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Linear Polyethyleneimine-Doxorubicin Conjugate for pH-Responsive Synchronous Delivery of Drug and MicroRNA-34a

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Abstract: Although stimuli-responsive co-delivery systems of chemotherapy drugs and microRNA (miRNA) can serve as a promising treatment strategy for cancer, to our best knowledge, pH-responsive nanocarriers for the co-delivery of chemical drugs and microRNAs (miRNAs) have not yet been reported. In this study, we synthesized doxorubicin (DOX)-tethered linear polyethylenimine (LPEI) conjugates linked *via* a pH-responsive hydrazone bond (LPEI-HZ-DOX) for the synchronous delivery of DOX and miRNA-34a. The free DOX was successfully released from the LPEI-HZ-DOX conjugates at acidic pH, which provided selective toxicity against cancer cells in a pH-sensitive manner. The resulting LPEI-HZ-DOX conjugates formed nano-sized complexes with chemically modified long-chain miRNAs with a size of ~200 nm, which exhibited synergistic toxicity and anti-proliferation activity against PC-3 cancer cells. This platform of cationic conjugates with high biocompatibility could serve as a pH-sensitive *in vitro* system for gene and drug delivery.

Keywords: doxorubicin, microRNA, co-delivery system, pH-responsive, linear polyethyleneimine.

Introduction

Tumor tissues are composed of heterogeneous cell populations and complicated microenvironments, which could hinder overcoming metastatic cancers completely by the use of simple medications.[1] Although several types of treatment strategies are currently being investigated, there are still few promising treatment options available.[2,3] One of the emerging treatment strategies for cancer is a combinational therapy, which modulates at least two molecular targets simultaneously and allows efficient treatment at lower doses.[4,5] Accordingly, a combinational therapy with chemotherapeutics and nucleic acid-based drugs has been intensively studied for efficient cancer therapy.[6-9] To deliver chemotherapeutic and nucleic acid-based drugs simultaneously into the same cells, the selection of proper nano-carrier systems, including cationic polymers and liposomes, seems to be crucial because of poor intracellular uptake of nucleic acid-based drugs.[10-12] In particular, to overcome complicated tumor microenvironments without causing toxicity to normal cells, stimuli-responsive drug delivery systems against external pH, temperature, chemicals, etc. seem to be favorable.[13-15] On-demand drug release in an acidic environment like that of tumor tissues using pH-responsive carrier systems allowed efficient treatment of cancer cells with negligible toxicity to normal cells.[16-18] In a previous study, the anticancer drug doxorubicin (DOX) was conjugated to a branched polyethylenimine (BPEI) with a large molecular weight of 25 kDa *via* a pH-responsive linker, which exhibited efficient co-delivery of DOX and small interfering RNA (siRNA) and high anti-proliferative activity against cancer cells.[19,20] In addition, a smaller amount of DOX was released at a neutral pH similar to that of normal tissues, which endowed good biocompatibility for normal cells. However, it should be noted that strong, high-molecular-weight (25 kDa) cationic carriers like BPEI cause severe cytotoxicity *via* disruption of plasma membranes and the onset of nonspecific apoptotic signaling pathways.[21-23] Thus, the development of biocompatible carriers with pH-responsive moieties is needed for the combinational delivery of gene and chemical therapies.

In cancer cells, the expression levels of mature microRNAs (miRNAs), small non-coding RNA molecules (approximately 22 nucleotides), are precisely regulated.[24,25] Mature miRNAs could modulate cellular gene expression by repressing translation and inducing the sequence-specific degradation of target mRNAs after binding to partially complementary sites in the 3′-untranslated region of target mRNAs. Indeed, it is well known that a wide range of miRNAs can regulate multiple signaling pathways related to tumor progression, metastasis, invasion, and chemoresistance.[25] Thus, miRNAs have received increasing attention as valuable biomarkers as well as therapeutic candidates for improved tumor treatment against cancer-related signaling networks. Recently, the co-delivery of chemotherapeutic drugs and miRNAs has been of great interest

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because of the synergistic effects of each therapeutic approach as well as its potential to provide a solution to overcome drug resistance.[26-28] Nevertheless, to our best knowledge, pH-responsive nanocarriers for the co-delivery of chemical drugs and miRNAs have not yet been reported.

In our present study, DOX was conjugated to low-molecular-weight (2.5 kDa) linear polyethylenimine (LPEI) *via* a pH-sensitive hydrazone bond for the synchronous delivery of DOX and miRNA-34a to cancer cells. The degree of modification in LPEI was measured by nuclear magnetic resonance (NMR) analysis and the Ellman assay, respectively. Cell viability after treatment with synthesized DOX-tethered LPEI conjugates linked *via* a hydrazone bond (LPEI-HZ-DOX) was comparatively examined for two types of cancer cells: the prostate cancer cell line PC-3 and the breast cancer cell line MCF-7. The release profile of DOX from the LPEI-HZ-DOX conjugate was quantitatively examined at different pH conditions for 4 days. Polyelectrolyte complexes of control DNA and miRNAs with LPEI-HZ-DOX were analyzed by gel electrophoresis. The morphology and size distribution of LPEI-HZ-DOX complexes with miRNAs were analyzed by scanning electron microscopy (SEM). Cell proliferation was investigated for cancer cells after treatment with LPEI-HZ-DOX complexes with miRNAs.

