicity greatly limit its applications. Many strategies have been developed for the delivery of free DOX to overcome these limitations. In this study, DOX was selected as a model drug to evaluate the potential applications of the obtained nanoparicles in drug delivery. Furthermore, the protonable amino group on DOX may contribute to pH-triggered drug release.

In this work, the preparation and characterization of mPEG-*b*-P(Glu-*co*-Phe) and mPEG-*b*-P(Lys-*co*-Phe) are presented. The physiochemical properties, self-assembly, stability, loading, and *in vitro* release of DOX and the charge-conversional behavior of the polyionic complex nanoparticles were assessed. The cytotoxicity of the DOX-loaded polyionic complex nanoparticles (DOX-NPs) was evaluated by MTT assay. The cell uptake of the DOX-NPs was further studied under different pH conditions by CLSM. Finally, the *in vivo* antitumor efficacy of the DOX-NPs was evaluated.

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EXPERIMENTAL SECTION

Materials. Poly(ethylene glycol) monomethyl ether (mPEG, M_n = 5000) was purchased from Aldrich and used without further purification. N^{ε}-Benzyloxycarbony-L-lysine (H-Lys(Z)-OH) was purchased from GL Biochem Co. Ltd. (Shanghai, China). γ-Benzyl-L-glutamate-*N*-carboxyanhydride (BLG-NCA) and amino-terminated poly(ethylene glycol) methyl ether (mPEG-NH_2) were synthesized as in our previous work.[29] N^{ε}-Benzyloxycarbonyl-L-lysine-*N*-carboxyanhydride (Lys(Z)-NCA) was synthesized as in the literature report.[30] L-Phenylalanine-*N*-carboxyanhydride (Phe-NCA) was purchased from Yeexin Biochem & Tech Co. Ltd. (Shanghai, China) and purified by recrystallization from *n*-hexane/tetrahydrofuran (1:1) before use. *N*,*N*-Dimethylformamide (DMF) was stored over calcium hydride (CaH_2) and purified by vacuum distillation with CaH_2. Doxorubicin hydrochloride (DOX·HCl) was purchased from Beijing Huafeng United Technology Corporation. Fluorescein isothiocyanate (FITC) was purchased from Aladdin.

Measurements. The ^{1}H NMR, critical micelle concentration (CMC), zeta potential, dynamic laser scattering (DLS), and transmission electron microscopy (TEM) measurements were performed as in our previous studies.[4,29] Molecular weight distributions (polydispersity index, PDI = M_w/M_n) of the mPEG-*b*-P(BLG-*co*-Phe) and mPEG-*b*-P(Lys(Z)-*co*-Phe) copolymers were determined by gel permeation chromatography (GPC) under the same test conditions as in our previous work.[29]

Synthesis of mPEG-*b*-P(Glu-*co*-Phe) Copolymers. mPEG-*b*-P(Glu-*co*-Phe) copolymer was synthesized through ROP of BLG-NCA and Phe-NCA monomers with mPEG-NH_2 as macroinitiator and the deprotection of benzyl groups established in our laboratory. In brief, mPEG-NH_2 (2 g, 0.4 mmol) was dissolved in dry DMF (20 mL) after an azeotropic dehydration process with toluene, then Phe-NCA (0.765 g, 4 mmol) and BLG-NCA (1.052 g, 4 mmol) dissolved in dry DMF (20 mL) were added via a syringe under argon. The reaction was maintained for 3 days at 35 °C under argon. Then, methoxy poly(ethylene glycol)-*b*-poly(γ-benzyl-L-glutamate-*co*-L-phenylalanine) (mPEG-*b*-P(BLG-*co*-Phe)) block copolymer was isolated by repeated precipitation from DMF into an excess amount of ice cold diethyl ether. Yield: 86%. ^{1}H NMR (400 MHz, TFA-*d*, ppm): δ 1.85−1.95 (−CH_2−CH$_2$−CO−), 2.26 (−CH$_2$−CH_2−CO−), 2.78 (C_6H_5−CH_2− of polyphenylalanine), 3.67 (PEG chain), 4.48−4.59 (C*H* of amide of polyglutamic acid and polyphenylalanine), 4.95 (C_6H_5−CH_2− of γ-benzyl groups), 6.82−7.01 (C_6H_5−).

Subsequently, mPEG-*b*-P(BLG-*co*-Phe) (3.0 g) was dissolved in dichloroacetic acid (30 mL). After 9 mL of HBr/acetic acid (33 wt %) was added, the solution was maintained at 25 °C under stirring for 1 h. Then the crude product was obtained by precipitation into excessive ice cold diethyl ether. After being dried under vacuum, the crude product was dialyzed (MWCO 3500 Da) against distilled water and lyophilized to give the mPEG-*b*-P(Glu-*co*-Phe) product, yielding a white solid. Yield: 81.0%. ^{1}H NMR (400 MHz, TFA-*d*, ppm): δ 1.75−2.01-(−CH_2−CH$_2$−COOH), 2.31 (−CH$_2$−CH_2−COOH),, 2.78 (C_6H_5−CH_2− of polyphenylalanine), 3.67 (PEG chain), 6.82−7.11 (C_6H_5−CH$_2$−), 4.38−4.66 (C*H* of amide of poly-(glutamic acid) and polyphenylalanine).

Synthesis of mPEG-*b*-P(Lys-*co*-Phe) Copolymers. mPEG-*b*-P(Lys-*co*-Phe) copolymer was synthesized through ROP of Lys(Z)-NCA and Phe-NCA monomers with mPEG-NH_2 as macroinitiator and then deprotected benzyloxycarbonyl groups. A similar procedure was used for mPEG-*b*-P(Glu-*co*-Phe). In brief, mPEG-NH_2 (2 g, 0.4 mmol) was dissolved in dry DMF (20 mL) after an azeotropic dehydration process with toluene, and then Phe-NCA (0.765 g, 4 mmol) and Lys(Z)-NCA (1.225 g, 4 mmol) were dissolved in dry DMF (25 mL) and added to the solution of mPEG-NH_2 via a syringe under argon. The reaction mixture was kept stirring for 3 days at 35 °C under a dry argon atmosphere. Then, methoxy poly-(ethylene glycol)-*b*-poly(N^{ε}-benzyloxycarbonyl-L-lysine-*co*-L-phenylalanine) (mPEG-*b*-P(Lys(Z)-*co*-Phe)) was obtained by repeated precipitation from DMF into excessive ice cold diethyl ether. Yield: 80.6%. ^{1}H NMR (400 MHz, TFA-*d*, ppm): δ 1.20−1.56 (−CH_2−CH_2−CH_2−CH$_2$−NH− of lysine), 3.02 (−CH_2−NH− of lysine), 2.79 (C_6H_5−CH_2− of polyphenylalanine), 3.69 (PEG chain), 4.34−4.62 (C*H* of amide of polylysine and polyphenylalanine), 4.97 (C_6H_5−CH_2− of γ-benzyl groups), 6.89−7.08 (C_6H_5−).

In order to remove benzyloxycarbonyl groups, mPEG-*b*-P(Lys(Z)-*co*-Phe) (2.9 g) was dissolved in trifluoroacetic acid (29 mL) at 0 °C. Then 8.7 mL of HBr/acetic acid (33 wt %) was added, and the solution was maintained at 25 °C under stirring for 1 h. The crude product was obtained through precipitation into excessive ice cold diethyl ether and then dialyzed (MWCO 3500 Da) against distilled water. The mPEG-*b*-P(Lys-*co*-Phe) white powder was obtained after lyophilization. Yield: 79.0%. ^{1}H NMR (400 MHz, TFA-*d*, ppm): δ 1.39−1.68 (−CH_2−CH_2−CH_2−CH$_2$−NH$_2$ of lysine), 3.07 (−CH_2−NH$_2$ of lysine), 2.81 (C_6H_5−CH_2− of polyphenylalanine), 3.69 (PEG chain), 4.44−4.67 (C*H* of amide of polylysine and polyphenylalanine), 6.68−7.06 (C_6H_5-).

Preparation of Polyionic Complex Nanoparticles. Blank polyionic complex nanoparticles were prepared by a dialysis method. Briefly, 100 mg of mPEG-*b*-P(Glu-*co*-Phe) and 100 mg of mPEG-*b*-P(Lys-*co*-Phe) were dissolved in 8.0 mL of DMF, the mole ratio of L-glutamic acid and lysine residues in the block copolymers was 1.10: 1, and the solution was allowed to stir at 25 °C for 2 h. Then, this solution was added slowly to 32.0 mL of deionized water and maintained at 25 °C under stirring for 12 h. Then DMF was removed by dialysis against distilled water to obtain the micelle solution and followed by lyophilization.

