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A pH sensitive co-delivery system of siRNA and doxorubicin for pulmonary administration to B16F10 metastatic lung cancer

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Pulmonary co-delivery to the lungs offers a potential therapy for pulmonary diseases. In this study, the doxorubicin was conjugated to polyethyleneimine by hydrazone bonds to form a pH sensitive conjugate (PEI-HZ-DOX). A co-delivery carrier was constructed by complexing Bcl2 siRNA with PEI-HZ-DOX. The complex particles could be used by pulmonary administration for metastatic lung cancer treatment. DOX release from PEI-HZ-DOX conjugate was in a pH-dependent pattern and accelerated by decreasing pH. The cell uptake of DOX and siRNA from PEI-HZ-DOX/siRNA and efficient intracellular release of DOX from PEI-HZ-DOX in B16F10 cells were further confirmed. Cell apoptosis and antitumor effects of the combined therapy were evaluated *in vitro*, the results showed that co-delivery of DOX and siRNA had better antitumor efficacy than mono-delivery of DOX or siRNA. Furthermore, the biodistribution results showed that pulmonary administration could improve the deposition amounts of DOX and siRNA in lungs and prolong the retention time compared with systemic administration, which demonstrated that the present delivery system are suitable for pulmonary co-delivery. Overall, pH sensitive PEI-HZ-DOX/siRNA complex nanoparticles exhibit great potential for clinical combination therapy of lung cancer by pulmonary administration in local delivery strategy.

Introduction

RNA interference (RNAi) has been widely used in medicine and biology.[1,2] Small interfering RNA (siRNA) can be incorporated into the RNA-induced silencing complex (RISC) and then degrade the complementary target mRNA, thus inhibit the expression of target protein at a post-transcriptional level. Moreover, doxorubicin (DOX, a most widely used anticancer drug) could inhibit the synthesis of RNA and DNA by inhibiting the progression of the topoisomerase II, which can induce the cell apoptosis.[4] Co-delivery of two or more therapeutic drugs with different anticancer mechanisms, which can effectively suppress the growth of tumors, has attracted more and more attention and has great potential for the therapeutic applications.[5-7] To enable the therapeutic use of siRNA and anticancer drug for achieving effective therapy, developing effective co-delivery systems is in great demand at present.

Currently, a number of researchers have developed different systems for co-delivery of DOX and siRNA[8,9] Generally, a safe and efficient co-delivery carrier is very crucial. The co-delivery systems should be designed to overcome hurdles, such as avoiding enzymatic degradation, carrying the drugs to the same cells, controlling the release of drugs and genes and decreasing the side effects of chemotherapy drugs.[10,11] To achieve these goals, nanoparticle platforms have provided advantages for co-delivery of chemotherapy drugs and siRNAs, such as liposomes,[12] inorganic materials[13] and polymers.[14] Some pH-sensitive linkages for connecting drugs with carriers were developed to enhance drug release in the slightly acidic microenvironments of tumor tissues, such as cis-aconityl[15,16] and hydrazone bonds.[17,18] Usually, positively charged materials are used for binding siRNAs and embedding the drugs. The perfect nanoparticles should have the advantages of high efficiency and low systemic toxicity when carrying therapeutic drugs and genes to the tumor regions. Base on this, it is necessary to develop co-delivery system and use effective delivery method, which would control the release of drugs and genes, quantify and sustain drugs and genes, decrease side effects, thus achieve an effective combination therapy.

Pulmonary delivery has become a widely used method to deliver drugs or genes for treatment of pulmonary or systemic disease, which presents several advantages such as high concentration level of drugs or genes in lungs, large alveolar surface area and extensive vascularization in the alveolar region for drugs or genes absorption, avoidance of first pass metabolism and reduction of the distribution in other normal tissues.[19-21] Using nanoparticles carriers for pulmonary co-delivery has gained lots of interests, which could result in prolonged residence time of drugs and genes.[22] There are some literatures about co-delivery systems for pulmonary administration based on porous microparticles and cationic liposome carriers,[23-26] however, co-delivery systems using polymer nanoparticles for pulmonary delivery have been rarely reported.[27] In the present study, a polymer nanoparticles system (PEI-HZ-DOX/Bcl2 siRNA) was prepared by electrostatic interaction between cationic PEI-HZ-DOX and anionic Bcl2 siRNA (Scheme 1). The DOX

