A Linear Polyethylenimine (LPEI) Drug Conjugate with Reversible Charge to Overcome Multidrug Resistance in Cancer Cells

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Abstract

Multidrug resistance (MDR) is an important hindrance to efficient cancer chemotherapy. Cancer cell lysosomes play an important role in intrinsic MDR by accumulating chemotherapy drugs and deactivating their therapeutic action. The cationic polymer polyethyleneimine (PEI) can disrupt the endosomal/lysosomal membrane via the proton-sponge effect (PSE). However, its positive charge makes it toxic and so it cannot be used *in vivo*. Here, linear PEI (LPEI) is used to demonstrate that a pH-triggered charge-reversal carrier can solve this problem. The imines are amidized by masking a lysosomal pH-active agent. LPEI regenerates its positive charge in the acidic endosomal/lysosomal cell compartments and disrupts the endosomal/lysosomal membrane, resulting in delivery of the drugs into the cytoplasm and nuclei where they exert their pharmacologic activity. Folic acid targeting groups are introduced into the polymer to increase its cancer-cell targeting capability. An anticancer drug camptothecin (CPT) conjugated to the carrier by intracellular cleavable disulfide bonds shows improved cytotoxicity over free CPT.

Keywords: linear polyethyleneimine (LPEI) drug conjugate; cancer-drug delivery; nuclear delivery; multidrug resistance; reversible charge; camptothecin

1. Introduction

Polymeric drug delivery systems (DDS), which include polymer drug conjugates,[1-3] dendrimers,[4-7] liposomes,[8, 9] polymer micelles and nanoparticles,[10-13] have attracted significant attention. These nano-sized DDS have several advantages over free drugs, including: 1) improved bioavailability through increasing the water solubility of hydrophobic drugs; [14] 2) increased stability and preserved drug activity during circulation in the blood compartments, [15] 3) prolonged circulation time in the blood, [16] 4) targeted accumulation in tumor tissue by passive targeting via the enhanced permeability and retention (EPR) effect[17] or by ligand (such

as folic acid) mediated active targeting [18, 19] and epidermal growth factor (EGF)[20]. However, the clinical efficacy of most of DDS is still low[21] and usually less than 1% of the administered active ingredient reaches the desired site[22] due to poor absorption and loss via metabolism and excretion.

DNA-interactive drugs, such as doxorubicin, camptothecin (CPT), and cisplatin, are some of the most effective anticancer drugs. They can intercalate, bind and distort DNA, block protein-associated DNA complexes or inactivate topoisomerase involved in DNA transcription, replication and repair to stop cancer cell growth.[23] Nuclear delivery of cancer therapy drugs is therefore the key to enhancing drug efficiency. However, drug-resistant cancer cells have many intracellular resistance mechanisms[24] to limit the access of cytosolic drugs to the nucleus. The drug carriers are typically taken up by target cells via endocytosis and trafficked through the endosomal-lysosomal pathway, which leads to degradation by lysosomal enzymes and loss of therapeutic activity.[25] Drug resistance cells also have transporter P-glycoprotein (Pgp) expressed in intracellular organelles such as lysosome, which actively sequester and trap drugs.[24] Only drugs that escape into the cytoplasm can go on to reach the nucleus, and endosomal escape remains a significant challenge to the nuclear delivery of cancer drugs.

Consequently, a variety of carrier system have been studied to enhance nuclear delivery, such as membrane-disruptive peptides and polymers,[26, 27] cell-specific targeting polymers and nuclear localization signal peptides (NLP)[28]. The cationic polymer, polyethyleneimine (PEI) is one of the most effective gene-delivery polymers.[29, 30] The relatively high gene-transfer activity of PEI is believed to be due in large part to efficient escape from the endosome via the proton-sponge effect.[29, 31] However, these highly positive charged peptides and polymers are

highly toxic. They can cause severe serum inhibition and are rapidly cleared from the plasma compartment, and thus cannot be used *in vivo*. [32]