Preparation of DOX-Loaded Polyionic Complex Nanoparticles. DOX-loaded polyionic complex nanoparticles (DOX-NPs) were prepared by a nanoprecipitation technique. Typically, the lyophilized polyionic complex nanoparticle powder (100 mg), DOX·HCl (20.0 mg), and triethylamine (7.2 mg) were dissolved in DMF (6.0 mL). The mixture solution was vigorously stirred at 25 °C for 6 h in the dark. Then, 12.0 mL of phosphate buffered saline (PBS, pH = 7.4) was added dropwise to the solution under stirring. The mixture was maintained at 25 °C under stirring for 12 h. The organic solvent and excess drug were removed by dialysis (MWCO 3500 Da) against deionized water for 48 h and then lyophilized to get DOX-NPs. The whole procedure was performed in the dark. To determine the drug loading content (DLC, wt %) and drug loading efficiency (DLE, wt %), the DOX-NPs were dissolved in DMF and measured by UV−vis spectrometer at 480 nm. DLC and DLE were calculated according to the following formulas:

$$\text{DLC} = (\text{amount of loaded DOX}/\text{amount of DOX} - \text{loaded nanoparticles}) \times 100\%$$

$$\text{DLE} = (\text{amount of loaded DOX}/\text{amount of feeding DOX}) \times 100\%$$

To obtain FITC-labeled DOX-loaded polyionic complex nanoparticles, the lyophilized polyionic complex nanoparticle powder (100 mg) and FITC (2 mg) were dissolved in DMF (4.0 mL) and stirred for 24 h at room temperature with protection from light. The mixture was dialyzed against distilled water to remove DMF and unreacted FITC, and then it was lyophilized to obtain a light yellow powder. DOX was loaded into FITC-labeled polyionic complex nanoparticles by the same procedure used for polyionic complex nanoparticles.

***In Vitro* Release of DOX.** The DOX release profiles were investigated in PBS at different pH conditions (7.4, 6.8, and 5.0) by the dialysis method. Briefly, the weighed freeze-dried DOX-NPs were dissolved in 5.0 mL of release medium and transferred into a dialysis bag (MWCO 3500 Da). The release experiment was initiated by placing the dialysis bag into 45.0 mL of release medium at 37 °C with continuous shaking at 100 rpm. At predetermined time, 3 mL of incubated solution was taken out and replaced with equal volume of fresh PBS. The amount of DOX released was determined using a UV−vis spectrometer at 480 nm. The release experiments were performed in triplicate.

Cell Cultures. The human lung carcinoma (A549) and human cervical carcinoma (HeLa) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with high glucose containing 10% fetal bovine serum, supplemented with 50 U mL^{-1} penicillin and 50 U mL^{-1} streptomycin, and incubated at 37 °C in 5% CO_2 atmosphere.

Confocal Laser Scanning Microscopy (CLSM) Observation. The cellular uptake of FITC-labeled DOX-NPs was investigated using CLSM on A549 cells. The cells were seeded on coverslip in 6-well plates with a density of 1×10^5 cells per well in 2 mL of DMEM and cultured for 24 h, and then the original medium was replaced with FITC-labeled DOX-NPs containing DMEM of pH 7.4 or 6.8, respectively. After 1 or 3 h incubation, the cells were washed and fixed with 4% formaldehyde. The cell nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI). The cellular localization and intracellular DOX release of DOX-NPs were observed using a laser scanning confocal microscope (Carl Zeiss LSM 700).

***In Vitro* Cytotoxicity Assay.** The *in vitro* cytotoxicities of mPEG-*b*-P(Glu-*co*-Phe), mPEG-*b*-P(Lys-*co*-Phe), polyionic complex nanoparticles, free DOX·HCl, and DOX-NPs were assessed by MTT assay against HeLa and A549 cells as in our previous studies.[4,29] Briefly, HeLa or A549 cells were seeded in 96-well plates (1×10^4 cells per well) in 100 μL of DMEM medium and incubated for 24 h, followed by removing culture medium and adding mPEG-*b*-P(Glu-*co*-Phe), mPEG-*b*-P(Lys-*co*-Phe), polyionic complex nanoparticles, free DOX·HCl, and DOX-NPs (200 μL DMEM) at the different concentrations. The cells were subjected to MTT assay after another 48 h incubation. The absorbency of the solution was measured on a Bio-Rad 680 microplate reader at 492 nm. The relative cell viability was calculated by $(A_{sample}/A_{control}) \times 100$, where A_{sample} and $A_{control}$ denoted absorbance of the sample well and control well, respectively. Data are presented as average ± SD (n = 3).

The pH effect on tumor cell proliferation inhibiton of DOX-NPs was evaluated against A549 and HeLa cells by MTT assay, which was similar to the above operations. Briefly, A549 or HeLa cells were seeded in 96-well plates (1×10^4 cells per well) in 100 μL of DMEM medium and incubated for 24 h. The culture medium was replaced with 200 μL of fresh medium containing DOX-NPs at pH 7.4 or 6.8. After 1.5 h treatment, the medium was replaced by 200 μL of fresh DMEM at pH 7.4. The cells were subjected to MTT assay after being incubated for another 46.5 h.

Hemolysis Assay. The hemolytic activities of polyionic complex nanoparticles, free DOX·HCl, and DOX-NPs were evaluated according to our previous work with minor modification.[4] Briefly, fresh rabbit blood was diluted with physiological saline, and then red blood cells (RBCs) were isolated by centrifugation. The remaining RBCs were diluted in PBS (pH = 7.4) after wash. Blank polyionic complex nanoparticles, free DOX·HCl, and DOX-NPs were dissolved in PBS (pH = 7.4) at different concentrations, and RBC suspension was added and then incubated at 37 °C in a thermostated water bath for 2 h. Then, RBCs were centrifuged and 100 μL of supernatant of each sample was measured with a Bio-Rad 680 microplate reader at 540 nm. PBS and pure water were used as negative and positive controls, respectively. All hemolysis experiments were carried out in triplicate. The hemolysis ratio (HR) of RBCs was calculated using the following formula: hemolysis (%) = $(A_{sample} - A_{negativecontrol})/(A_{positivecontrol} - A_{negativecontrol}) \times 100$, where A_{sample}, $A_{negativecontrol}$, and $A_{positivecontrol}$ were denoted as the absorbencies of samples and negative and positive controls, respectively.

Animals. Male Balb/C nude mice (6−8 weeks old) were purchased from the Experimental Animal Center, Chinese Academy of Sciences (Shanghai, China). Kunming mice (6−8 weeks old, male) were purchased from Laboratory Animal Center, Jilin University (Changchun, China). All the animals were maintained under required conditions in accordance with guidelines evaluated (e.g., pathogen-free condition for nude mice, free access to food and water) and approved by the Animal Care and Use Committee of Jilin University.

Maximum Tolerated Dose (MTD). The Kunming mice were randomly divided into 10 groups (n = 3) and administered intravenously with free DOX·HCl or DOX-loaded polyionic complex nanoparticles (5, 10, 15, 20, 25 mg of DOX/kg body weight) for a single dose. Changes in body weight and survival of mice were followed daily for ten days. The maximum tolerated dose (MTD) was defined as the dose that leads to neither animal death nor larger than 15% of body weight loss or other remarkable changes in the general appearance within the entire period of the experiments.

***In Vivo* Antitumor Efficiency.** A human non-small cell lung cancer xenograft tumor model was established by subcutaneous injection of A549 cells (1.5×10^6) in the right flank of each mouse. Mice were randomly divided into 5 groups when the tumor volume reached about 30−50 mm^3, and this day was designated as day 0. Mice were injected intravenously with PBS, free DOX·HCl (2 mg kg^{-1}), free DOX·HCl (4 mg kg^{-1}), DOX-NPs (2 mg kg^{-1}), and DOX-NPs (4 mg kg^{-1}) via tail vein four times at day 0, day 3, day 7, and day 10. The tumor volume (mm^3) was calculated using $V = ab^2/2$, where a and b represented the longest and shortest diameter of the tumors. The body weight was measured to indicate the systemic toxicity. At the end of the treatment (day 17), animals

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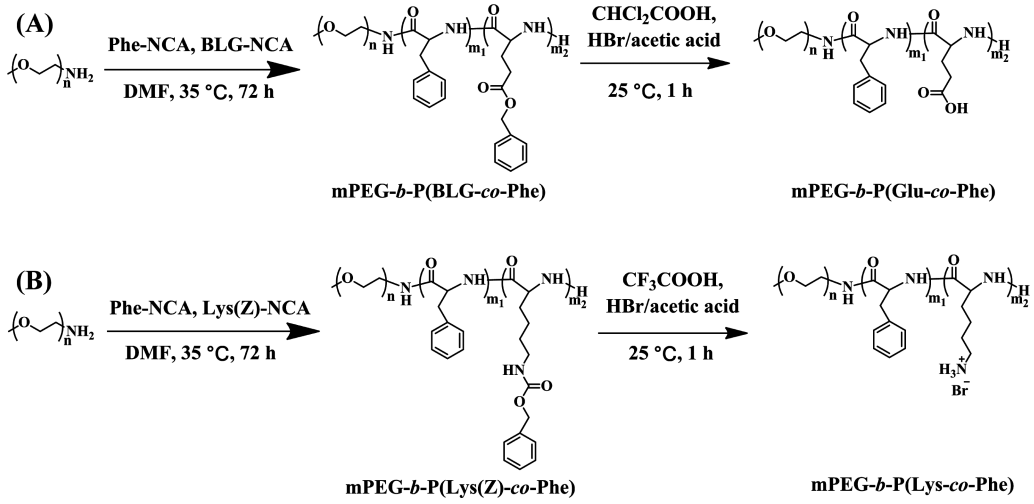
Scheme 1. Synthesis Routes of mPEG-*b*-P(Glu-*co*-Phe) (A) and mPEG-*b*-P(Lys-*co*-Phe) (B)