Experimental

Materials. Thiol-modified miRNA-34a at both 3′- and 5′-ends (miRNA, 5′-UGGCAGUGUCUUAGCUGGUUGU-3′) and complementary miRNA-34a* (miRNA*, 5′-CAAUCAGCAAGUAUACUGCCCU-3′) were purchased from Bioneer (Daejeon, Korea). Salmon sperm DNA (~2000 bp), propylene sulfide, and BPEI (molecular weight 25 kDa) were obtained from Sigma (St. Louis, MO, USA). Dithiobismaleimidoethane (DTME) was purchased from Thermo Scientific (Rockford, IL, USA). LPEI (MW 2.5 kDa) was obtained from Polysciences, Inc. (Warrington, PA, USA). DOX was purchased from Wako (Japan). 3-Maleimidopropionic acid hydrazide (MPH) was obtained from Speedchemical Co. (Shanghai, China). Roswell Park Memorial Institute (RPMI) 1640 medium and penicillin/streptomycin were obtained from Invitrogen (Carlsbad, CA, USA). Fetal bovine serum (FBS) was purchased from Gibco BRL (Grand Island, NY, USA). Cell counting kit-8 (CCK-8) was obtained from Dojindo Laboratories (Kumamoto, Japan). Ellman's reagent was from Thermo Scientific (Rockford, IL, USA). All other chemicals and reagents were of analytical grade.

Synthesis of the LPEI-HZ-DOX Conjugate. Thiol-modified LPEI (LPEI-SH) was prepared according to a previous study.[29] Briefly, after LPEI (20 μmol) in deionized water (DW, 2 mL) was evaporated under reduced pressure, the resulting product was dissolved in methanol (3 mL) with nitrogen purging for 5 min. To adopt thiol groups to LPEI, propylene sulfide (280 μmol) was added to the resulting solution and reacted for 24 h at 50 °C under a nitrogen atmosphere. The degree of thiol in LPEI was measured using Ellman's reagent. After mixing Ellman's reagent solution (325 μM, 150 μL) with each sample (20 μL) and incubating for 15 min, the absorbance of each sample was measured at a wavelength of 412 nm using a spectrophotometer (SpectraMAX, Molecular Devices; Sunnyvale, CA, USA). A calibration curve was plotted using cysteine hydrochloride solutions as a control. For the synthesis of LPEI-MPH, LPEI-SH (20 μmol) in methanol was reacted with MPH (280 μmol) in methanol. After 2 h, the reaction mixture was evaporated under reduced pressure and the product was hydrated with DW. For purification, the reactant was dialyzed using a membrane with a molecular weight cut-off (MWCO) 2 kDa overnight and lyophilized. The degree of remnant thiol groups in LPEI-MPH was measured using Ellman's reagent. The LPEI-MPH (12.3 μmol) in methanol was reacted with DOX (17.2 μmol) in the presence of triethylamine (68.8 μmol) for 24 h at 50 °C under a nitrogen atmosphere while being protected from light to form the LPEI-HZ-DOX conjugate. The unreacted DOX and other chemicals were removed by dialysis against DW for 4 h (MWCO 2 kDa). The purified LPEI-HZ-DOX conjugate was lyophilized and stored at -20 °C until further use.

DOX modification in the LPEI-HZ-DOX conjugate was characterized by ^{1}H NMR spectra (BRUKER AVANCE II-500 spectrometer operating at 500 MHz; Switzerland) using D_2O/CD_3OD mixture (50/50 volume ratio) as a solvent. The DOX content in LPEI-HZ-DOX was determined by measuring the absorbance at 490 nm using a spectrophotometer (SpectraMAX, Molecular Devices; Sunnyvale, CA, USA). A calibration curve was constructed by measuring known concentrations of standard DOX solutions. The relative loading amount of DOX in the LPEI-HZ-DOX conjugate was determined using the following equation; [DOX amount]/[LPEI-HZ-DOX amount] × 100.

Cell Viability Assay. PC-3 cells (human prostate cancer cells) and MCF-7 cells (human breast cancer cells) were maintained in RPMI supplemented with 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin at 37 °C under a humidified atmosphere of 5% CO_2. PC-3 cells and MCF-7 cells were plated on a 96-well plate at a density of 5×10^3 cells/well for 24 h. Three types of cationic polymers (LPEI, LPEI-MPH, and BPEI 25 kDa) were administered at different concentrations to cells in the absence of serum for 5 h. After incubation, the medium was changed and replaced with fresh RPMI medium supplemented with 10% FBS, and cells were further incubated for 24 h. Cell viability was determined using the CCK-8 assay according to the manufacturer's protocol.

Release Profile of DOX from the LPEI-HZ-DOX Conjugates. The LPEI-HZ-DOX conjugates (0.3 μmol) in 1 mL of DW were placed in a dialysis bag (MWCO 2000). The dialysis bags were then immersed in 15 mL of two different buffer solutions: phosphate buffer (pH 7.4) and acetate buffer (pH 5.2) at 37 °C. The buffer solutions were changed with fresh

buffer solutions at predetermined time intervals. The amount of DOX released in the buffer solutions was determined by measuring fluorescence intensity using a spectrofluorophotometer (GeminiEM, Molecular Devices; Sunnyvale, CA, USA) at excitation/emission wavelengths of 480/595 nm.