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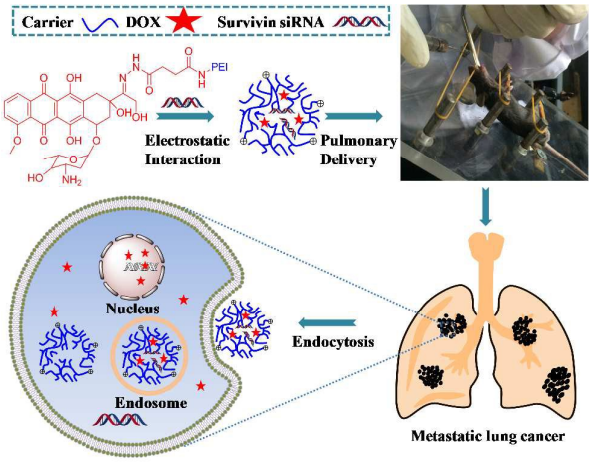
release from the acid-sensitive PEI-HZ-DOX with pH-responsive hydrazine bond was evaluated in different pH environment. The intracellular uptake, cell apoptosis and cytotoxicity of PEI-HZ-DOX/Bcl2 siRNA complex nanoparticles were evaluated *in vitro* studies. Furthermore, the biodistribution of PEI-HZ-DOX/Bcl2 siRNA complex nanoparticles *in vivo* in B16F10 metastatic lung cancer were investigated via pulmonary and systemic administration.



Scheme 1. The schematic illustration of pulmonary co-delivery of DOX and siRNA to the cells.

Experimental

Materials

Branched PEI (25,000 Da) and tert-Butyl carbazate were obtained from Sigma-Aldrich (St. Louis, MO, USA). Doxorubicin hydrochloride (DOX·HCl) was purchased from Beijing Huafeng United Technology Co., Ltd. (Beijing, China). Succinic anhydride (SA) was purchased from Alfa Aesar (Lancashire, UK). Dulbecco's modified Eagle's medium (DMEM), trypsin-EDTA solution and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, USA). Methyl thiazolyl tetrazolium (MTT) was from Amresco (Solon, Ohio, USA). AnnexinV-FITC/PI apoptosis detection kit was obtained from Nanjing KeyGEN Biotech Co. Ltd., China. FAM labeled siRNA, negative control siRNA (Nc siRNA) and two Bcl-2 siRNA duplexes (Bcl2-1 and Bcl2-2) were obtained from Genepharma (Suzhou, China). The sequences of Nc siRNA, Bcl2-1 and Bcl2-2 are as follows: Nc siRNA (sense) 5'-UUC UCC GAA CGU GUC ACG UTTdTdT-3', (antisense) 5'-ACG UGA CAC GUU CGG AGA ATTdTdT-3'; Bcl2-1 siRNA (sense) 5'-UGU GGA UGA CUG AGU ACC UGAdTdT-3', (anti-sense) 5'-UCA GGU ACU CAG UCA UCC ACAdTdT-3'; Bcl2-2 siRNA (sense) 5'-GUA CAU CCA UUA UAA GCU GUCdTdT-3', (anti-sense) 5'-GAC AGC UUA UAA UGG AUG UACdTdT-3'. The mixture of Bcl2-1 siRNA and Bcl2-2 siRNA in equal molar ratio was named Bcl2 siRNA. FAM-labeled siRNA and cy5-labeled siRNA were purchased from RiboBio (Guangzhou, China). Other reagents were obtained from Beijing Chemical Works (Beijing, China).

Synthesis of PEI-HZ-DOX and Characterization

The synthesis of PEI-HZ-DOX was described as the following steps. Firstly, SA (0.5 g) and tert-Butyl carbazate (0.7 g) were dissolved in 50-60 mL ethyl acetate. The mixture reacted for 4 h with constant stirring. After purification, SA-HZ-Boc was obtained. Then the mixture of SA-HZ-Boc, NHS and EDC were dissolved in dimethyl sulfoxide (DMSO) with stirring for 12 h, and the reaction solution was added to the PEI solution. The mixture proceeded with magnetic stirring for 24 h and the PEI-SA-HZ-Boc was obtained. After deprotection, PEI-SA-HZ (100 mg) and DOX (50 mg) were dissolved in 20-25 mL methanol, and the mixture was allowed to proceed at room temperature for 48 h in the dark. PEI-HZ-DOX was purified by dialysis method (MWCO 3,500 Da) and lyophilized. The chemical structures of PEI-HZ-DOX were conducted by ^{1}H NMR spectra at 400MHz (in D_2O, Bruker, Ettlingen, Germany). The measurements were carried out at room temperature in D_2O.