A drug delivery system for *in vivo* tumor-targeted nuclear drug delivery should have slow interactions with the blood serum and red blood cells (RBCs) to ensure a long circulation time, be able to bind tumor cells, be quickly taken up once in the tumor, escape from the lysosome, traverse to the nucleus and finally release the drug. [33, 34] Amides with β -carboxylic acid groups are acid labile. [35-37] We used this type of amide to reversibly modify the amine groups and develop pH-triggered charge-reversal, which is ideal for tumor nucleus drug delivery. [18, 28, 38] In our former study, we developed a targeted charge-reversal Poly(L-lysine) (PLL)/amide polymer conjugate to demonstrate this ideal. These negatively charged drug carriers had very low toxicity and low interaction with cells, and may therefore be used *in vivo*. However, once inside cancer cells' acidic lysosomes, the acid-labile amides hydrolyze into primary or secondary amines. The regenerated positively charged carrier can escape from the lysosomes and traverse into the nucleus and thereby enhance the drug efficacy. [38] The hydrolytic kinetics of the acid-labile amides are crucial to the design of pH-triggered charge-reversal drug carriers. The hydrolytic kinetics of acid-labile imides and their potential use in charge-reversal drug carriers has not been studied. **LPEI with imides on its backbond has a better endosome/lysosome-escaping ability than PLL. LPEI could escape early from acidic compartments into cytosol which may improve the drug delivery efficacy.** [39, 40] Here, LPEI was employed as a drug carrier with unique imine groups in the back bond, which was capable of escaping from the endosome (Scheme 1). The hydrolytic kinetics of the acid-labile imides of LPEI were studied and an optimal LPEI/amide was synthesized for use in *in vivo* nuclear drug delivery. Its tumor targeting

and cellular uptake were enhanced by a targeting group folic acid. The anticancer drug CPT conjugated to the LPEI/amide by a disulfide bond was much more cytotoxic than free CPT.

Scheme 1. Targeted charge-reversal LPEI conjugate structure and its acid-triggered charge reversal (A) and nuclear drug delivery (B). The negatively charged drug conjugate accumulates in cancer tissues via the enhanced permeation and retention effect. It is taken up by the cancer cells via folic acid receptor-mediated endocytosis, transferred into an endosome and then a lysosome. The labile amides hydrolyze in the acidic tumor microenvironment (pH < 7.0), endosome (pH = 6.0-6.5) and lysosome (pH = 4.5-5.5) to regenerate the LPEI as the carrier. The regenerated LPEI carrier ruptures the lysosomal membrane to escape into the cytosol and traverse into the nucleus where it releases the carried drug.

2. Experimental Section

2.1. Materials.

2,3-Dimethylmaleic anhydride (98%) (DM), 1,2-dicarboxylic-cyclohexene anhydride 95% (DCA), 1,2-cyclohexanedicarboxylic anhydride 97% (CA), sodium deuterioxide (40wt% solution in D₂O, 99+% D), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) (commercial grade), folic acid (FA) (98%) and fluorescein isothiocyanate (FITC) (90+%), (S)-(+)-camptothecin (CPT) (95%), 2-methyl-2-oxazoline (98%), methyl *p*-toluenesulfonate (>98%), hydrochloride (37%), dimethyl sulfoxide (DMSO, >99%) and diethyl ether (99.7%) were purchased from Sigma-Aldrich and used as received. 3-Tritylsulfanylpromionic acid and N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP) was obtained according to the literature, respectively.[41, 42]

2.2. Preparation of linear poly(ethyleneimine) (LPEI).

To a solution of methyl *p*-toluenesulfonate (146 mg, 0.807 mmol) in acetonitrile (100 mL) was added 2-methyl-2-oxazoline (20 ml, 233 mmol). The reaction mixture was sealed in the flask and stirred for 3 days. The mixture was then precipitated in ether and further purified by repeated precipitation. The precipitate was dried in vacuum for 48 h. ¹H NMR (400 MHz, D₂O, δ) of poly(2-methyl-2-oxazoline): δ 7.60 (d, 1H), 7.28 (d, 1H), 3.42 (m, 4H), 1.98 (s, 3H). Poly(2-methyl-2-oxazoline) (10 g, M_{n(NMR)} = 10 kDa) was added into a mixture of 40 ml water and 30 ml concentrated (37%) hydrochloric acid. The solution was stirred at 110 °C overnight. The solution was then cooled down to room temperature. The precipitate was collected to give LPEI in a hydrochloride salt form. ¹H NMR (400 MHz, D₂O, δ) of LPEI·HCl: 3.48 (s, 4H). The hydrochloride on LPEI was removed by neutralizing with NaOH followed with dialysis (MWCO = 1,000) against water. LPEI was obtained by freeze drying.

2.3. Preparation of LPEI/DM, LPEI/DCA and LPEI/CA.

A typical procedure to amidize LPEI using anhydrides is as follows: LPEI (30 mg, 0.68 mmol NH groups) was dissolved into 2 ml of deionized water and the solution pH was adjusted to 8.5. DM (0.43 g, 3.4 mmol) was gradually added to the solution and the pH was kept as 8.5 by adding 3 M NaOH during the reaction. The reaction was allowed to continue for an additional half hour after the DM was completely added. The solution was dialyzed against water at pH 8.5. The product (LPEI/DM) was obtained after freeze drying. LPEI amidized by the other anhydrides (LPEI/DCA, LPEI/CA), other amidized products (FLPEI/DM), and the charge-reversal conjugate (FLPEI/DM-CPT) were prepared similarly. ^1H NMR (400 MHz, D_2O , δ) at pH 11:

LPEI/DM: 3.0-3.6 (br, 2H, $\text{CONCH}_2\text{CH}_2$), 2.5-2.8 (br, 2H, NHCH_2CH_2), 1.74 (s, 3H, CH_3).