PC-3 cells and MCF-7 cells were plated on a 96-well plate at a density of 5×10^3 cells/well for 24 h. LPEI-HZ-DOX conjugates that had been incubated for 3 days at pH 5.2 and 7.4 were added to PC-3 and MCF-7 cells, respectively. Free DOX and LPEI-HZ-DOX conjugates were added to the cells at different DOX concentrations for 48 h. Cell viability was determined using the CCK-8 assay, according to the manufacturer's protocol.

Preparation and Characterization of LPEI-HZ-DOX Complexes. The binding affinity of LPEI-HZ-DOX to long-chain nucleic acids and salmon sperm DNA (control DNA) was determined compared to that of LPEI used as a control. The salmon sperm DNA (0.5 μg) was mixed with LPEI or LPEI-HZ-DOX at different weight ratios (0, 0.01, 0.1, 1, 2, and 5) and incubated for 20 min at room temperature. The resulting samples were loaded on a 1% agarose gel followed by electrophoresis at a constant voltage of 100 V for 30 min in 0.5% TAE buffer. The DNAs were visualized by staining with ethidium bromide.

A long-chain microRNA-34a conjugate (lc-miRNA) was prepared as described in our previous study.[30] Briefly, miRNA-34a (20 nmol) thiol-functionalized at both the 3′- and 5′-ends was incubated with 1 M dithiothreitol as reducing agent overnight at pH 8.0. The reactant was subsequently purified *via* a desalting column (MWCO 7 kDa). The resulting miRNA-34a with de-protected thiol groups was reacted with 20 nmol DTME overnight. To prepare the miRNA duplex and the lc-miRNA duplex, common miRNA and conjugated lc-miRNA were annealed with miRNA* for 1 h at 37 °C. The prepared lc-miRNA was loaded onto 15% polyacrylamide gels and gel electrophoresis was performed for 45 min at 180 V. The miRNA within the gels was stained with ethidium bromide and visualized by an ultraviolet transilluminator.

To prepare the LPEI-HZ-DOX/lc-miRNA complex, the duplex form of miRNA and lc-miRNA (1 μg) in diethylpyrocarbonate (DEPC)-treated DW was mixed with the LPEI-HZ-DOX conjugate at various weight ratios (0, 1, 2, 5, and 10) and incubated for 20 min at room temperature. The complexes were loaded on a 1% agarose gel and electrophoresed at 100 V for 30 min in 0.5% TAE buffer. Each duplex form of miRNA was visualized by staining with ethidium bromide. To observe the morphology of LPEI-HZ-DOX/lc-miRNA complexes, LPEI-HZ-DOX (19 μg) and lc-miRNA (1.9 μg) were mixed in DW (100 μL) for 20 min at room temperature. The LPEI-HZ-DOX/lc-miRNA complexes were mounted onto a silicon wafer and dried using N_2 gas at room temperature. The samples were visualized by scanning electron microscopy (SEM, Hitachi S-4800, Japan). To determine the particle size, over 100 LPEI-HZ-DOX/lc-miRNA complexes were randomly picked out of complexes in the SEM images and analyzed using the Image J software program (National Institutes of Health; Bethesda, MD, USA; http://rsb.info.nih.gov/ij/).

***In vitro* Anti-Proliferative Activity.** The extent of cell proliferation was evaluated in PC-3 cells *in vitro* using the CCK-8 assay. A total of 5×10^2 cells per well were plated in 96-well plates and incubated for 24 h prior to treatment with the samples. Free DOX, LPEI-HZ-DOX, and LPEI-HZ-DOX/lc-miRNA at a DOX concentration of 5.6 μM and 11.1 μM were administered to cells in the absence of serum for 5 h. For the preparation of LPEI-HZ-DOX/lc-miRNA, LPEI-HZ-DOX conjugates at a DOX concentration of 5.6 and 11.1 μM were mixed with lc-miRNA at a polymer/RNA weight ratio of 20, which corresponded to an RNA concentration of 66 and 132 nM, respectively. Multimerized GFP siRNA was prepared according to a previous study to function as a long-chain control RNA[6] and added to the cells. After 5 h incubation, the media were changed with fresh RPMI media containing 10% FBS. After a 7-day incubation, 10 μL of CCK-8 solution was added to each well, followed by incubation for 45 min, and the absorbance was measured at 450 nm using a spectrophotometer (SpectraMAX, Molecular Devices, CA, USA).