Drug Release Studies

Drug release studies were carried out according to previous method.[28] Briefly, 2 mL of PEI-HZ-DOX (the concentration of DOX was 50 μg/mL) was prepared and put into a dialysis bag (MWCO 7,000 Da), and then 58 mL of PBS (pH 7.4, 6.8 and 5.0) was added into the solution. 2 mL PBS was taken out from the release medium and then 2 mL fresh PBS was added at predetermined time points. DOX concentration was detected at an excitation of 480 nm and emission wavelength of 590 nm.

Preparation of PEI-HZ-DOX/siRNA Complex Nanoparticles

PEI-HZ-DOX and siRNA were dissolved in diethyl pyrocarbonate (DEPC) water at a 5/1 (wt/wt) ratio, then the solution was vortexed and incubated at room temperature for 30 min. PEI-HZ-DOX/siRNA complex nanoparticles were obtained. The sizes and the zeta potentials of PEI-HZ-DOX/siRNA complex nanoparticles were determined at 25 ^{o}C by a zeta potential/BI-90Plus particle size analyzer (Brookhaven, USA).

Cell Uptake Studies

The simultaneous delivery of DOX and siRNA was observed by confocal laser scanning microscope (CLSM, ZEISS LSM 780, Germany). B16F10 cells were seeded at a density of 1.0×10^{5}cells/well in six-well plate overnight. The cells were transfected with PEI-HZ-DOX/siRNA complex nanoparticles at a 5/1 (wt/wt) ratio for 3 h and 24 h, respectively. The cells were washed with cold PBS five times, and the cells were fixed with 4 % paraformaldehyde solution for 20 min at room temperature. The cell nuclei were stained by 4'-6-diamidino-2-phenylindole (DAPI, 1 mg/mL, 1 L/well) for 2 min. The cells were imaged by CLSM to observe the internalization of DOX and siRNA.

Cell Apoptosis Assay

B16F10 cells (2×10^{5} cells/well) were treated with Bcl2 siRNA, free DOX, free DOX/ Bcl2 siRNA, PEI-HZ-DOX/Nc siRNA and PEI-HZ-DOX/Bcl2 siRNA (5/1, wt/wt, 2 μg of siRNA/well) for 48 h and then harvested. Cells without treatment were used as the control. Cell apoptosis was detected with an Annexin V-FITC/PI apoptosis analysis kit. After treatment and harvesting, cells were rinsed by cold PBS and trypsinized. And then cells were suspended in binding buffer and stained with Annexin V-FITC and propidium iodide for 15 min in the dark. The apoptosis cells were performed using a flow cytometer (BD, USA).

Cytotoxicity Assay

The cytotoxicity of PEI/Nc siRNA, PEI/Bcl2 siRNA, PEI-HZ-DOX/Nc siRNA and PEI-HZ-DOX/Bcl2 siRNA (5/1, wt/wt, 2 μg of siRNA/well) was assessed with MTT against B16F10 cells. B16F10 cells (10,000 cells/well) were seeded in a 96-well plate at 37 °C for 24 h. The cells were incubated with different nanoparticles for 24 h and 48 h, respectively. Then 20 μL of MTT (5 mg/mL) was added into each well for extra 4 h. Finally, the medium was removed and each well was added with 160 μL of DMSO for dissolving the formazan crystals. The plate was measured at 492 nm using a Bio-Rad 680 Microplate Reader.