LPEI/DCA: 3.0-3.6 (br, 2H, $\text{CONCH}_2\text{CH}_2$), 2.5-2.8 (br, 2H, NHCH_2CH_2), 2.28-2.30 (t, 4H, CH_2CH_2), 1.48-1.56 (m, 4H, CH_2CH_2).

LPEI/CA: 3.0-3.6 (br, 2H, $\text{CONCH}_2\text{CH}_2$), 2.5-2.8 (br, 2H, NHCH_2CH_2), 2.6-2.7 (m, 2H, CHCH_2), 1.63-1.64 (m, 4H, CH_2CH_2), 1.35-1.42 (m, 4H, CH_2CH_2).

2.4. Preparation of folate-linear poly(ethyleneimine) (FLPEI) and FLPEI/DM.

Folic acid (112 mg, 0.25 mmol) was dissolved in anhydrous dimethyl sulfoxide (6 ml) and then EDC (674 mg, 3.5 mmol) was added to this solution while stirring in the dark at room temperature for 1 h. The solution was added to the LPEI·HCl (1 g, 12.5 mmol NH groups) solution in 20 ml of water at pH 8.5 (pH was adjusted by 2 M NaOH). The solution was stirred for 2 days in the dark. The mixture was extensively dialyzed (MWCO 3,500 Da) against water (2 L $\times$ 4) for 2 days, and was lyophilized with a yield of 75%. The product was confirmed by ^1H NMR spectroscopy and the folate content was determined by UV ($\epsilon_{363} = 6200$). ^1H NMR (400

1 MHz, D₂O, δ) of FLPEI: 8.56 (s, 1H), 7.56 (d, 2H), 6.74 (d, 2H), 3.01 (s, 4H). FLPEI/DM was
2 prepared similarly as described in 2.3.

3 2.5. Preparation of FLPEI-FITC and FLPEI/DM-FITC.

4 FLPEI (0.6 g, 13.6 mmol NH groups) was dissolved in 15 ml ethanol. FITC (90 mg,
5 0.23mmol) was added to this solution, and the solution was stirred for 24 h in the dark. The
6 solution was then dialyzed (Spectra/Pro MWCO=3,500) against water to remove the unreacted
7 FITC. Finally, the purified solution was lyophilized to obtain a yellow solid with a yield of 78%.
8 ¹H NMR (400 MHz, D₂O, δ) of FLPEI-FITC: 8.56 (s, 1H), 7.0-7.8 (br, 5H), 6.3-6.8 (br, 7H),
9 3.01 (s, 4H). FLPEI/DM-FITC was prepared similarly as described in 2.3.

10 2.6. Preparation of thiolated CPT.[38]

11 CPT (348 mg, 1.0 mmol), 3-tritylsulfanylpropionic acid (348 mg, 1.0 mmol), DMAP (134 mg,
12 1.1 mmol) and EDC (382 mg, 2.0 mmol) were mixed in anhydrous dichloromethane (50 ml).
13 The solution was stirred overnight at room temperature. The solution turned to clear. The solvent
14 was removed by rotary evaporation. The residue was directly dissolved in 14 ml of anhydrous
15 dichloromethane, and triethylsilane (2 ml) was added followed by trifluoroacetic acid (4 ml).
16 After 2 h stirring at room temperature, dichloromethane and TFA were removed by evaporation.
17 The crude product was redissolved in dichloromethane and the solution was poured into an
18 excess of cold diethyl ether. The precipitate was isolated and dried under vacuum. A yellowish
19 product with a purity of 92% (according to ¹H NMR) was obtained with a yield of 80%. To
20 obtain CPT-SH with purity > 99%, silica gel column chromatography was applied using
21 chloroform and methanol (1:0.05) as eluent and the yield was 72%. ¹H NMR (400 MHz, CDCl₃,
22 δ) 8.42 (s 1H), 8.25-8.23 (d, 2H), 7.98-7.96 (d, 1H), 7.88-7.84 (t, 1H), 7.72-7.70 (t, 1H), 5.74-

5.70 and 5.46-5.41 (dd, 2H), 5.32 (s, 2H), 2.91-2.88 (m, 2H), 2.83-2.80 (m, 2H), 2.35-2.12 (m, 2H), 1.78-1.74 (t, 1H), 1.04-1.00 (t, 3H).