Results and Discussion

Synthesis and Characterization of LPEI-HZ-DOX. The synthesis scheme of the LPEI-HZ-DOX conjugate is shown in Figure 1(a). LPEI with low molecular weight (2.5 k) was used for the synthesis of DOX conjugation due to its biocompatibility in this study. LPEI-SH was prepared by a ring-opening reaction of the propylene sulfide with secondary amines of LPEI.[29,31] Incorporated thiol groups in LPEI-SH were reacted with maleimide moieties of MPH *via* the Michael-type addition to synthesize LPEI-MPH.[32,33] The degree of thiol groups and MPH substitution in LPEI were characterized by the Ellman's assay. The number of grafted thiol groups in LPEI-SH and MPH in LPEI-MPH were 1.5±0.1 and 1.4±0.1, respectively. The hydrazide groups in LPEI-MPH were reacted with the carbonyl groups of DOX, generating the LPEI-HZ-DOX conjugate. It should be noted that DOX was immobilized to the conjugates *via* a hydrazone bond that is well known to be stable at neutral pH but vulnerable at acidic pH. In an acidic environment, free DOX can be easily released from the LPEI-HZ-DOX conjugate.[34-37] Figure 1(b) shows a schematic illustration of the formation of LPEI-HZ-DOX polyelectrolyte complexes with miRNAs and their intracellular delivery to cancer cells. In this study, lc-miRNA was prepared *via* a simple maleimide-thiol coupling reaction according to previous studies, which is appropriate for the formation of polyelectrolyte complexes with cationic carriers.[30,38,39] Excellent endocytosis of LPEI-HZ-DOX/lc-miRNA complexes is followed by the strong electrostatic complexation between lc-miRNA and LPEI-HZ-DOX-produced nano-sized particles. Dissociation of hydrazone bonds in the acidic environment like lysosomes/endosomes

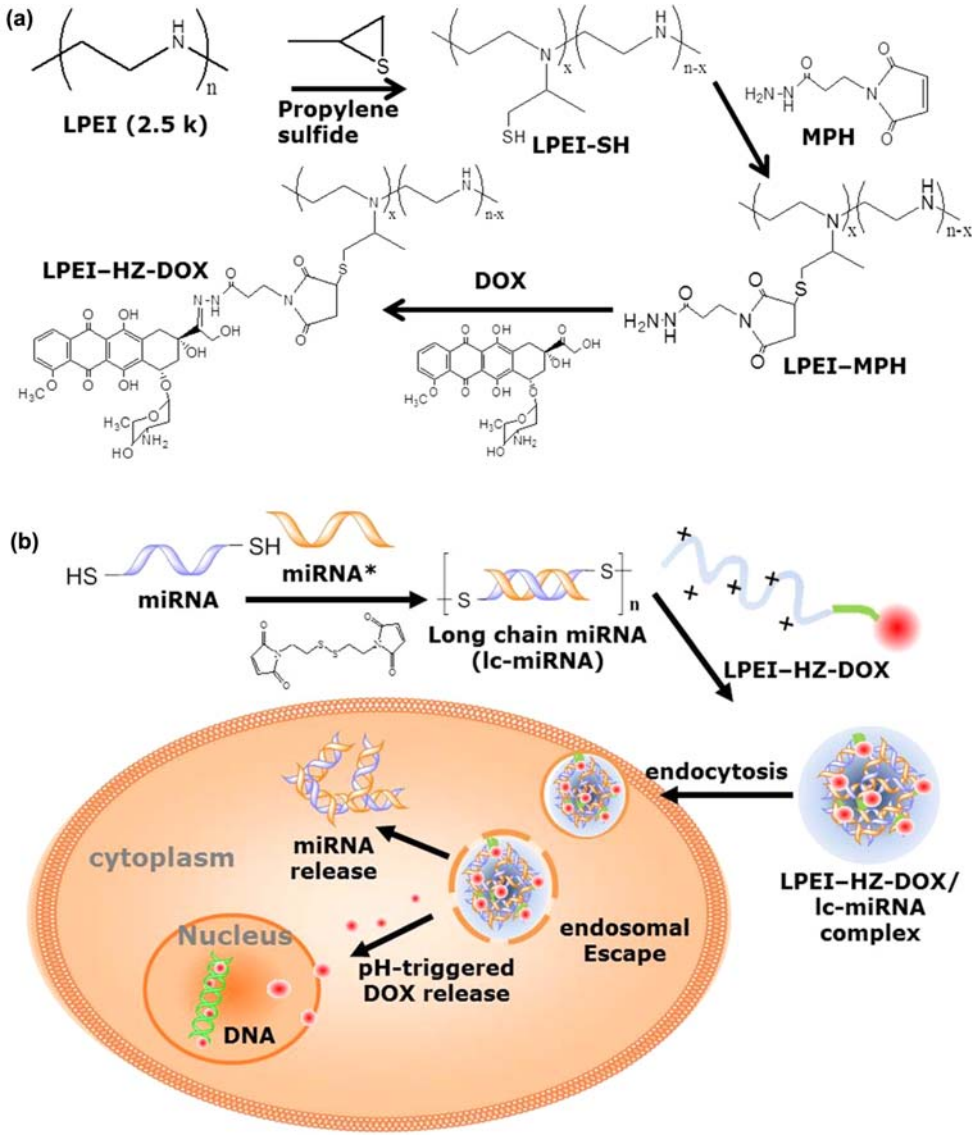

Figure 1. (a) The synthetic scheme of the LPEI-HZ-DOX conjugate and the pH-responsive DOX release from LPEI-HZ-DOX. (b) Illustrations of the preparation of lc-miRNA *via* chemical conjugation and cellular uptake of the LPEI-HZ-DOX/lc-miRNA complex for the synergistic delivery of miRNA and DOX in cancer cells by acid-triggered release in endosomes.