***In vivo* Imaging After Pulmonary Administration**

All animals were performed in compliance with the guidelines established by the School of Life Sciences Animal Care and Use Committee of Northeast Normal University, and all procedures were approved by the Animal Care and Use Committee of Northeast Normal University. Pulmonary administration was carried out according to the modification of previously described method.[29] Briefly, mice were administered pentobarbital sodium (Sigma, USA) by intraperitoneal injection at the dose of 70 mg/kg body weight. PEI-HZ-DOX/siRNA complex nanoparticles (DOX 10 μg, Cy5 labeled siRNA 20 μg) were directly sprayed to the lungs using a liquid aerosol device (MicroSprayer® Aerosolizer, Penn-Century, Philadelphia, PA). Lungs of mice were excised at fixed time intervals. The fluorescence at excitation and emission wavelengths of 523 and 560 nm for DOX distribution and of 650 and 670 nm for Cy5 siRNA distribution were imaged by measuring bioluminescence with a Maestro *in vivo* Imaging System (Cambridge Research & Instrumentation, Inc., USA) according to the manufacturer's instructions. Data were analyzed by a commercial software (Maestro 2.4). To evaluate the distribution of DOX and siRNA by pulmonary administration, mice were injected with the same PEI-HZ-DOX/siRNA complex nanoparticles via tail intravenous injection.

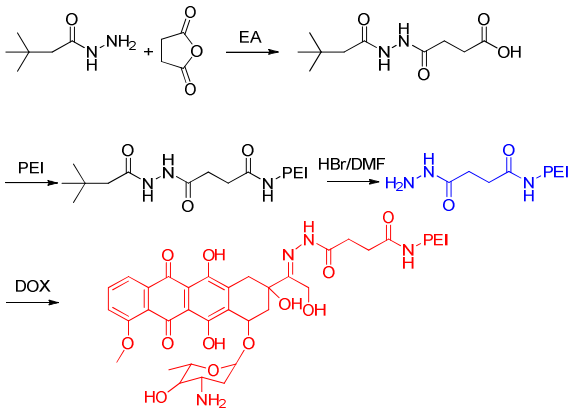
Statistical Analysis

All data are presented as the mean ± standard deviation (SD) of at least three independent experiments. Statistical analysis were performed using Student's t test (*P < 0.05, **P < 0.01, ***P < 0.001).

Results and discussion

Synthesis and Characterizations of PEI-HZ-DOX Conjugates

Hydrazone bond contained PEI-HZ-DOX was conveniently prepared according to the process shown in Scheme 2. The chemical structure of synthesized PEI-HZ-DOX was confirmed by ^{1}H NMR (Figure 1), the characteristic peaks at 2.5-3.5 ppm was assigned to the protons in PEI and at 7.0-8.0 ppm was the protons in DOX (a, b, c).[30] The ^{1}H NMR results showed that DOX was successfully conjugated to PEI via hydrazone bonds. The DOX contents (%) in PEI-HZ-DOX conjugates is 9.7 % calculated by ^{1}H NMR spectra and UV-vis spectrophotometry analysis.



Scheme 2. Synthesis procedures of PEI-HZ-DOX.

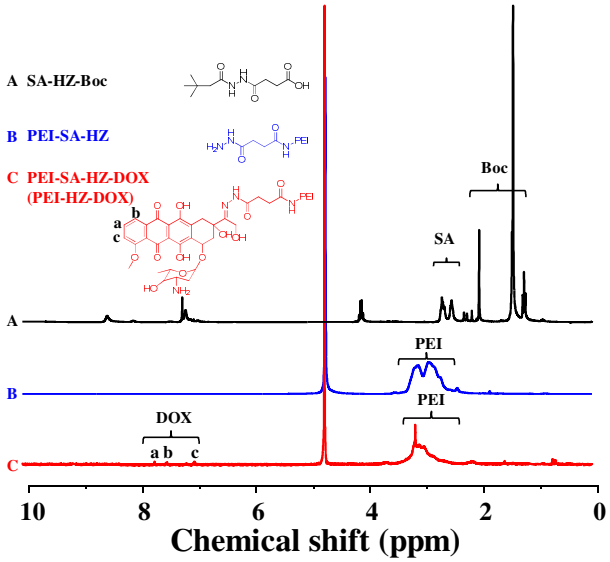


Figure 1. ^{1}H NMR spectra of SA-HZ-Boc, PEI-SA-SA and PEI-HZ-DOX in D2O.