2.7. Preparation of FLPEI-CPT and FLPEI-CPT/DM (Scheme 2).

CPT was introduced to the side chain of linear PEI using a heterobifunctional reagent SPDP and the thiolated CPT. A typical synthetic procedure is described as follows for the FLPEI-CPT: FLPEI (136 mg, 3.09 mmol NH groups) was dissolved in water (6 ml), SPDP (57.5 mg, 0.185mmol) in methanol (1.2 ml) was dropped into the solution. The reaction was kept in dark for 2 h, and then the solution was dialyzed (Spectra/Pro MWCO=1,000) against water. The final solution was dried to get 98 mg of FLPEI-PDP. The degree of the substitution was determined from the peak intensity ratio of the methylene protons of LPEI (NHCH_2CH_2 , $\delta = 3.01$ ppm and the pyridyl protons ($\text{C}_5\text{H}_4\text{N}$, $\delta = 7.6$ ppm) of the pyridyldithiopropionyl (PDP) groups in ^1H NMR measurement in D_2O and the absorbance of 2-thiopyridone ($\lambda_{\text{max}} = 343$ nm; $\epsilon = 7.06 \times 10^3$) released after the addition of DTT. The obtained FLPEI-PDP (1.2 mol% substitution; 98 mg, 0.025 mmol PDP) was dissolved in 8 ml DMSO. Thiolated CPT (20.2 mg, 0.05 mmol) in 1 ml DMSO was added into the solution and stirred for 2 h. De-ionized water (15 ml) was added to the solution to precipitate out the unreacted CPT. After filtration, the solution was dialyzed (Spectra/Pro MWCO=3,500) against acetonitrile ($200 \text{ ml} \times 3$) and water ($1 \text{ L} \times 3$). Finally, the solution was lyophilized to give 50 mg of yellow solid. The CPT content was determined by HPLC. ^1H NMR (400 MHz, D_2O) of FLPEI-PDP, δ 8.59 (s 1H), 8.29 (m, 1H), 7.70 (m, 2H), 7.58 (d, 2H), 7.23 (m, 1H), 6.75 (d, 2H), 3.3-3.6 (m, 2H, $\text{CONCH}_2\text{CH}_2$), 2.5-3.0 (br, 2H, NHCH_2CH_2).

FLPEI/DM-CPT was prepared similarly as described in 2.3. FLPEI/DM-CPT: (6.0-9.0) (week, CPT and FA), 3.9-4.1 (br, CONCH₂CH₂), 3.0-3.6 (br CONCH₂CH₂), 2.5-2.8 (br, NHCH₂CH₂), 1.74 (s, CH₃).

Scheme 2. Synthesis of CPT-SH (A) and the targeted charge-reversal conjugate FLPEI/DM-CPT (B).

2.8. Reverse-phase HPLC.

Ion-pairing reverse-phase HPLC (RP-HPLC) was performed on a RP-C18 HPLC column (250 × 4.6mm², 5 micrometers). The gradient elution was used to characterize the products and study the drug release. Eluant A was 0.05% TFA and B was acetonitrile containing 0.05% TFA. The mobile phase was run 20% B to 50% B within 10 min and followed with isocratic elution of 50%

B for 20 min at a flow rate of 1.3 ml/min. The volume of each injection was 100 µl, and the detection of eluted samples was performed at 360 nm wavelength.

2.9. Amide hydrolysis studied by ^1H NMR spectroscopy.

The hydrolysis of amidized LPEI was monitored by ^1H NMR spectroscopy as follows. LPEI/DM was dissolved in PBS buffer (0.1 M) at pH 7.4, 6.0 or 5.0, respectively. The solution was dialyzed against PBS buffer (0.1 M) at the same pH of the solution at 37 °C. Samples were taken at different time interval and pH was adjusted to 8.5 to stop the hydrolysis. After freeze drying, samples were dissolved in D_2O , and the solution pH was adjusted to 11.0 using a D_2O solution of NaOD. The ^1H NMR spectra of the samples were recorded on a Bruker Avance DRX-400 spectrometer.

2.10. Zeta-potential measurements.

The ζ -potentials of the polymers were measured using phase-analysis light-scattering technology. The polymer was dissolved in PBS buffer (0.1 M) at a pH of 7.4, 6.0, or 5.0 to reach a concentration of 5 mg/ml. The sample was dialyzed at PBS buffer (0.1M) at the same pH of solution at 37 °C. Samples were taken at timed intervals and their zeta-potentials were measured using the Zeta-Nanosizer (ZEN3600, Malvern Instruments Ltd, Worcestershire, UK), which was routinely calibrated with a -50-mV zeta-potential standard (Malvern Instruments). The attenuator was set at 9 and the F (Ka) value was set at 1.5. Each measurement was performed for 30 runs, and the results were processed with DTS software version 3.32.