within a cell generates free DOX, which could allow its intercalation into chromosomes and therapeutic effects.[36,40,41] Simultaneously, released miRNAs also elicit biological functions like anti-proliferation and apoptosis in cancer cells.[25]

To confirm the DOX conjugation to LPEI-MPH, LPEI-HZ-DOX conjugates were analyzed by ^{1}H NMR (Figure 2). In the ^{1}H NMR spectra, the prominent peaks for the -CH_3 (0.9 ppm), -CH_2- (1.1-1.3 ppm), and aromatic rings (7.2-8.1 ppm) of DOX, and -CH_2CH_2- (2.4-2.8 ppm) of LPEI were observed in LPEI-HZ-DOX conjugates. The number of grated DOX molecules per LPEI-HZ-DOX was also determined by ultraviolet-visible spectrophotometry. Around 1.1±0.1 of DOX molecules per single LPEI-HZ-DOX conjugate were attached, which corresponds to 17.4±1.6% of the DOX loading amount in LPEI-HZ-DOX conjugates. The MW of the LPEI-HZ-DOX conjugate calculated based on its structure was 3414.0 Da. In a previous study, DOX was conjugated to BPEI (MW 25 kDa) with ~13% of the DOX/polymer loading amount linked *via* a hydrazone bond.[20] Considering its high MW, BPEI has severe cytotoxicity compared with that of LPEI (MW 2.5 kDa).

Cell Viability Assay. The cell viabilities of LPEI- and LPEI-MPH-treated PC-3 cells and MCF-7 cells were compared with those treated with the conventional cationic polymer for gene delivery, BPEI (25 kDa). Figure 3(a) shows that the relative cell viabilities of PC-3 cells after treatment with BPEI at concentrations of 5 and 10 μg/mL were 20.2±1.2% and 2.9±0.3%,

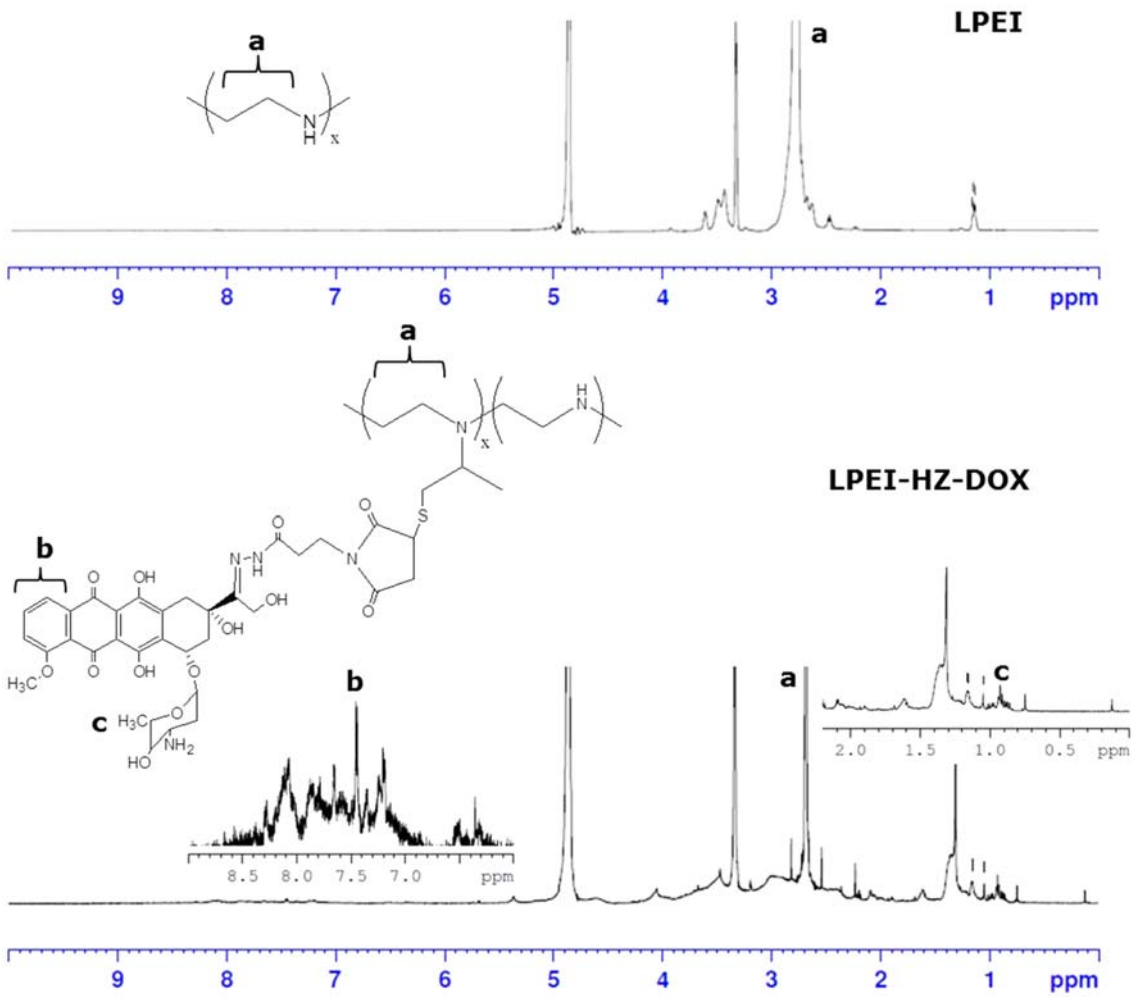

Figure 2. ^{1}H NMR spectra of LPEI and LPEI-HZ-DOX in a D_2O/CD_3OD mixture solution.