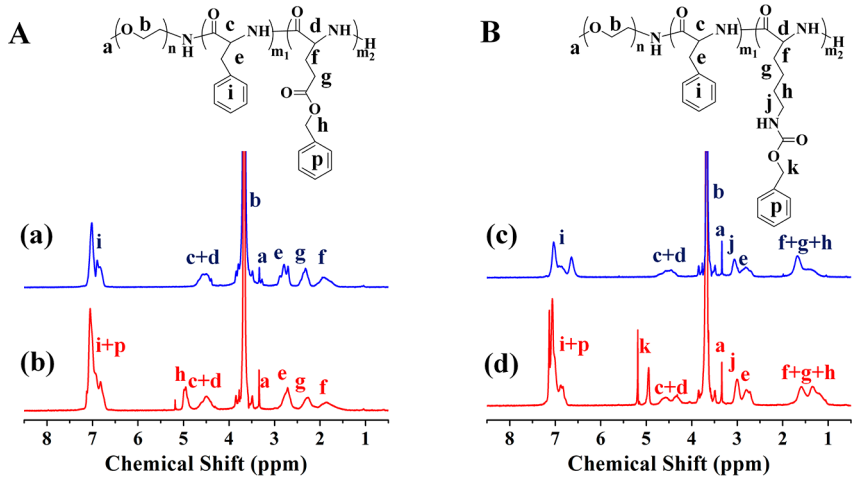


Figure 1. ^{1}H NMR spectra of mPEG-*b*-P(Glu-*co*-Phe) (a), mPEG-*b*-P(BLG-*co*-Phe) (b), mPEG-*b*-P(Lys-*co*-Phe) (c), and mPEG-*b*-P(Lys(Z)-*co*-Phe) (d) in CF_3COOD.

were sacrificed and the tumors were excised for histopathology analyses.

Histopathology and Immunohistochemical Evaluation. The histopathological damage evaluation was performed by the hematoxylin and eosin (H−E) method just as in our previous work.[4] Tumor sections stained with hematoxylin and eosin were observed by microscope (Nikon TE2000U).

The immunohistochemical evaluation was performed using rabbit monoclonal primary antibody for cleaved PARP (Abcam, Cambridge, MA, USA) and PV-6000 two-step immunohistochemistry kit (Zhongshan Goldbridge Biotechnology, Beijing, China) as the literature report.[31]

***In Situ* Terminal Deoxynucleotidyl Transferase-Mediated UTP End Labeling (TUNEL) Assay.** The paraffin-embedded tumor slices were prepared as described above. TUNEL assay was carried out using a FragEL DNA fragment detection kit (colorimetric-TdT Enzyme method) according to the manufacturer's protocol (EMD chemicals Inc., Darmstadt, Germany) with minor modification (briefly, hematoxylin was utilized to replace methyl green as the counterstain).

Statistical Analysis. All experiments were performed at least three times and expressed as means ± SD. Statistical significances were determined using Student's *t* test. $P < 0.05$ was considered statistically significant, and $P < 0.01$ was considered highly significant.

RESULTS AND DISCUSSION

Preparation of Oppositely Charged Block Copolymers. Since synthesis and polymerization of cyclic and highly reactive α-amino acid *N*-carboxyanhydrides (NCAs) were first reported by Hermann Leuchs, a great number of poly(amino acid) (PAA) polymers were developed by the polymerization of α-amino acid NCAs with a primary amine initiator in a controlled manner.[26,32,33] In this study, mPEG-*b*-P(Glu-*co*-Phe) and mPEG-*b*-P(Lys-*co*-Phe) block copolymers were synthesized via the one-pot ring-opening copolymerization of BLG-NCA or Lys(Z)-NCA with Phe-NCA using mPEG-NH_2 as the macroinitiator, followed by deprotecting the protection groups in HBr/acetic acid (Scheme 1). The structures of copolymers were determined by ^{1}H NMR and GPC. The typical ^{1}H NMR spectra of the resulting copolymers in trifluoroacetic acid-*d* (TFA-*d*) are shown in Figure 1, and all peaks were well assigned. The resonances at δ 4.95 or 4.97 ppm disappeared in the mPEG-*b*-P(Glu-*co*-Phe) (Figure 1a) and mPEG-*b*-P(Lys-*co*-Phe) (Figure 1c), indicating the complete deprotection of the γ-benzyl groups ($C_6H_5CH_2$−O−). The degrees of polymer-

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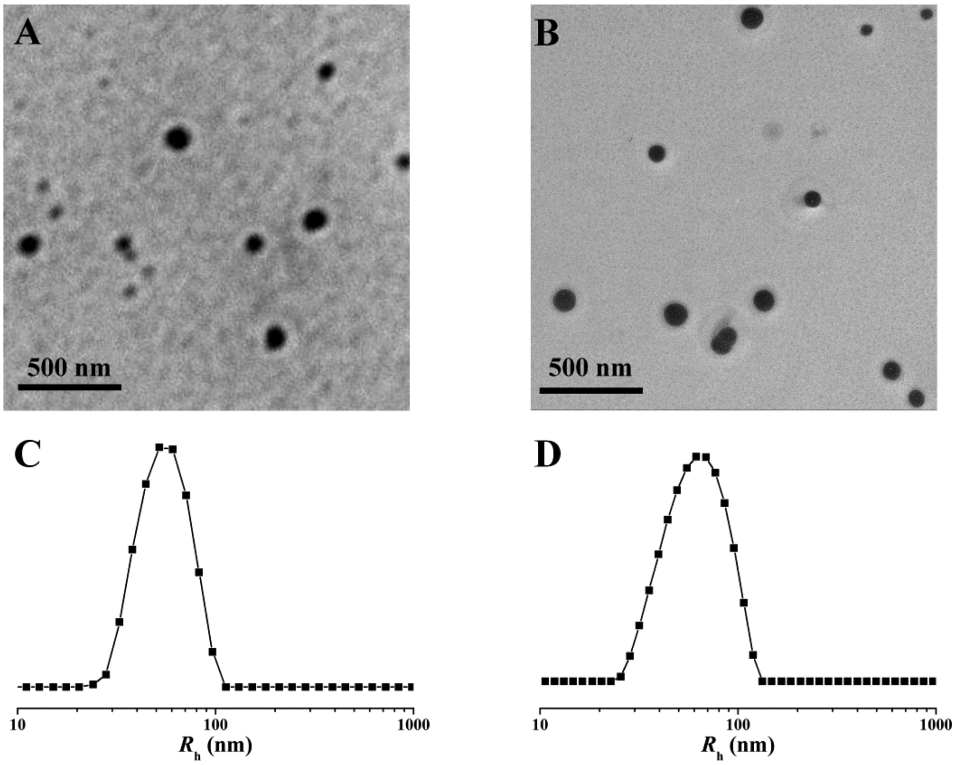

Figure 2. Typical micrographs and hydrodynamic radii (R_h) of blank polyionic complex nanoparticles (A and C) and DOX-loaded polyionic complex nanoparticles (B and D) estimated by TEM and DLS.

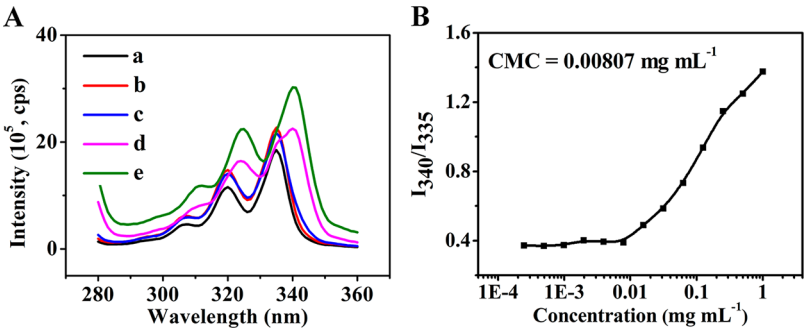

Figure 3. Excitation spectra of pyrene in blank polyionic complex nanoparticle solutions (PBS) at different concentrations [(a) 2.44×10^{-4}, (b) 1.95×10^{-3}, (c) 1.56×10^{-2}, (d) 2.50×10^{-1}, (e) 1.00 mg mL^{-1}, λ_{em} = 390 nm) (A), and the dependence of excitation fluorescence intensity ratio (I_{340}/I_{335}) on the logarithmic concentration of blank polyionic complex nanoparticles (B).

ization (DP) of BLG and Phe blocks in mPEG-*b*-P(BLG-*co*-Phe) were calculated to be 10 and 10, respectively, by comparing the integration of the methylene peak of the glutamate (−COCH_2−) and phenylalanine units (C_6H_5−CH_2−) with that of the methylene peak of PEG (−CH_2−CH_2−). After deprotection of γ-benzyl, DP of Glu and Phe blocks in mPEG-*b*-P(Glu-*co*-Phe) were calculated to be 10 and 10, respectively. Similarly, both calculations for mPEG-*b*-P(Lys(Z)-*co*-Phe) and mPEG-*b*-P(Lys-*co*-Phe) by ^{1}H NMR gave the same DP values of lysine units (DP = 9) and phenylalanine units (DP = 10). The above results suggested that the deprotection process did not lead to the chain scission of poly(amino acid) backbones. The M_n values of mPEG-*b*-P(Glu-*co*-Phe) and mPEG-*b*-P(Lys-*co*-Phe), calculated by ^{1}H NMR, were 7760 and 8351 g mol^{-1}, respectively. Gel permeation chromatography (GPC) analyses showed that both mPEG-P(BLG-Phe) and mPEG-*b*-P(Lys(Z)-*co*-Phe) copolymers had a narrow molecular weight distribution (PDI = 1.10 and 1.08, respectively). The narrow distribution may be attributed to the choice of starting PEG with a narrow molecular weight distribution (PDI = 1.04) and the living feature of the ROP of the NCA monomer.