2.11. Hemolysis assay.

The membrane activity of polymers was measured using a red blood cell (RBC) hemolysis assay.[43] Sheep blood was collected in a heparin-containing tube and centrifuged at 1000 g for 5 minutes to separate the red blood cells (RBCs) from the plasma. The RBCs were dispersed in

anticoagulant–blood preservative Alsever's Buffer (Sigma). The suspension (280 μ L) was washed five to six times by centrifuge (1000 g, 3 minutes) until the cell suspension became clear, and then equally divided into two tubes and resuspended in gelatin veronal buffer–EDTA (GVB) buffer (Sigma) at pH 7.4 as the RBC stock solution. LPEI/DM was prehydrolyzed at pH 6.0 for 0 h, 12 h, or 24 h, respectively. The polymers were collected. Each of them was dissolved in PBS at pH 7.4 at concentrations of 10 μ g/ml, 50 μ g/ml, 100 μ g/ml, and 400 μ g/ml, respectively. The polymer solution (100 μ l), GVB buffer (200 μ l), and RBC stock solution (100 μ l) were added to a tube. The tube was incubated at 37 $^{\circ}$ C for 1 h. Then, 2 ml of 0.15 M NaCl was added to the tube. The tube was centrifuged (1,000 $\times$ g, 3 min) to separate the intact RBCs. The supernatant solution was collected. Absorbance of hemoglobin in the supernatant was measured at 412 nm using a UV-VIS spectrophotometer. The observed hemolysis of RBC in PBS solutions (pH 7.4) and in Milli-Q water was used as negative and positive controls, respectively. The observed hemolytic activity of a given polymer at a given concentration value was normalized to that of the positive control, Milli-Q water. All hemolysis experiments were carried out in triplicate.

2.12. Cell culture.

SKOV-3 adenocarcinoma cells and MCF-7 cells were purchased from American Type Culture Collection (Rockville, MD). Cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS), 10 μ g/ml insulin, and 1% antibiotic/antimycotic solution (Sigma A9909) at 37 $^{\circ}$ C in a 5% CO₂ environment. The cells used for cellular uptake and *in vitro* cytotoxicity assay of the folate-functionalized LPEI or conjugate were cultured in folic-free medium (Invitrogen Corp.) for at least two weeks before use.

2.13. Cellular uptake and intracellular colocalization of the polymers and conjugate.

A confocal scanning laser microscope (Leica TCS SP2 microscope) was used to observe the cellular uptake and intracellular localization of the FITC labeled polymers. For evaluating the role of surface folic acid in enhancing cellular uptake of FLPEI/DM-FITC, cells were incubated with 2.0 μ M of FLPEI/DM-FITC for 8 h, in the presence or absence of 20 μ M free folic acid. Cells were washed with PBS at 4 °C three times and images were taken. For the intracellular colocalization study, cells were incubated with 2.0 μ M of FLPEI/DM-FITC for 10 or 22 h. LysoTracker® Red DND-99 (Molecular Probes, Eugene, OR) (150 nM) was then added and cells were incubated for 2 h. Cells were washed with PBS at 4 °C three times and images were taken. For studying nuclear localization of the polymer, cells were incubated with 2.0 μ M of FLPEI/DM-FITC for 24 h. Images were taken after adding DRAQ 5 nuclei dye (1 μ l) (Biostatus, 5 mM in diluted acid). LysoTracker was excited with 543 nm laser and the emission wavelength from 523 to 563 nm was observed. DRAQ 5 nuclei dye was excited with 633 nm laser and the emission wavelength from 660 to 760 nm was observed. FLPEI/DM-FITC was excited with 488 nm laser and the emission wavelength from 505 to 530 nm was observed. To test the accumulation of free dye in the cells from the polymer solution, 1.6 ml of the polymer solution was filtered through a Centricon filter with a 1,000-molecular-weight cutoff (Amicon, Bedford, MA, no. YM-3) at 2600 g for 1 h. Unconjugated dye should pass through the filter and into the filtrate while polymer-conjugated FITC would be retained on the filter. The filtrate was then applied to the cells and the cells were observed similarly.

2.14. In-vitro cytotoxicity assay.

The cytotoxicity assay was carried out using the (3-(4, 5- dimethylthiazolyl)-2, 5- diphenyltetrazolium bromide) (MTT) cell proliferation assay kit (ATCC, Manassas, VA) according to the modified manufacturer's protocol. SKOV-3 and MCF-7 cells were cultured in

folic-free medium (Invitrogen Corp., Carlsbad, CA) for at least two weeks before use. They were then seeded onto 96-well plates at a density of 10,000 cells per well and incubated for 24 h. The original medium (200 μ l) was removed and replaced with the polymer, conjugate or free CPT solutions at different concentrations and incubated for 24 h. The medium in each well was then replaced with fresh cell culture medium and further incubated for 24 h. MTT reagent (10 μ l) was then added to each well and incubated for 6 h until purple precipitates were visible. Finally, the detergent reagent (100 μ l) was added to each well, and the plates were incubated at 37 $^{\circ}$ C for 18 h until all the crystals were dissolved. The absorbance intensity at 570 nm was recorded and the cytotoxicity was expressed as a percentage of the control.