***In vitro* Drug Release**

The DOX release behaviors from PEI-HZ-DOX conjugates *in vitro* were determined in PBS at pH 7.4, 6.8 and 5.0, mimicking the physiological, intratumoral and intracellular acidic microenvironments. As shown in Figure 2, there was no significant initial burst release of DOX at all pH value within 24 h. The amounts of DOX release increased significantly with the pH decreased from 7.4 to 5.0. At physiological pH (pH 7.4), approximately only 35.4 % DOX was released from PEI-HZ-DOX conjugates after 72 h. However, 52.2 % and 78.5 % DOX were released at pH 6.8 and 5.0, respectively. The release rate of DOX increased prominently (about higher 43 %) when the pH value changed from 7.4 to 5.0. Notably, when PEI-HZ-DOX conjugates entered into the acidic tumor tissue at pH 6.5-7.2 and even tumor cell endosome at pH 5.0-6.2, more and more DOX was released from PEI-HZ-DOX conjugates.[31] These results was attributed to the acid-sensitive hydrazone bonds in PEI-HZ-DOX conjugates.[32,33]

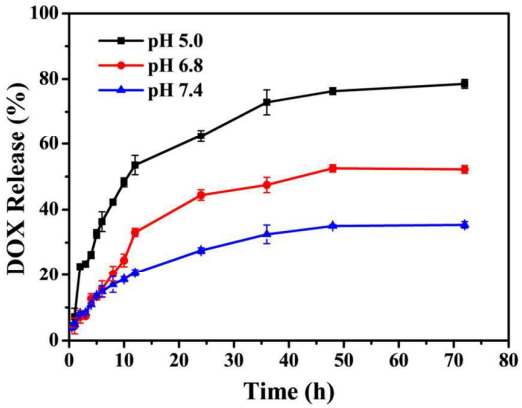


Figure 2. DOX release profiles for PEI-HZ-DOX in PBS at pH 7.4, 6.8 and 5.0. Results are shown as mean ± standard deviation (n=3).

Particle Sizes and Zeta Potentials

The hydrodynamic diameter of PEI-HZ-DOX/siRNA complex nanoparticles was about 78.2±4.1 nm (Figure 3). The size of nanoparticles is a crucial factor in designing an ideal cancer-targeting delivery carrier, too small or too large sizes of nanoparticles are not suitable for selecting tumor distribution of drugs. Generally, the nanoparticles should be small enough to penetrate into tumor region via the leaky vasculatures (<200 nm), as well as large enough to reduce the renal filtration (>20 nm). The size of PEI-HZ-DOX/siRNA complex nanoparticles in our study could facilitate intracellular uptake in tumors and avoid the reticuloendothelial system-mediated clearance.[34,35] Furthermore, the zeta potentials of PEI-HZ-DOX/siRNA complex nanoparticles were +20.4±1.9 mV, which would increase cell uptake via electrostatic interactions with cell membrane.[36]

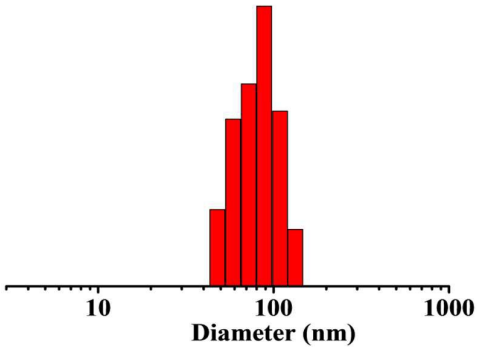


Figure 3. Particle size of PEI-HZ-DOX/siRNA at ratio of 5/1 (wt/wt).

Intracellular Distribution

The intracellular distribution of PEI-HZ-DOX/siRNA complex nanoparticles was investigated by CLSM in B16F10 cells. As shown in Figure 4, green (FAM-siRNA) fluorescence was caught in the cytoplasm at both 3 h and 24 h incubation. Furthermore, most of the DOX fluorescence was found in the cytoplasm after 3 h of incubation, but DOX fluorescence was located both in nucleus and cytoplasm after 24 h of incubation. These results showed that siRNA and DOX were co-delivered into the same cells, and DOX could release from the complex nanoparticles in acidic environment, which could be attributed to that the complex nanoparticles entered to cells probably via endocytosis mechanism, and then, the complex nanoparticles escaped from the lysosomes induced by the proton sponge effect of PEI, thus siRNA released from the complex nanoparticles, and DOX released from the PEI-HZ-DOX conjugate after cleavage of the hydrazone bonds.[32,37]