Fabrication and Solution Behaviors of Polyionic Complex Nanoparticles. Amphiphilic block copolymers with both hydrophilic and hydrophobic segments can self-assemble in aqueous solution.[1,2] In the study, mPEG-*b*-P(Glu-*co*-Phe) and mPEG-*b*-P(Lys-*co*-Phe) polyionic complex could self-assemble into the micellar-type nanoparticles in the aqueous phase. Both blank polyionic complex nanoparticles (BNPs) and DOX-loaded polyionic complex nanoparticles (DOX-NPs) were prepared by a nanoprecipitation technique. To obtain DOX-NPs, DOX·HCl was first neutralized by excess triethylamine to remove the hydrochloride. DOX-NPs gave a DLC of 14.0 wt % and a DLE of 87.5%, indicating that DOX·HCl was successfully loaded into the nanoparticles through hydrophobic/aromatic interactions with the poly(L-phenyl-

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alanine) component of the copolymers. The sizes and morphologies of the nanoparticles were studied by DLS and TEM measurements. As shown in Figures 2C and 2D, the hydrodynamic radii (R_h) of BNPs and DOX-NPs measured by DLS were 56.6 ± 16.0 and 65.9 ± 18.1 nm, respectively. TEM images revealed that both BNPs and DOX-NPs had a uniformly spherical morphology.

CMC is one of the key parameters to describe the stability of micellar-type nanoparticles. In the present study, CMC is determined by fluorescence spectroscopy using pyrene as a probe, which has a peak shift in its excitation spectrum when it is entrapped into a hydrophobic inner core.[34] The CMC value was obtained from the plot of fluorescence intensity ratio of I_{340}/I_{335} (Figure 3) versus logarithm concentration of the nanoparticles. The blank polyionic complex nanoparticles were found to have a low CMC value of 0.00807 mg mL^{-1} (Figure 3B), indicating the great stability of polyionic complex nanoparticles against dilution. In addition to the hydrophobicity of polypeptide backbones, the $\pi-\pi$ stacking of phenylalanine units and the electrostatic interaction between $[COO^-]$ and $[NH_3^+]$ groups within the nanoparticles may also contribute to the low CMC value. In addition, such a low CMC value will guarantee the self-assembled nanoparticles to retain their original morphology under highly diluted conditions (e.g., bloodstream) before reaching the tumor site, which is one of the important requirements for drug delivery.

Charge-Conversional Property of Polyionic Complex Nanoparticles. The dissociation degree of carboxyl groups and amino groups in charged polymers is significantly influenced by environmental pH.[28] As the environmental acidity increases from neutral to slightly acidic, the protonation degree of the carboxylic acid groups and amino groups will be enhanced, and thus leads to the decreased dissociation degree of carboxyl groups and the increased dissociation degree of amino groups. Therefore, by adjusting the feed ratio of mPEG-*b*-P(Glu-*co*-Phe) and mPEG-*b*-P(Lys-*co*-Phe) in the polyionic complex, the polyionic complex nanoparticles might display charge-conversion behavior at the tumor extracellular condition (Scheme 2). In the present study, the mole ratio of L-glutamic acid and lysine residues in the block copolymers was 1.10:1, which could guarantee the self-assembled nanoparticles to stay negatively charged in blood and normal tissues (pH 7.4). As shown in Figure 4, both BNPs and DOX-NPs were negatively charged above pH 7.0. The zeta potential of NPs increased significantly when environmental acidity increased and became positive at pH 6.8 (about 10 mV). Considering the negatively charged cell membranes, the charge-conversion performance of DOX-NPs at the tumor extracellular environment will be conducive to their uptake by tumor cells.[20,21]

Release Behavior of Charge-Conversional DOX-NPs. The drug release behavior of DOX-NPs was investigated using a dialysis method at 37 °C in phosphate buffered saline (PBS) at different pH values (7.4, 6.8, and 5.0). As shown in Figure 5, the results clearly demonstrated that the environmental acidity had a significant effect on the DOX release rate from the polyionic complex nanoparticles. DOX-NPs displayed a much slower DOX release at pH 7.4; 17 wt % loaded DOX was released from the nanoparticles over the test duration (156 h). The relatively slower release at pH 7.4 may be attributed to the robust interaction between the carriers and DOX. Both mPEG-*b*-P(Glu-*co*-Phe) and mPEG-*b*-P(Lys-*co*-Phe) copolymers had a poly(phenylalanine) domain. In addition to hydrophobic interaction with DOX, $\pi-\pi$ stacking between DOX molecules

Scheme 2. Schematic Illustrations of Charge-Conversional Behavior (A) and *in Vivo* Performance (B) of DOX-NPs

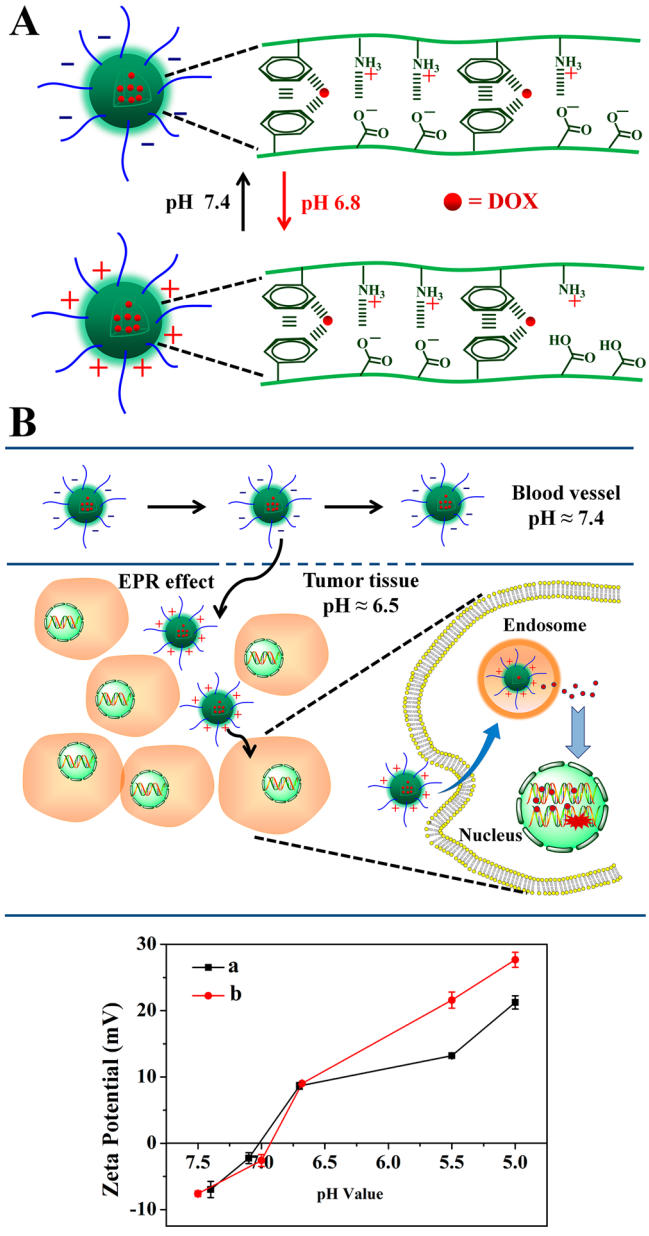

Figure 4. Zeta potential changes of blank polyionic complex nanoparticles (a) and DOX-loaded polyionic complex nanoparticles (b) at different pH values. Each point was an average of six measurements.

and phenylalanine units also contributes to the overall carrier/DOX interaction. The release of DOX at pH 6.8 slightly increased and less than 30% of DOX was released within the same period. When DOX-NPs were incubated at pH 5.0, more than 65% of DOX were released at 156 h, indicating that the release of DOX-NPs was sensitive to endo/lysosomal pH. The pH-sensitive release of DOX from DOX-NPs might be attributed to the increased hydrophilicity of DOX·HCl in acid condition, which hampers the hydrophobic/aromatic interactions between DOX and phenylalanine units. Such a pH-triggered release behavior of DOX from DOX-NPs will decrease the drug loss during blood circulation and improve

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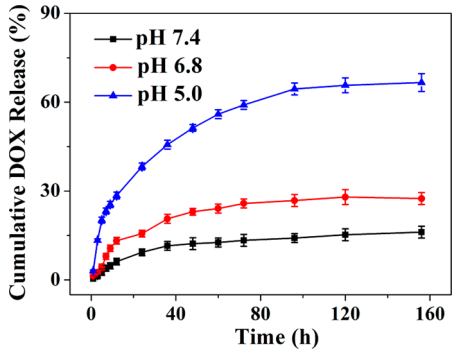

Figure 5. DOX release profiles of DOX-loaded polyionic complex nanoparticles in PBS at various pH values (7.4, 6.8, and 5.0) at 37 °C. Each point was an average of three measurements.

intracellular drug release at tumor cells, which will contribute to the overall therapeutic efficacy.