3. Results and Discussion

3.1. Optimizing charge-reversible LPEI for nuclear delivery

Fig. 1. Structure of LPEI/CA, LPEI/DCA and LPEI/DM.

The optimal LPEI/amide drug carrier should be negatively charged at physiological pH (pH 7.4), which inhibits its interaction with cells, and quickly hydrolyzed to regenerate the cationic LPEI at the tumor extracellular pH (pH < 7) or the endosomal/lysosomal pH (pH = 4.5-6.5)[44, 45] for cellular uptake, endosomal/lysosomal escape, and nuclear localization (Scheme 1). The β -carboxylic acid-amide structures were first optimized by comparing the acid-triggered charge-reversal of the amides resulting from the various anhydrides (Fig. 1). LPEI was amidized with

CA, DCA and DM. The structures of the LPEI/amide were characterized by ^1H NMR. The surface charges of the LPEI/amide were characterized by measuring their ζ -potentials. Each β -carboxylic amide in the LPEI/amide has a β -carboxylic acid group and thus the LPEI/amide should be negatively charged at pH 7.4. As the amides hydrolyze to regenerate the secondary amines, the polymer should gradually become positively charged as shown in Fig. 2. LPEI/CA was stable and hardly hydrolyzed at all in the acidic solution, thus the surface charge was negative during the experiment. LPEI/DCA was less stable and could slowly hydrolyze in an acidic solution. After hydrolyzing for 33 h at pH 5.0, the surface charge became slightly positively charged. LPEI/DM was found to have the fastest charge-reversal; at pH 5.0 and 6.0, the time needed to become positively charged (ζ -potential higher than zero) was 1.0 and 7.3 h, respectively. At pH 7.4, LPEI/DM was still negatively charged after hydrolyzing for 48 h. Of the three LPEI/amides, LPEI/DM was always negatively charged at physiological pH but became positively charged after 7.3 h at pH 6 and fully positively charged within 1.0 h at pH 5.0. Therefore, LPEI/DM is ideal as a charge-reversal carrier for tumor-targeted nuclear drug delivery.

Fig. 2. ζ -potential as a function of time for LPEI/CA (A), LPEI/DCA (B) and LPEI/DM (C) (2 mg/mL) in 0.1 M PBS at pH 5.0, 6.0 and 7.4 (n=3).

The hydrolysis of LPEI/DM was further studied by ^1H NMR (Fig. 3). The peaks of the methylene proton in CH_2NH and the amide CH_2NCO overlapped over a wide pH range making it difficult to determine the rate of hydrolysis. Interestingly, at $\text{pH} \geq 11$, the chemical shift of these methylene protons was well differentiated as two broad peaks at 2.64 and 3.38 ppm, respectively (Fig. 3A). LPEI/DM is stable in basic conditions, so it is appropriate to adjust pH to 11 at the designated time interval to determine hydrolysis. The intensity ratio of the two peaks was used to calculate the amidization and the percentage of hydrolyzed amide. The typical intensity change of the two peaks during the hydrolysis of LPEI/DM at pH 5.0 is shown in Fig. 3B. The intensity of the CH_2 -amide peak decreased with hydrolysis whereas that of the CH_2 -imine increased. The hydrolytic rates of the LPEI/DM at different pH values are shown in Fig. 4. The amide hydrolyzed quickly at an acidic pH, more slowly as the pH increased and was stable in basic conditions. At pH 5.0 and 6.0, the time needed for 50% of the amide to hydrolyze was 6.1 and 13.1 h, respectively, but at pH 7.4, only 27% of the amide was hydrolyzed after 40 h. The hydrolysis study of LPEI/DM by ^1H NMR further confirmed that LPEI/DM is ideal for lysosomal pH (~ 4 -5) triggered hydrolysis and charge-reversal for tumor-targeted nuclear drug delivery.

Fig. 3. ^1H NMR spectra of LPEI/DM at different pH (A) and the hydrolysis of LPEI/DM at pH 5.0 detected by ^1H NMR spectroscopy (pH 11) (B).

Fig. 4. Hydrolysis kinetics of LPEI/DM at pH 5.0, 6.0, 7.4 and 11.0.