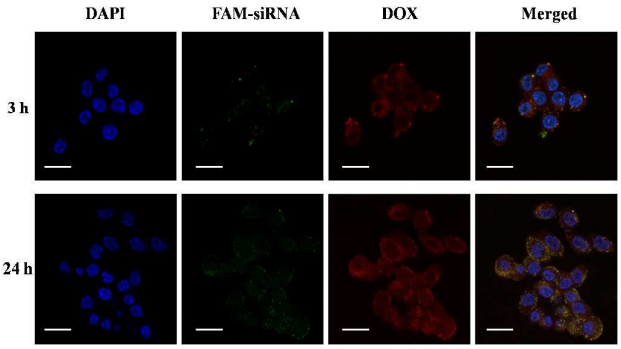


Figure 4. CLSM images of B16F10 cells incubated with PEI-HZ-DOX/siRNA complex nanoparticles for 3 h and 24 h. DAPI: cell nucleus (blue); FAM: siRNA (green); DOX (red). White scale bars=20 μm.

Apoptosis Assay

The apoptosis of the B16F10 cells treated with Bcl2 siRNA, free DOX, free DOX/ Bcl2 siRNA, PEI-HZ-DOX/Nc siRNA and PEI-HZ-DOX/Bcl2 siRNA was measured by flow cytometry (FCM). As shown in Figure 5, there were about 8.58 % and 46.47 % apoptosis cells when treated with Bcl2 siRNA and free DOX, respectively. The cells incubated with PEI-HZ-DOX/Bcl2 siRNA induced about 63.33 % apoptosis cells, higher about 11 % and 13 % than free DOX/ Bcl2 siRNA (52.03 %) and PEI-HZ-DOX/Nc siRNA (50.61 %). These results indicated that the co-delivery system of DOX and siRNA could lead to more effective apoptosis than that of mono-delivery system or free DOX and siRNA.

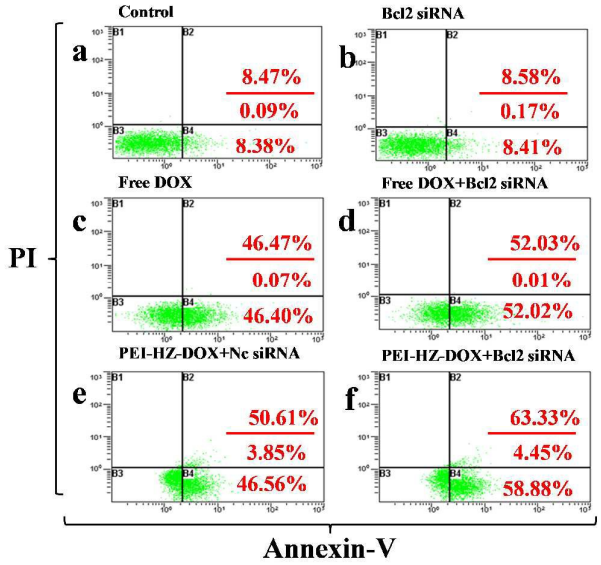


Figure 5. The apoptosis of B16F10 cells treated with Bcl2 siRNA (b), free DOX (c), free DOX/ Bcl2 siRNA (d), PEI-HZ-DOX/Nc siRNA (e)

and PEI-HZ-DOX/Bcl2 siRNA (f) and without treatment as control (a), respectively, for 48 h by flow cytometry.