Enhanced Cellular Uptake of Charge-Conversional DOX-NPs. To further demonstrate whether the charge-conversion behavior of DOX-NPs will benefit their internalization by cells at tumor extracellular pH, the cellular uptake behaviors of DOX-NPs at pH 7.4 and 6.8 were investigated in A549 cells by CLSM. DOX-NPs were labeled by FITC (green). As shown in Figure 6, the significantly different cellular uptake level was observed for DOX-NPs at varied pH conditions. After 1 h incubation, DOX-NPs were successfully uptaken at pH 6.8, whereas the green fluorescence was rarely observed in the cells incubated with DOX-NPs at pH 7.4. When the incubation period increased to 3 h, DOX-NPs internalized by cells at pH 7.4 and 6.8 both increased. And extremely strong green fluorescence was observed in cells after incubation at pH 6.8, compared with that at pH 7.4. The different cell uptake level may be a result of the positive surface charge of DOX-NPs at pH 6.8, which leads to the enhanced cellular internalization due to the strong interaction of the nanoparticles with cells. On the contrary, DOX-NPs remains negatively charged at pH 7.4, which may hamper the cellular internalization to a certain degree. These results directly confirmed that DOX-NPs exhibited greatly enhanced cellular uptake at tumor extracellular pH. The intracellular DOX release behaviors of DOX-NPs at pH 7.4 and 6.8 were also investigated in A549 cells by CLSM. As expect, at the same incubation period, enhanced intracellular DOX uptake was observed in cells after treatment with DOX-NPs at pH 6.8 (Figure 6), which was probably attributed to the high level of cell uptake at slightly acidic conditions. Additionally, at pH 6.8, DOX fluorescence was mainly observed in the cytosol after 1 h of incubation. However, after 3 h incubation, DOX was dominantly localized in the cell nucleus, indicating the efficient release of DOX promoted by the increased acidity in subcellular compartments.

***In Vitro* Cytotoxicity Studies.** The *in vitro* cytotoxicities of mPEG-*b*-P(Glu-*co*-Phe), mPEG-*b*-P(Lys-*co*-Phe), blank polyionic complex nanoparticles, free DOX, and DOX-NPs were evaluated using MTT assay. HeLa (human cervical cancer) and A549 (human lung adenocarcinoma) cell lines were utilized. As shown in Figures 7A and 7B, mPEG-*b*-P(Lys-*co*-Phe) was found to be obviously toxic to the cells at higher concentration (500 μg mL^{-1}), which was attributed to the positive surface charge induced by lysine residues.[35] However, the cell viabilities of HeLa and A549 cells treated with mPEG-*b*-P(Glu-*co*-Phe) copolymers and blank polyionic complex nanoparticles were around 85 to 100%, respectively, at all test concentrations up to 500 μg mL^{-1}, indicating that the polyionic complex nanoparticles had excellent safety and biocompatibility. DOX-NPs exhibited a dose- and time-dependent cell proliferation

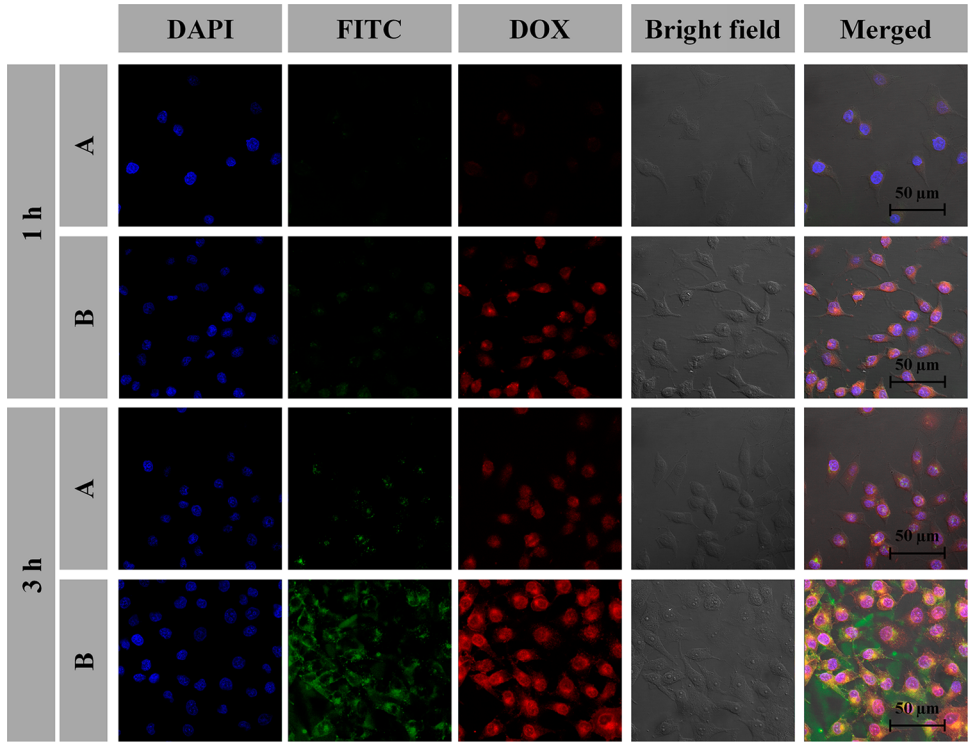

Figure 6. Confocal laser scanning microscopic observation of A549 cells after incubation with FITC-labeled DOX-loaded polyionic complex nanoparticles at pH 7.4 (A) or 6.8 (B) for 1 h and 3h.

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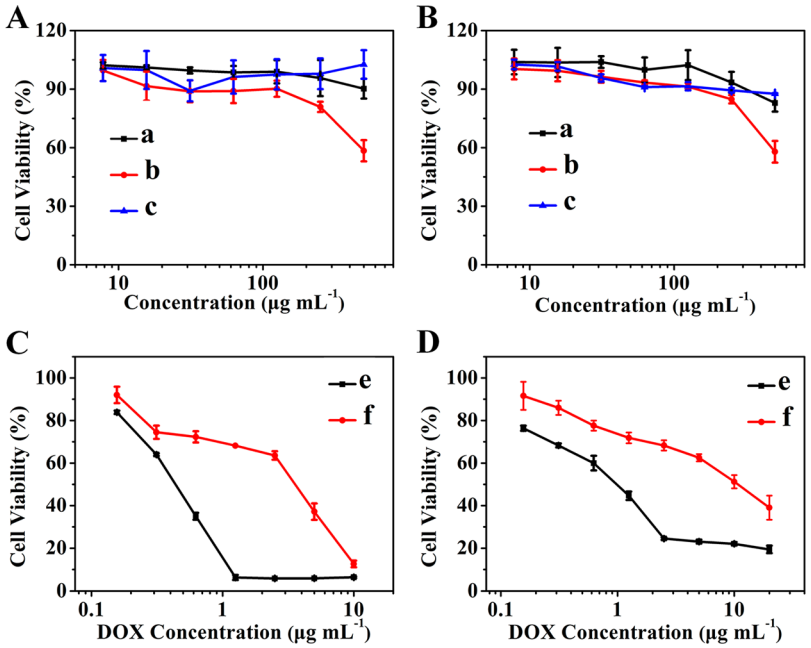

Figure 7. Cell viabilities of HeLa (A and C) and A549 (B and D) cells incubated with mPEG-*b*-P(Glu-*co*-Phe) (a), mPEG-*b*-P(Lys-*co*-Phe) (b), blank polyionic complex nanoparticles (c), free DOX (e), and DOX-loaded polyionic complex nanoparticles (f) for 48 h. Data are presented as the average ± standard deviation (n = 3).

inhibition behavior for both HeLa and A549 cells (Figures 7C and 7D). The results also showed that DOX-NPs revealed lower cell proliferation inhibition activity as compared with free DOX at the same drug concentration, which might be attributable to the poor cell uptake and slow drug release of DOX-NPs at pH 7.4.