respectively, which indicates significant cytotoxicity. On the other hand, the cell viabilities after treatment with LPEI and LPEI-MPH at a polymer concentration of 5 μg/mL were 81.3±1.7% and 90.7±6.1%, respectively. Considering that the cytotoxicity of PEI was influenced by MW and increased branching, the low cytotoxicity of LPEI (2.5 kDa) and LPEI-MPH might be attributed to their low MW and linear type of PEI.[42] Figure 3(b) shows the relative cell viability of MCF-7 cells after treatment with different cationic polymers. LPEI (2.5 kDa) and LPEI-MPH also exhibited significantly reduced cytotoxicity against PC-3 cells. An approximately 3.1-fold higher viability was observed for cells treated with LPEI-MPH at a polymer concentration of 10 μg/mL, compared with those treated with BPEI.

Release Profile of DOX from the LPEI-HZ-DOX Conjugate. To assess whether pH variation influenced the cleavage of the hydrazone bond of LPEI-HZ-DOX, the release behavior of DOX from the LPEI-HZ-DOX conjugate was investigated at different pH conditions, *i.e.*, pH 7.4 and pH 5.2. The release behavior of DOX was examined at increasing incubation times and DOX concentrations. Figure 4(a) shows that 75.1±23.1% and 42.7±4.8% of the conjugated DOX was released from LPEI after 3 days at pH 5.2 and at pH 7.4, respectively. These results evidently demonstrate that the hydrazone bond of LPEI-HZ-DOX was relatively stable at the neutral pH but was cleaved in the acidic environment. The pH-responsive release behavior of DOX is advantageous for an effective cancer therapy because this conjugate will release more DOX after reaching the acidic tumor site while it will be stable during its circulation in the bloodstream at pH 7.4.[43,44] The release profile of DOX from the LPEI-HZ-DOX conjugate we found is comparable with that of other groups. For example, Dong *et al*. reported on a BPEI conjugated with DOX *via* a pH-responsive hydrazone linker and showed that 39% of DOX was released at pH 7.4 and 90% of DOX was released at pH 5.0 after 45 h.[19] The release profile of our DOX is slightly slower compared with that of Dong *et al*.,[19] but the tendency toward a pH-dependent DOX release was not remarkably different. This result suggests that free DOX could be selectively released at acid pH environments like tumor tissues and intracellular endo-

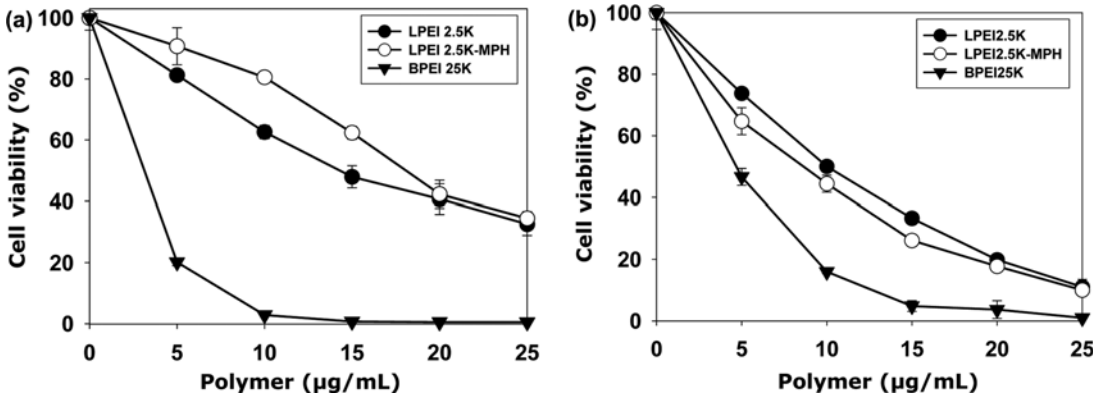

Figure 3. The biocompatibility of LPEI, LPEI-MPH, and BPEI at various concentrations of the polymers with (a) PC-3 cells and (b) MCF-7 cells after 24-h incubation. Error bars mean the standard deviation for three different samples.