Cell Cytotoxicity

MTT assay was conducted to determine the toxicities of PEI/Nc siRNA, PEI/Bcl2 siRNA, PEI-HZ-DOX/Nc siRNA and PEI-HZ-DOX/Bcl2 siRNA against B16F10 cells. As shown in Figure 6A, the cell viability reduced to 67.2 % for PEI-HZ-DOX/Nc siRNA and the value was 57.6 % for PEI-HZ-DOX/Bcl2 siRNA at 24 h. While, a significant decrease in cell viability was detected, and the cell viability reduced to 53.8 % for PEI-HZ-DOX/Nc siRNA and 39.6 % for PEI-HZ-DOX/Bcl2 siRNA at 48 h (Figure 6B). Co-delivery DOX and Bcl2 siRNA of PEI-HZ-DOX/Bcl2 siRNA complex nanoparticles displayed significant anti-proliferation effects, higher than that of mono-delivery of Bcl2 siRNA of PEI/Bcl2 siRNA or DOX of PEI-HZ-DOX/Nc siRNA, whereas the negative control PEI/Nc siRNA showed very low cytotoxicity at a siRNA concentration of 1 μg/mL. The above data showed that co-delivery of DOX and Bcl2 siRNA could effectively increase cytotoxicity against B16F10 cells, which may have better tumor inhibition capability *in vivo*.

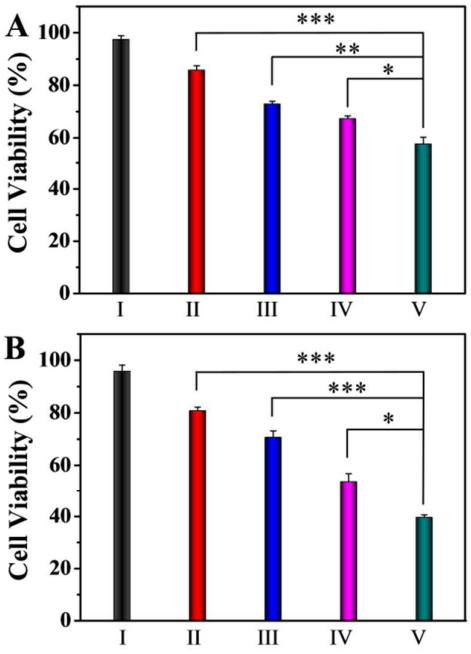


Figure 6. In vitro cell viability of B16F10 cells following incubation with control (I), PEI/Nc siRNA (II), PEI/Bcl2 siRNA (III), PEI-HZ-DOX/Nc siRNA (IV) and PEI-HZ-DOX/Bcl2 siRNA (V) nanoparticles for 24 h and 48 h, respectively. Indicated values are mean ± SD (n=6). (*P < 0.05, **P < 0.01, ***P < 0.001).

Biodistribution

For biodistribution determinations, the mice were treated with PEI-HZ-DOX/Cy5 siRNA by pulmonary administration and in comparison with systemic administration. After different time of post-injection, mice were sacrificed and the lungs of the mice were isolated. As shown in Figure 7A, the upper row was DOX fluorescence by pulmonary administration (PD), the lower row was fluorescence intensity of DOX by systemic administration (SD). The fluorescence intensity of DOX treated with PEI-HZ-DOX by pulmonary administration was significantly higher than treated by systemic administration, especially after 6 h post-injection, and the strong fluorescence signal sustained for about 7 days. Furthermore, *in vivo* distribution of Cy5 siRNA (Red) was also observed after pulmonary administration or systemic administration (Figure 7B), and the results were similar to the fluorescence intensity distribution of DOX. From the above results we could conclude that the most amount of DOX and siRNA could accumulate in lungs and sustain for at least 7 days after pulmonary administration, thus which may have better tumor inhibition capability for lung cancer *in vivo* than that via systemic administration.

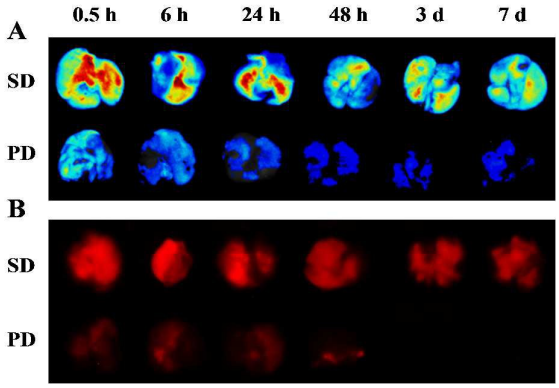


Figure 7. Distribution of DOX and Cy5 labeled siRNA in lungs at 0.5 h, 6 h, 24 h, 48 h, 3 d and 7 d after systemic delivery (SD) and pulmonary delivery (PD).