To verify the feasibility of the charge-conversional DOX-NPs for cancer therapy *in vitro*, A549 and HeLa cells were treated with DOX-NPs in DMEM at pH 7.4 and 6.8 and cell viabilities were measured by MTT assay. As shown in Figure 8, DOX-

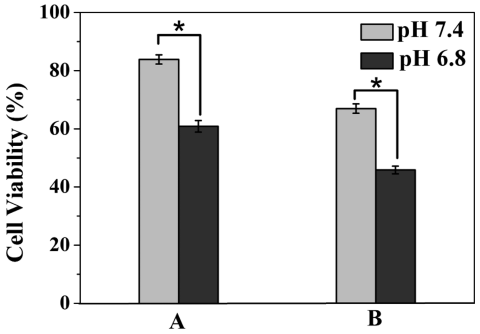

Figure 8. Cell viabilities of A549 (A) and HeLa (B) cells after incubation with DOX-loaded polyionic complex nanoparticles (DOX-NPs) at the DOX concentration of 40 μg mL^{-1} at different pH conditions. Cells were first incubated with DOX-NPs in culture media for 1.5 h at pH 7.4 or 6.8, and then the media were replaced with fresh DMEM at pH 7.4 and further incubated for 48 h. * p < 0.001.

NPs showed the obviously enhanced antitumor activity at pH 6.8 compared to that at pH 7.4 (p < 0.001). For A549 cells, cell viabilities at pH 7.4 and 6.8 were 83.9 and 60.9%, respectively. Cell viabilities for HeLa cells at pH 7.4 and 6.8 were 67.0 and 45.9%, respectively. The enhanced cell-proliferation inhibition of DOX-NPs at pH 6.8 is mainly attributed to the high level of cell uptake. In addition, the slightly enhanced DOX release at acidic conditions may contribute to the enhanced cell-proliferation inhibition to some extent.

Hemolysis Assay. The excellent blood compatibilities of the nanoscaled drug delivery systems are among the basic requisites because these nanomedicines will be finally be administrated via intravenous injection into blood vessels. The blood compatibilities of blank polyionic complex nanoparticles, free DOX·HCl, and DOX-NPs were evaluated using hemolysis assay. As shown in Figure 9, blank polyionic complex nanoparticles showed inappreciable hemolysis toxicity (~0%) to RBCs even at a concentration of 10 mg mL^{-1} (Figure 9A), revealing the outstanding blood compatibility of this material. DOX-NPs also exhibited a lower hemolysis activity as compared with free DOX·HCl (Figure 9B). Such low hemolytic activity of the nanocarrier might be attributed to the outer hydrophilic PEG shell and the negative surface charge of these nanoparticles, which will benefit their *in vivo* applications.

Maximum Tolerated Dose Study. Base on the above results, the maximum tolerated doses of DOX-NPs and free DOX for a single dose were investigated in tumor-free Kunming mice before the *in vivo* antitumor study. The mice were treated with different doses of DOX-NPs or free DOX by intravenous injection, and the drug effects were investigated by daily observation of body weight changes and survival rates. As shown in Figure 10, free DOX could be tolerated at a dose of 5 mg kg^{-1}, while increasing the DOX dose to 10 mg kg^{-1} led to the death of 2 mice among the 3 treated mice, indicating that the single iv MTD for free DOX was about 5 mg kg^{-1}. MTD for free DOX was consistent with the reported LD_{50} of DOX (the lethal dose of killing 50% of the test animals within a designated period), which is around 12 mg kg^{-1}.[8] On the contrary, no obvious body weight loss and no toxic death were observed for the mice treated with DOX-NPs at all designed DOX dosages from 5 to 25 mg kg^{-1}, revealing that MTD for DOX-NPs was above 25 mg kg^{-1}. The high MTD for DOX-NPs may be attributed to the slow release kinetics of DOX

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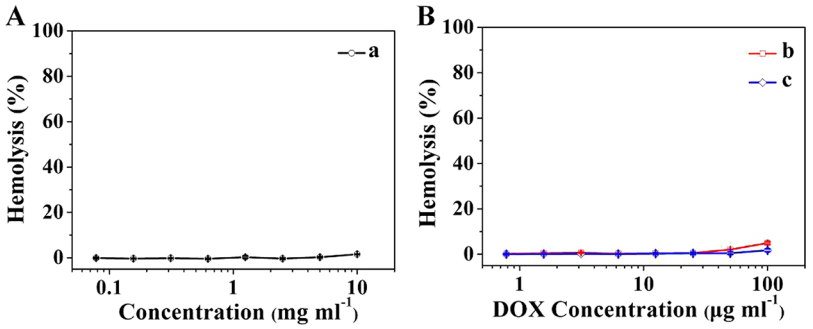

Figure 9. Hemolytic activities of blank polyionic complex nanoparticles (a), free DOX·HCl (b), and DOX-NPs (c) on rabbit red blood cells.

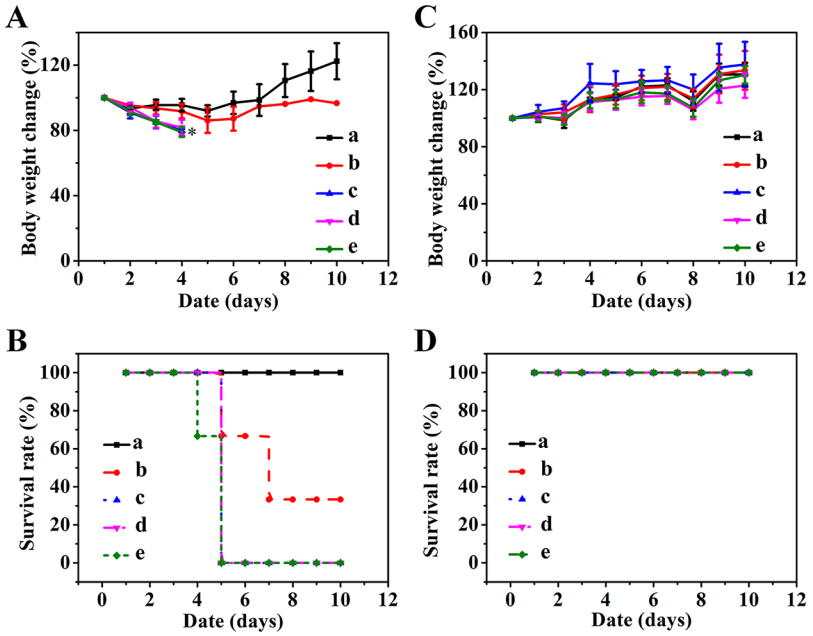

Figure 10. Maximum tolerated dose (MTD) studies on body weight change and survial rate for free DOX (A and B) and DOX-NPs (C and D) in tumor-free Kunming mice at different DOX doses [(a) 5 mg kg^{-1}, (b) 10 mg kg^{-1}, (c) 15 mg kg^{-1}, (d) 20 mg kg^{-1}, (e) 25 mg kg^{-1}].

under normal physiological conditions (Figure 5) and the remarkable safety of the polyionic complex nanoparticles. In addition, the negative surface charge of DOX-NPs would also contribute to the high MTD, since it has been reported that the slightly negative surface charge could decrease the uptake by RES and enhance the blood compatibility.[23] The wide therapeutic window for DOX-NPs will greatly benefit the final *in vivo* application.

***In Vivo* Antitumor Efficiency.** The *in vivo* antitumor activity and systemic toxicity of DOX-NPs were carried out in mice bearing A549 xenograft. Mice were treated with PBS, free DOX·HCl, and DOX-NPs. The tumor volumes and the body weights were measured every two or three days. As shown in Figure 11, tumor volume of PBS treatment group increased rapidly, while all DOX formulation treatment groups were effective in inhibiting the tumor growth. At the end of the treatment (day 17), the average tumor volumes of mice treated with free DOX·HCl (2 mg kg^{-1}), free DOX·HCl (4 mg kg^{-1}), DOX-NPs (2 mg kg^{-1}), and DOX-NPs (4 mg kg^{-1}) were 23.1%, 14.2%, 15.4%, and 10.7% of that in the control group, respectively ($p < 0.001$), demonstrating the dose-dependent antitumor activities of both free DOX·HCl and DOX-NPs. Even though the charge-conversional DOX-NPs were less cytotoxic than free DOX·HCl *in vitro*, the *in vivo* performance of DOX-NPs was better than that of free DOX in tumor inhibitation. The mice treated with DOX-NPs had a significantly lower mean tumor volume ($p < 0.01$) than the mice in the free DOX groups at the dose of 2 mg kg^{-1} (Figure 11B). The enhanced tumor inhibition of DOX-NPs may be attributed to the prolonged circulation time, the efficient cell uptake after accumulation at the tumor site *via* the EPR effect, and the accelerated drug release in subcellular compartments. With the increase in DOX dose, there was no significant difference in the tumor volumes of the free DOX·HCl and DOX-NPs treatment groups, which was most probably because of the severe body damage and malnutrition of animals induced by higher dose of free DOX·HCl. Body weight change is a clear reflection of drug-related systemic toxicity. As shown in Figure 11C, the treatment with free DOX at 4 mg kg^{-1} resulted in the greatest body weight loss (24%), compared with free DOX at 2 mg kg^{-1} (14%), indicating the seriously toxic effects of DOX caused by the slightly increasing dose. In contrast, the treatment with DOX-NPs at 2 or 4 mg kg^{-1} appeared to be well-tolerated and did not induce any significant body weight loss. These results demonstrated that DOX-NPs exhibited enhanced antitumor efficacy over free DOX with minimal drug-related toxicity, suggesting that the charge-conversional