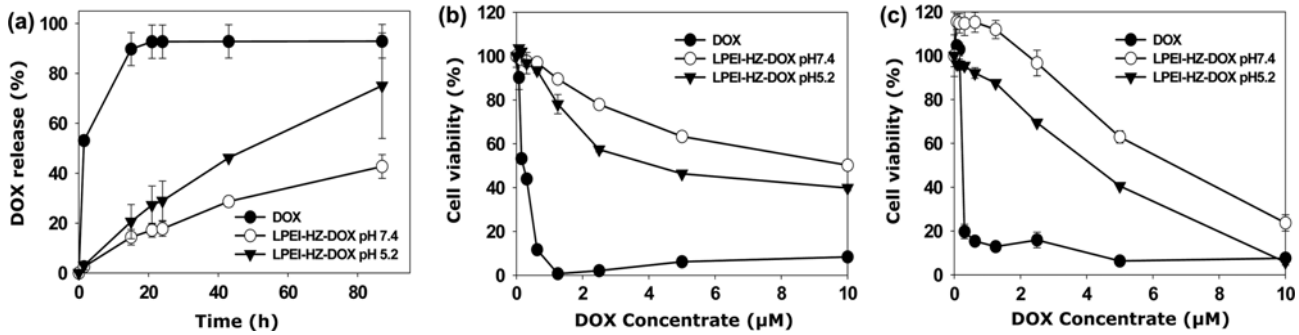

Figure 4. (a) The release profile of free DOX from LPEI-HZ-DOX conjugates at different pH values (pH 5.2 and pH 7.4). Error bars mean the standard deviation for two different samples. (b-c) Cell viabilities of (b) PC-3 cells and (c) MCF-7 cells after treatment with LPEI-HZ-DOX incubated at the two pH values for 3 days. Error bars mean the standard deviation for three different samples.

somes/lysosomes.[18]

In addition, the cytotoxicity was investigated after incubation of LPEI-HZ-DOX at different pH values to examine whether the release of DOX from LPEI-HZ-DOX in a pH-responsive manner could be toxic to cancer cells. Various concentrations of free DOX and LPEI-HZ-DOX were administered to PC-3 cells and MCF-7 cells after a 3-day incubation period at pH 5.2 and 7.4. As shown in Figure 4(b)-(c), free DOX was more cytotoxic than LPEI-HZ-DOX, which was due to the uncleaved DOX from the polymer conjugates. LPEI-HZ-DOX showed higher toxicity against cancer cells after incubation at pH 5.2 than at pH 7.4. This result might be attributed to the larger amount of DOX that was released from LPEI-HZ-DOX in the acidic environment than at neutral pH by the selective cleavage of the hydrazone bond of LPEI-HZ-DOX. In addition, the cytotoxicity of LPEI-HZ-DOX at pH 5.2 was significantly elevated at increasing amounts of DOX. The viability of MCF-7 cells treated with free DOX, LPEI-HZ-DOX at pH 5.2, and LPEI-HZ-DOX at pH 7.4 was 15.9±3.6, 96.6±5.9, and 69.4 ±0.2% at a concentration of 2.5 μM, respectively. Reduced toxicity by LPEI-HZ-DOX at pH 5.2, compared to free DOX, might be attributed to incomplete cleavage of DOX from the conjugate.

Preparation and Characterization of the Complexes. Salmon sperm DNA (control DNA) with ~2000 base pairs was used to evaluate the affinity of LPEI-HZ-DOX to long-chain nucleic acids *via* a gel retardation assay after incubation of DNA with LPEI-HZ-DOX and LPEI at various weight ratios. LPEI-HZ-DOX formed a stable complex with salmon sperm DNA at a higher weight ratio than did LPEI, which was probably due to the substitution of secondary amines in LPEI with MPH and DOX (Figure 5(a)). To examine binding affinities of two kinds of miRNAs, conventional miRNA and chemically conjugated lc-miRNA, were prepared and analyzed by gel electrophoresis. Figure 5(b) shows significantly retarded migration of lc-miRNAs compared with miRNAs, which indicates that miRNAs were successfully converted into lc-miRNAs as described in our previous study.[30] The binding affinities for LPEI-HZ-DOX were assessed using the two kinds of miRNAs at different weight ratios. The lc-miRNA successfully interacted and condensed with LPEI-HZ-DOX at an LPEI-HZ-DOX/lc-miRNA weight ratio of 5 (Figure 5(c)), indicating a similar behavior as that with salmon sperm DNA. It seemed that both miRNA and lc-miRNA formed stable complexes at a weight ratio of 5. However, more free miRNAs than lc-miRNAs existed after the formulation of LPEI-HZ-DOX/miRNA

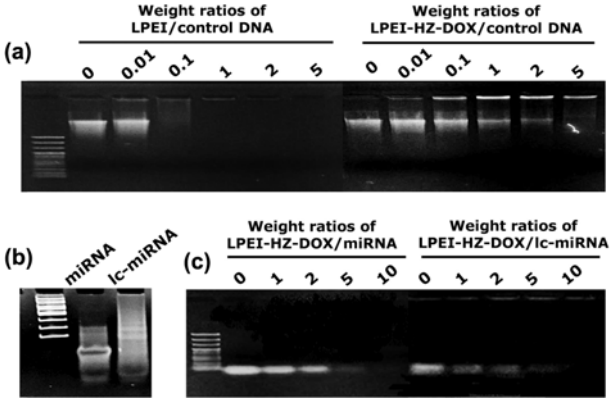

Figure 5. (a) Gel retardation assays of salmon sperm DNA with two kinds of polymers (LPEI-HZ-DOX and LPEI) at various polymer/salmon sperm DNA weight ratios. (b) Gel electrophoresis of common miRNA and lc-miRNA using a 15% acrylamide gel. (c) Gel retardation assays of miRNAs and lc-miRNA after incubation with LPEI-HZ-DOX at various LPEI-HZ-DOX/miRNA weight ratios.

at the same weight ratios of 1 and 2. This result might be attributed to the fact that the lc-miRNAs condensed more successfully with LPEI-HZ-DOX due to their strong ionic interaction with the cationic ions of LPEI-HZ-DOX compared with that observed for conventional miRNAs.[38] Unlike gel images shown in Figure 5(b), migration profiles of miRNA and lc-miRNA were not significantly different in Figure 5(c), which might be attributed to different resolution between agarose gels and polyacrylamide gels.[30] However, lc-miRNA bands were much fainter than miRNA bands at same loading amount in agarose gel probably due to heterogeneous size of lc-miRNAs.