Conclusions

In summary, a pH sensitive co-delivery of DOX and siRNA system for the pulmonary delivery was developed. The obtained PEI-HZ-DOX/siRNA complex nanoparticles were formed spontaneously in aqueous solution with a diameter of 78 nm and zeta potentials of +20 mV. The particle size was suitable for selecting intratumoral accumulation and high cell uptake via electrostatic interactions with cell membrane. PEI-HZ-DOX/Bcl2 siRNA complex nanoparticles could efficiently deliver DOX and siRNA into the same cells. Furthermore, The PEI-HZ-DOX/Bcl2 siRNA showed significant combination therapy on cell apoptosis and tumor cytotoxicity in vitro. Notably, the DOX and siRNA fluorescence intensity treated with PEI-HZ-DOX/siRNA by pulmonary administration were significantly higher than treated by systemic administration, which may have good tumor inhibition capability for metastatic lung cancer in vivo.

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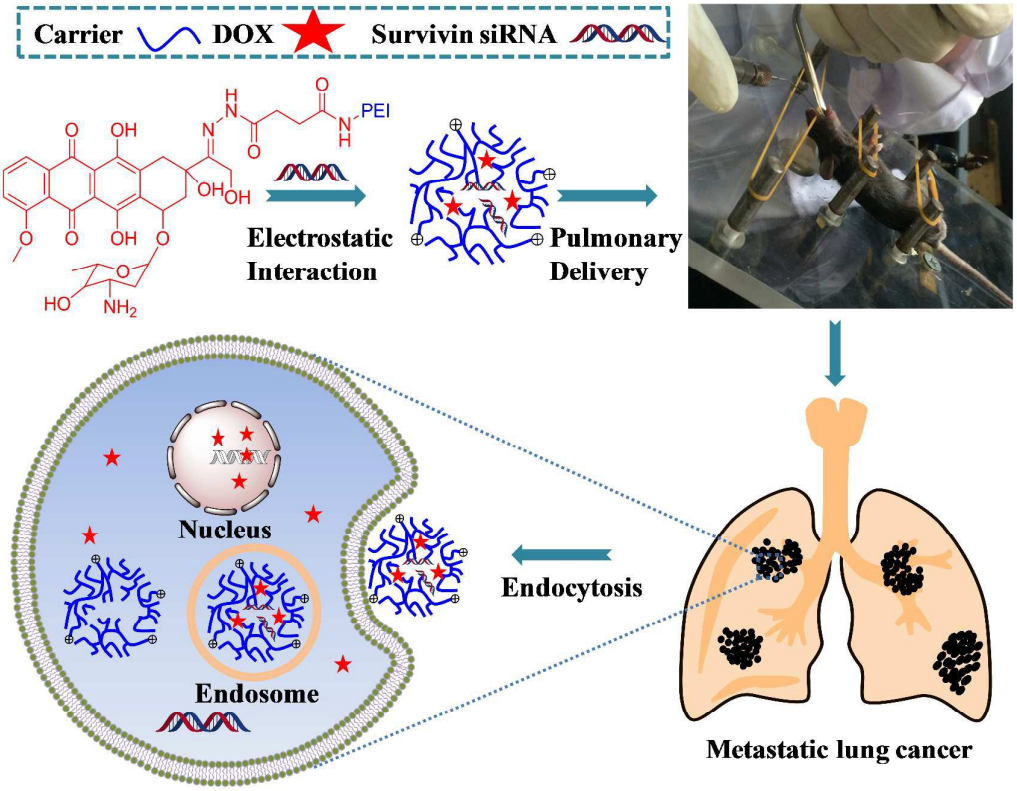
Graphical Abstract



The schematic illustration of pulmonary co-delivery of DOX and siRNA to the cells.

In this study, the doxorubicin was conjugated to polyethyleneimine by hydrazone bonds to form a pH sensitive conjugate (PEI-HZ-DOX). A co-delivery carrier was constructed by complexing Bcl2 siRNA with PEI-HZ-DOX. The complex particles could be co-delivered to cancer cells by pulmonary administration for metastatic lung cancer treatment. In the B16F10 tumor-bearing mice models, PEI-HZ-DOX/Cy5 siRNA could be significantly accumulated in lungs via the pulmonary delivery, which may have good tumor inhibition capability for metastatic lung cancer in vivo.