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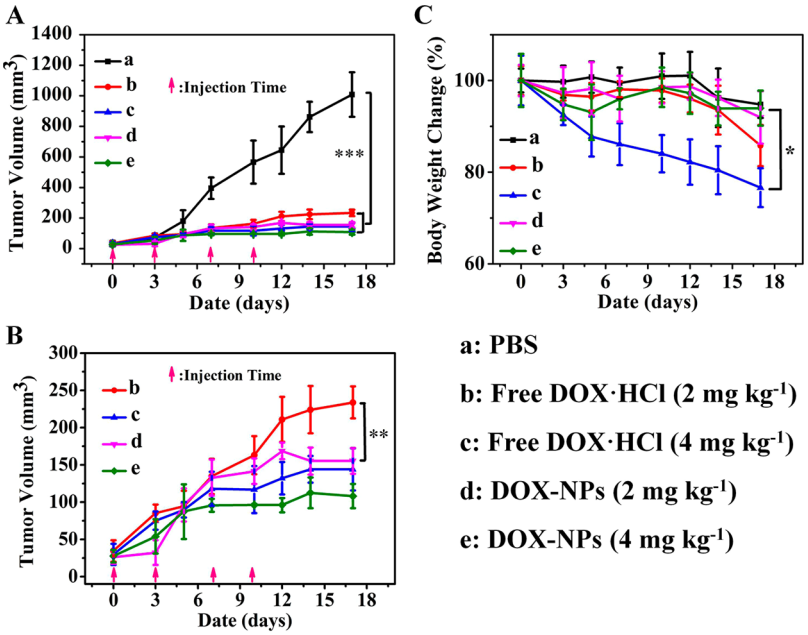

Figure 11. *In vivo* antitumor efficacy of free DOX and DOX-loaded polyionic complex nanoparticles in the A549 tumor bearing nude mice. (A) Tumor volume of the mice as a function of time; (B) amplificatory figure of tumor volume; (C) body weight change with the time of tumor bearing mice. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

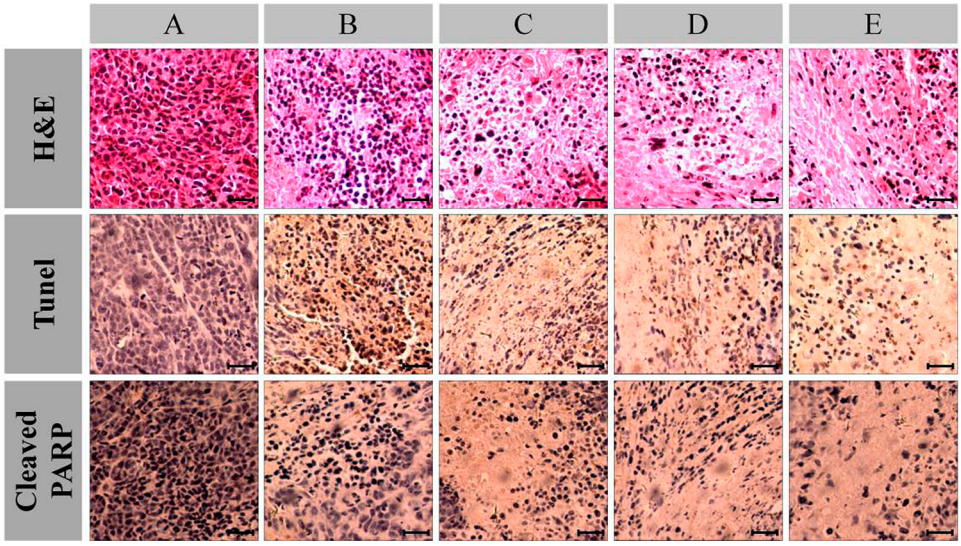

Figure 12. Histopathological analysis of tumors in A549 human lung cancer xenograft-bearing nude mice: (A) PBS; (B) free DOX (2 mg kg^{-1}); (C) DOX-NPs ((2 mg kg^{-1}); (D) free DOX (4 mg kg^{-1}); and (E) DOX-NPs (4 mg kg^{-1}). Nuclei were stained bluish violet while extracellular matrix and cytoplasm were stained pink in H&E staining. Brown and blue stains indicated apoptotic and normal cells, respectively, in TUNEL analysis; brown and blue stains indicated cleaved PARP1 and nuclei, respectively, in immunohistochemical assay. Scale bars: 100 μm.

polyionic complex is a promising drug delivery system for cancer therapy.

Histological and Immunohistochemical Analyses. To further evaluate the antitumor efficacy of the charge-conversional DOX-NPs, A549 tumor bearing nude mice were sacrificed at the end of the treatment and the tumor sections were prepared and stained with H&E, TUNEL, and PARP for pathology analysis (Figure 12).

For H&E staining, the normal tumor cells had large nuclei with spherical or spindle shape and more chromatin, whereas the necrotic cells did not have clear cell morphology, and the chromatin became darker and pyknotic or absent outside the cell. Poly-ADP-ribose polymerase (PARP) was one of the essential substrates cleaved by both caspase-3 and -7. The presence of cleaved PARP1 could further detect DNA strand breaks in many cell types. The TUNEL assay could detect DNA fragmentation in the nuclei of tumor cells. As shown in Figure 12, the various degrees of tissue necrosis and cell apoptosis were observed in the free DOX·HCl and DOX-NPs treated groups at 2 and 4 mg kg^{-1} dose, and the highest level of tumor necrosis and apoptosis was observed in the group treated with DOX-NPs at 4 mg kg^{-1}, which was consistent with *the in vivo* tumor growth inhibition study. These results clearly confirmed that the charge-conversional DOX-NPs provided a higher antitumor efficacy as compared with free DOX.

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CONCLUSION

A polypeptide-based pH-responsive charge-conversional polyionic complex nanoparticle system was developed for promoting the tumor cell uptake and DOX delivery in cancer treatment. The charge-conversional polyionic complex nanoparticles were prepared by simply mixing a pair of oppositely charged block copolymers which could load DOX with a decent drug loading content. DOX-NPs could reverse their surface charge from the negatively charged form into the positively charged form in the slightly acidic tumor extracellular environment. CLSM and *in vitro* cytotoxicity studies confirmed that the charge-conversional DOX-NPs exhibited remarkably enhanced cellular uptake and antitumor efficiency at the tumor extracellular pH. MTD study demonstrated that DOX-NPs had an excellent safety profile compared with the free DOX. The *in vivo* study on A549 lung tumor bearing mice demonstrated that DOX-NPs exhibited much lower toxicity and higher antitumor efficacy compared to free DOX·HCl at the same drug concentration. With convenient fabrication, robust self-assembling structure, excellent cytocompatibility and biocompatibility, favorable charge-conversional feature, and promoted intracellular DOX release behavior, the charge-conversional polyionic complex nanoparticles provide a useful and versatile platform for efficient cancer chemotherapy.

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Notes

The authors declare no competing financial interest.

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REFERENCES

(1) Kim, S.; Shi, Y.; Kim, J. Y.; Park, K.; Cheng, J.-X. Overcoming the barriers in micellar drug delivery: loading efficiency, in vivo stability, and micelle-cell interaction. *Expert Opin. Drug Delivery* **2010**, *7* (1), 49−62.

(2) Peer, D.; Karp, J. M.; Hong, S.; FarokHzad, O. C.; Margalit, R.; Langer, R. Nanocarriers as an emerging platform for cancer therapy. *Nat. Nanotechnol.* **2007**, *2* (12), 751−760.

(3) Barenholz, Y. Doxil (R) - The first FDA-approved nano-drug: Lessons learned. *J. Controlled Release* **2012**, *160* (2), 117−134.

(4) Li, M. Q.; Song, W. T.; Tang, Z. H.; Lv, S. X.; Lin, L.; Sun, H.; Li, Q. S.; Yang, Y.; Hong, H.; Chen, X. S. Nanoscaled Poly(L-glutamic acid)/Doxorubicin-Amphiphile Complex as pH-responsive Drug Delivery System for Effective Treatment of Nonsmall Cell Lung Cancer. *ACS Appl. Mater. Interfaces* **2013**, *5* (5), 1781−1792.

(5) Uchino, H.; Matsumura, Y.; Negishi, T.; Koizumi, F.; Hayashi, T.; Honda, T.; Nishiyama, N.; Kataoka, K.; Naito, S.; Kakizoe, T. Cisplatin-incorporating polymeric micelles (NC-6004) can reduce nephrotoxicity and neurotoxicity of cisplatin in rats. *Br. J. Cancer* **2005**, *93* (6), 678−687.

(6) Wagner, E. Programmed drug delivery: nanosystems for tumor targeting. *Expert Opin. Biol. Ther.* **2007**, *7* (5), 587−593.