3.2 Cellular uptake and lysosome escape

Folic acid groups were conjugated to LPEI/DM for tumor targeting and cellular uptake because folic acid receptors are overexpressed in many malignant tumor cells but in few normal cells. The average folic acid receptor on LPEI was about 2 per molecule as determined by ^1H NMR and UV. LPEI/DM was also labeled with a fluorescence dye, FITC, for cellular uptake and intracellular trafficking experiments. The average FITC attached to LPEI/DM was 2.5 per molecule as determined by UV. The folate receptor-mediated endocytosis and intracellular trafficking of FLPEI/DM-FITC was observed with confocal laser light fluorescence. The cellular uptake of FLPEI/DM-FITC in the presence and absence of excess folic acid was studied (Fig. 5A-C). The uptake of folate acid functionalized polymer by SKOV-3 cells was greatly decreased in the presence of excess folic acid. This study indicated the role of folic acid functionalization in enhancing the uptake of polymer drug conjugate. A subcellular compartment labeling method

1 was used to observe the subcellular distribution of FLPEI/DM-FITC (Fig. 5D-F). The
2 fluorescence of FITC in FLPEI/DM-FITC was green. LysoTracker was used to label late
3 endosomes/lysosomes and displayed as red. The overlay of the two fluorescence images shows
4 yellow spots within the 12 h incubation period, which indicates that FLPEI/DM-FITC was
5 localized in late endosomes/lysosomes. Green spots were shown within the 24 h incubation
6 period, which indicates that some of hydrolyzed FLPEI/DM-FITC escaped from
7 endosomes/lysosomes (Supporting Fig. 1). The conjugate in a late endosome/lysosome
8 regenerates its positive charges, and is expected to lyse the lysosomal membrane and escape
9 from it (Scheme 1). The lysosome-lyzing ability of LPEI/DM was estimated using a hemolytic
10 assay of RBCs, a measure of the drug carrier's ability to rupture lysosomes.

Fig. 5. Cellular uptake and subcellular localization of polymer conjugate observed by confocal laser scanning fluorescence microscopy. (A-C) Observation of folate-targeting groups enhancing cellular uptake: SKOV-3 cells were incubated for 8 h with FLPEI/DM-FITC (2 μ M), in the presence (A, FLPEI/DM-FITC; B, overlay of A with nuclear dye) or absence of 20 μ M free folic acid (C, overlay of FLPEI/DM-FITC with nuclear dye). Observation of polymer conjugate in endosomes/lysosomes (D-F) and nuclei (G-I): (D-F) FLPEI/DM-FITC (2 μ M) was cultured with the cells for 12 h. FLPEI/DM-FITC (D), LysoTracker (E), overlay of D and E with the transmittance channel (F). (G-I) FLPEI/DM-FITC (2 μ M) was cultured with the cells for 24 h. FLPEI/DM-FITC (G), overlay of G with images of nuclear dye and transmittance images (H), and enlarged image of the selected part in H (I). **Scale bars: 25 μ m.**

Table 1. Hemolysis [mean $\pm$ SD] of sheep RBCs after incubation with different concentrations of the polymers at 37 $^{\circ}$ C for 120 min [n = 3].

Concentration (μ g/mL)	LPEI/DM	LPEI/DM[a]	LPEI/DM[b]	LPEI
10	1.41 $\pm$ 0.54	3.82 $\pm$ 0.28	2.15 $\pm$ 0.57	6.05 $\pm$ 0.98
50	1.25 $\pm$ 0.39	2.43 $\pm$ 0.31	5.01 $\pm$ 0.47	9.83 $\pm$ 0.41
100	2.55 $\pm$ 0.36	1.60 $\pm$ 0.27	11.60 $\pm$ 0.68	24.7 $\pm$ 0.32
400	1.35 $\pm$ 0.61	8.29 $\pm$ 0.40	22.5 $\pm$ 0.89	45.2 $\pm$ 0.97

[a] hydrolysis at pH 6.0 for 12 h, [b] hydrolysis at pH 6.0 for 24 h.

Sheep RBCs were incubated with four polymer concentrations in the range of 10-400 μ g/mL (Table 1). In 2 h LPEI caused 6.1% and 9.8% hemolysis at 10 and 50 μ g/mL, respectively, and 24-45% at higher concentrations. LPEI/DM without hydrolysis showed no hemolytic ability and with hydrolysis for 12 h at pH 6.0 showed 8.3% hemolysis only at concentrations as high as 400 μ g/mL. After being hydrolyzed at pH 6.0 for 24 h LPEI/DM showed a stronger hemolytic activity and caused 11.6% and 22.5% hemolysis at 100 and 400 μ g/mL, respectively. The results

indicate that LPEI/DM will have similar lysosome-lyzing ability to LPEI after regeneration in more acidic lysosomes (pH 4-5) so that the conjugate can escape from the lysosome. The subsequent nuclear localization of FLPEI/DM was confirmed by observing the colocalization of FLPEI/DM-FITC and DRAQ-5 labeled nuclei (blue) using confocal microscopy (Fig. 5G-I and Supporting Fig. 2). When the cells were exposed to FLPEI/DM-FITC for 12 h, FLPEI-FITC (green) regenerated from FPLPEI/DM-FITC was clearly observable in the nuclei and 12.7% of the polymer conjugate was localized in the nucleus as calculated by ImageJ (Supporting Fig. 3). This result suggests that this polymer conjugate was capable of accumulation in the nucleus.