The morphology and size of the LPEI-HZ-DOX/lc-miRNA complexes were examined by SEM. As shown in Figure 6(a), spherical and homogeneous particles were successfully formulated. The average size of the LPEI-HZ-DOX/lc-miRNA complexes was 217.6±25.3 nm. Considering that a particle size of ~200 nm is favorable for intracellular uptake, these LPEI-HZ-DOX/lc-miRNA complexes might be successfully delivered within cells *via* endocytosis.[45,46]

Anti-Proliferative Activity *In vitro*. In previous studies, miRNA-34a has been shown to inhibit cellular proliferation *via* p53-dependent pathways.[47] We examined if LPEI-HZ-DOX could deliver miRNAs and had biological effects on cancer cells. The extent of cancer cell proliferation after treatment with LPEI-HZ-DOX, LPEI-HZ-DOX/lc-miRNA, and LPEI-HZ-DOX/lc-RNA control was quantitated in PC-3 cells. After incubation of each sample for 7 days, the amount of cells was measured using the CCK-8 assay. Negligible changes in cell proliferation were observed after treatment of the samples with 5.6 μM DOX. Cell proliferation was clearly reduced to 48.0±4.3%, 54.1±3.2%, and 32.7±1.5% by treatment with LPEI-HZ-DOX, LPEI-HZ-DOX/lc-RNA, and LPEI-HZ-DOX/lc-miRNA at a DOX concentration of 11.1 μM, respectively. LPEI-HZ-DOX caused reduced cell viability due to the cytotoxicity of the released DOX. However, LPEI-HZ-DOX/lc-RNA exhibited significantly higher suppression of cell proliferation than observed for LPEI-HZ-DOX and LPEI-HZ-DOX/lc-RNA, which evidently shows the presence of synergistic effects probably due to intracellular miRNA-34a processing. These biological effects were likely due to the successful intracellular location of the complexes and the intracellular release of DOX and the miRNAs.

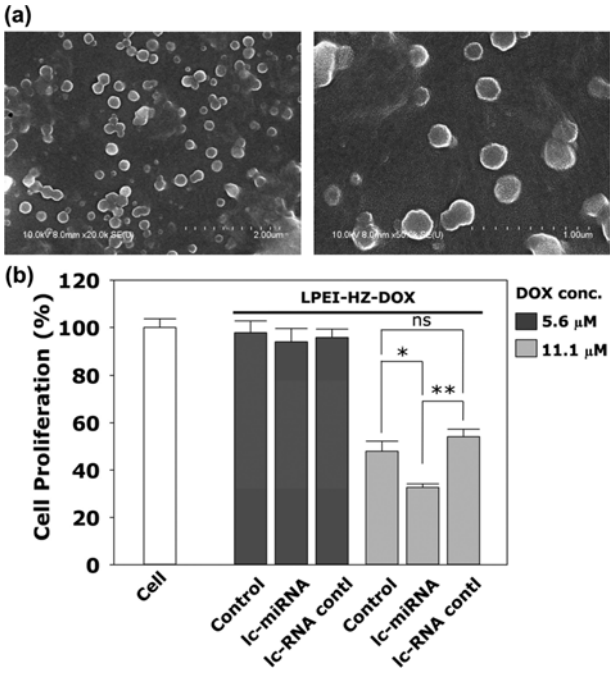

Figure 6. (a) Scanning electron microscopy (SEM) images of LPEI-HZ-DOX/lc-miRNA-d complexes. (b) The relative amount of cell proliferation using PC-3 cells evaluated by CCK-8 assay after treatment with LPEI-HZ-DOX/lc-miRNA complexes at two kinds of DOX concentrations, 5.6 and 11.1 μM (*p<0.05; **p<0.001; ns=not significant). Error bars mean the standard deviation for three different samples.

Conclusions

In conclusion, a novel pH-responsive DOX-conjugated polymer, LPEI-HZ-DOX, was synthesized for co-delivery of DOX and miRNA34a to cancer cells. Secondary amines in the low-MW LPEI used as a backbone were substituted for DOX *via* hydrazone bonds. The free DOX was successfully released from the LPEI-HZ-DOX conjugates at an acidic pH, which provided selective toxicity against cancer cells in a pH-sensitive manner. The fabricated LPEI-HZ-DOX conjugates formed nano-sized complexes with chemically modified lc-miRNAs with a size of ~200 nm, which exhibited synergistic toxicity and anti-proliferation activity against PC-3 cancer cells. This platform of cationic conjugates with high biocompatibility could serve as a pH-sensitive delivery route of miRNAs and chemical drugs *in vitro*.

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