(7) Song, W.; Tang, Z.; Li, M.; Lv, S.; Yu, H.; Ma, L.; Zhuang, X.; Huang, Y.; Chen, X. Tunable pH-Sensitive Poly(beta-amino ester)s Synthesized from Primary Amines and Diacrylates for Intracellular Drug Delivery. *Macromol. Biosci.* **2012**, *12* (10), 1375−1383.

(8) Bae, Y.; Nishiyama, N.; Fukushima, S.; Koyama, H.; Yasuhiro, M.; Kataoka, K. Preparation and biological characterization of polymeric micelle drug carriers with intracellular pH-triggered drug release property: Tumor permeability, controlled subcellular drug distribution, and enhanced in vivo antitumor efficacy. *Bioconjugate Chem.* **2005**, *16* (1), 122−130.

(9) Yu, S.-f.; Wang, Z.; Wu, G.-l.; Wang, Y.-n.; Gao, H.; Ma, J.-b. Anti-Tumor Drug Delivery of a pH-Sensitive Poly(aspartic acid)-Containing Block Copolymer. *Acta Polym. Sin.* **2012**, *4*, 427−432.

(10) Zheng, X. M.; Jiang, T.; He, F. Synthesis and Characterization of PNIPAM-b-polycarbonate Copolymers as Self-Assembled Thermosensitive Micelles for Drug Delivery. *Acta Polym. Sin.* **2011**, *8*, 895−902.

(11) Lee, E. S.; Oh, K. T.; Kim, D.; Youn, Y. S.; Bae, Y. H. Tumor pH-responsive flower-like micelles of poly(L-lactic acid)-b-poly(ethylene glycol)-b-poly(L-histidine). *J. Controlled Release* **2007**, *123* (1), 19−26.

(12) van Sluis, R.; Bhujwalla, Z. M.; Raghunand, N.; Ballesteros, P.; Alvarez, J.; Cerdan, S.; Galons, J. P.; Gillies, R. J. In vivo imaging of extracellular pH using (1)H MRSI. *Magn. Reson. Med.* **1999**, *41* (4), 743−750.

(13) Leeper, D. B.; Engin, K.; Thistlethwaite, A. J.; Hitchon, H. D.; Dover, J. D.; Li, D. J.; Tupchong, L. Human Tumor Extracellular Ph as a Function of Blood-Glucose Concentration. *Int. J. Radiat. Oncol.* **1994**, *28* (4), 935−943.

(14) Ojugo, A. S. E.; McSheehy, P. M. J.; McIntyre, D. J. O.; McCoy, C.; Stubbs, M.; Leach, M. O.; Judson, I. R.; Griffiths, J. R. Measurement of the extracellular pH of solid tumours in mice by magnetic resonance spectroscopy: a comparison of exogenous F-19 and P-31 probes. *NMR Biomed.* **1999**, *12* (8), 495−504.

(15) Chen, W.; Meng, F. H.; Cheng, R.; Zhong, Z. Y. pH-Sensitive degradable polymersomes for triggered release of anticancer drugs: A comparative study with micelles. *J. Controlled Release* **2010**, *142* (1), 40−46.

(16) Huang, W. C.; Chiang, W. H.; Huang, Y. F.; Lin, S. C.; Shih, Z. F.; Chern, C. S.; Chiang, C. S.; Chiu, H. C. Nano-scaled pH-responsive polymeric vesicles for intracellular release of doxorubicin. *J. Drug Targeting* **2011**, *19* (10), 944−953.

(17) Manocha, B.; Margaritis, A. Controlled Release of Doxorubicin from Doxorubicin/gamma-Polyglutamic Acid Ionic Complex. *J. Nanomater.* **2010**, DOI: 10.1155/2010/780171.

(18) Dadsetan, M.; Taylor, K. E.; Yong, C.; Bajzer, Z.; Lu, L. C.; Yaszemski, M. J. Controlled release of doxorubicin from pH-responsive microgels. *Acta Biomater.* **2013**, *9* (3), 5438−5446.

(19) Xu, P. S.; Van Kirk, E. A.; Zhan, Y. H.; Murdoch, W. J.; Radosz, M.; Shen, Y. Q. Targeted charge-reversal nanoparticles for nuclear drug delivery. *Angew. Chem., Int. Ed.* **2007**, *46* (26), 4999−5002.

(20) Cho, E. C.; Xie, J. W.; Wurm, P. A.; Xia, Y. N. Understanding the Role of Surface Charges in Cellular Adsorption versus Internalization by Selectively Removing Gold Nanoparticles on the Cell Surface with a I-2/KI Etchant. *Nano Lett.* **2009**, *9* (3), 1080−1084.

(21) Gratton, S. E. A.; Ropp, P. A.; Pohlhaus, P. D.; Luft, J. C.; Madden, V. J.; Napier, M. E.; DeSimone, J. M. The effect of particle design on cellular internalization pathways. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105* (33), 11613−11618.

(22) Oupicky, D.; Ogris, M.; Howard, K. A.; Dash, P. R.; Ulbrich, K.; Seymour, L. W. Importance of lateral and steric stabilization of polyelectrolyte gene delivery vectors for extended systemic circulation. *Mol. Ther.* **2002**, *5* (4), 463−472.

(23) Xiao, K.; Li, Y.; Luo, J.; Lee, J. S.; Xiao, W.; Gonik, A. M.; Agarwal, R. G.; Lam, K. S. The effect of surface charge on in vivo biodistribution of PEG-oligocholic acid based micellar nanoparticles. *Biomaterials* **2011**, *32* (13), 3435−3446.

(24) Yoon, S. R.; Yang, H. M.; Park, C. W.; Lim, S.; Chung, B. H.; Kim, J. D. Charge-conversional poly(amino acid)s derivatives as a drug

delivery carrier in response to the tumor environment. *J. Biomed. Mater. Res., Part A* **2012**, *100A* (8), 2027−2033.
(25) Du, J. Z.; Du, X. J.; Mao, C. Q.; Wang, J. Tailor-Made Dual pH-Sensitive Polymer-Doxorubicin Nanoparticles for Efficient Anticancer Drug Delivery. *J. Am. Chem. Soc.* **2011**, *133* (44), 17560−17563.
(26) Bae, Y.; Kataoka, K. Intelligent polymeric micelles from functional poly(ethylene glycol)-poly(amino acid) block copolymers. *Adv. Drug Delivery Rev.* **2009**, *61* (10), 768−784.
(27) Shah, N. B.; Vercellotti, G. M.; White, J. G.; Fegan, A.; Wagner, C. R.; Bischof, J. C. Blood-Nanoparticle Interactions and in Vivo Biodistribution: Impact of Surface PEG and Ligand Properties. *Mol. Pharmaceutics* **2012**, *9* (8), 2146−2155.
(28) Harada, A.; Kataoka, K. Formation of Polyion Complex Micelles in an Aqueous Milieu from a Pair of Oppositely-Charged Block-Copolymers with Poly(Ethylene Glycol) Segments. *Macromolecules* **1995**, *28* (15), 5294−5299.
(29) Ding, J.; Zhuang, X.; Xiao, C.; Cheng, Y.; Zhao, L.; He, C.; Tang, Z.; Chen, X. Preparation of photo-cross-linked pH-responsive polypeptide nanogels as potential carriers for controlled drug delivery. *J. Mater. Chem.* **2011**, *21* (30), 11383.
(30) van Dijk-Wolthuis, W. N. E.; van de Water, L.; van de Wetering, P.; van Steenbergen, M. J.; Kettenes-van den Bosch, J. J.; Schuyl, W. J. W.; Hennink, W. E. Synthesis and characterization of poly-L-lysine with controlled low molecular weight. *Macromol. Chem. Phys.* **1997**, *198* (12), 3893−3906.
(31) Bressenot, A.; Marchal, S.; Bezdetnaya, L.; Garrier, J.; Guillemin, F.; Plenat, F. Assessment of Apoptosis by Immunohistochemistry to Active Caspase-3, Active Caspase-7, or Cleaved PARP in Monolayer Cells and Spheroid and Subcutaneous Xenografts of Human Carcinoma. *J. Histochem. Cytochem.* **2009**, *57* (4), 289−300.
(32) Leuchs, H. Glycine-carbonic acid. *Ber. Dtsch. Chem. Ges.* **1906**, *39*, 857−861.
(33) Miyata, K.; Christie, R. J.; Kataoka, K. Polymeric micelles for nano-scale drug delivery. *React. Funct. Polym.* **2011**, *71* (3), 227−234.
(34) Zhao, C. L.; Winnik, M. A.; Riess, G.; Croucher, M. D. Fluorescence Probe Techniques Used to Study Micelle Formation in Water-Soluble Block Copolymers. *Langmuir* **1990**, *6* (2), 514−516.
(35) Deng, J. Z.; Gao, N. N.; Wang, Y. A.; Yi, H. Q.; Fang, S. T.; Ma, Y. F.; Cai, L. T. Self-Assembled Cationic Micelles Based on PEG-PLL-PLLeu Hybrid Polypeptides as Highly Effective Gene Vectors. *Biomacromolecules* **2012**, *13* (11), 3795−3804.