3.3 Design and synthesis of nuclear localizing charge-reversal LPEI drug conjugate

A charge-reversal conjugate (FLPEI/DM-CPT) was designed using LPEI/DM as the drug carrier and CPT as the anticancer drug (Scheme 2). To achieve specific intracellular drug delivery, CPT was anchored to the carrier by a glutathione (GSH)-reactive disulfide bond, which is cleavable in the strongly reducing intracellular environment ($(\sim 0.5-10) \times 10^{-3}$ M GSH) but stable in the blood ($(\sim 20-40) \times 10^{-3}$ M GSH)[46-48]. As shown in scheme 2, folic acid was first introduced to LPEI by the reaction of its EDC-activated γ -carboxylic acid with the LPEI secondary amine. Thiolated CPT (CPT-SH) was then attached to LPEI by the heterobifunctional coupling reagent SPDP. (Fig. 6)

Fig. 6. ^1H NMR spectra of FLPEI-PDP (A), FLPEI-CPT (B) and FLPEI/DM-CPT (C) in D_2O .

Subsequently, the LPEI were amidized by DM in water at pH 8.5. The structure of the polymers was characterized by ^1H NMR (Fig. 6), UV (Fig. 7) and HPLC (Fig. 8). The typical absorbance of the UV spectra and the single peak on HPLC indicate the successful synthesis of all the polymers. The CPT content was determined by HPLC as 5.0 wt%.

Fig. 7. UV spectra of CPT (a), Folic acid (b), FLPEI-CPT (c), FLPEI/DM-CPT (d), LPEI (e), FLPEI (f), FLPEI-FITC (g) and FLPEI/DM-FITC (h).

Fig. 8. HPLC of CPT (a), CPT-SH (b), FLPEI (c), FLPEI-CPT (d) and FLPEI/DM-CPT.

3.4. In vitro cytotoxicity

Fig. 9. MTT of LPEI and LPEI/DM on SKOV-3 cells (A), FLPEI/DM-CPT and CPT on folic acid-free SKOV-3 cells (B) and folic acid-free MCF-7 cells (C).

The cytotoxicity of free CPT, LPEI/DM and FLPEI/DM-CPT to SKOV-3 and MCF-7 cells was evaluated using the 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT)

1 assay. They were cultured with cells for 24 h, and then the cells were post-cultured for 24 h to
2 allow the damaged cells to undergo apoptosis. The results are presented in Fig. 9. LPEI is very
3 toxic because of its positive charge, as mentioned. However, the negatively charged LPEI/DM
4 had a very low cytotoxicity. Its IC_{50} was higher than 73 $\mu\text{g/mL}$. The IC_{50} of the CPT in the
5 FLPEI-CPT to SKOV-3 and MCF-7 cells was 3.4 and 1.1 $\mu\text{g/mL}$, respectively. The FLPEI/DM-
6 CPT showed much greater cytotoxicity than free CPT, which may be attributable to its nuclear
7 drug delivery.

8 **4. Conclusions**

9 We have demonstrated cancer cell nucleus-targeted drug delivery by a LPEI-based drug
10 conjugate. Compared with the polylysine-based charge-reversal drug conjugate, LPEI has
11 secondary amines that affect the hydrolytic kinetics of the LPEI/amide, and thus has a different
12 charge-reversal profile. The hydrolysis of the β -carboxylic acid amide of the secondary amine
13 was slower than that of the primary amines, and thus DM was chosen for amidizing LPEI to give
14 optimal charge-reversal kinetics. The resulting LPEI-based amide hydrolyzed under acidic
15 conditions to regenerate the amines, giving rise to a negative-to-positive charge reversal. The
16 recovered LPEI was able to overcome the intracellular drug resistance of cancer cells and deliver
17 the anticancer drug to the cell nucleus to enhance the drug efficiency.

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Targeted charge-reversal LPEI conjugate structure and its acid-triggered charge reversal (A) and nuclear drug delivery (B): The negatively charged drug conjugate accumulates in cancer tissues via the enhanced permeation and retention effect; it is taken up by the cancer cells via folate receptor-mediated endocytosis, transferred into an endosome and then a lysosome; the labile amides hydrolyze at the acidic tumor microenvironment (pH < 7.0), endosome (pH = 6.0-6.5) and lysosome (pH = 4.5-5.5) to regenerate the LPEI as the carrier. The regenerated LPEI carrier ruptures the lysosomal membrane to escape into the cytosol and traverse into the nucleus and releases the carried drug there.