

See discussions, stats, and author profiles for this publication at: <https://www.researchgate.net/publication/278968966>

Oncolytic Adenovirus Coated with Multidegradable Bioreducible Core–Cross-Linked Polyethylenimine for Cancer Gene Therapy

ARTICLE in BIOMACROMOLECULES · JUNE 2015

Impact Factor: 5.75 · DOI: 10.1021/acs.biomac.5b00538 · Source: PubMed

CITATIONS

2

READS

28

6 AUTHORS, INCLUDING:



Joung-Pyo Nam

University of Utah

23 PUBLICATIONS 70 CITATIONS

[SEE PROFILE](#)



Kihoon Nam

University of Utah

46 PUBLICATIONS 1,178 CITATIONS

[SEE PROFILE](#)



Chae-Ok Yun

Hanyang University

170 PUBLICATIONS 3,298 CITATIONS

[SEE PROFILE](#)



Sung Wan Kim

Kyung Hee University

72 PUBLICATIONS 303 CITATIONS

[SEE PROFILE](#)

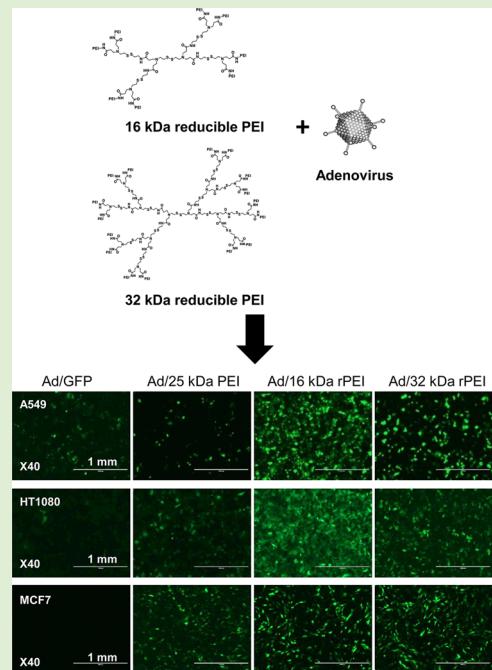
Oncolytic Adenovirus Coated with Multidegradable Bioreducible Core-Cross-Linked Polyethylenimine for Cancer Gene Therapy

Joung-Woo Choi,[†] Joung-Pyo Nam,[†] Kihoon Nam,[†] Young Sook Lee,[†] Chae-Ok Yun,^{*,‡} and Sung Wan Kim^{*,†,‡}

[†]Center for Controlled Chemical Delivery (CCCD), Department of Pharmaceutics and Pharmaceutical Chemistry, University of Utah, Salt Lake City, Utah 84112, United States

[‡]Department of Bioengineering, College of Engineering, Hanyang University, 222 Wangsimni-ro, Seongdong-gu, Seoul 133-791, Republic of Korea

ABSTRACT: Recently, adenovirus (Ad) has been utilized as a viral vector for efficient gene delivery. However, substantial immunogenicity and toxicity have obstructed oncolytic Ad's transition into clinical studies. The goal of this study is to generate an adenoviral vector complexed with multidegradable bioreducible core-cross-linked polyethylenimine (rPEI) polymer that has low immunogenicity and toxicity while having higher transduction efficacy and stability. We have synthesized different molecular weight rPEIs and complexed with Ad at varying molar ratios to optimize delivery of the Ad/polymer complex. The size and surface charge of Ad/rPEIs were characterized. Of note, Ad/rPEIs showed significantly enhanced transduction efficiency compared to either naked Ad or Ad/25 kDa PEI in both coxsackievirus and adenovirus receptor (CAR) positive and negative cancer cells. The cellular uptake result demonstrated that the relatively small size of Ad/16 kDa rPEIs (below 200 nm) was more critical to the complex's internalization than its surface charge. Cancer cell killing effect and viral production were significantly increased when oncolytic Ad (RdB/shMet, or oAd) was complexed with 16 kDa rPEI in comparison to naked oAd-, oAd/25 kDa PEI-, or oAd/32 kDa rPEI-treated cells. This increased anticancer cytotoxicity was more readily apparent in CAR-negative MCF7 cells, implying that it can be used to treat a broad range of cancer cells. Furthermore, A549 and HT1080 cancer cells treated with oAd/16 kDa rPEI had significantly decreased Met and VEGF expression compared to either naked oAd or oAd/25 kDa PEI. Overall, these results demonstrate that shMet expressing oncolytic Ad complexed with multidegradable bioreducible core-cross-linked PEI could be used as efficient and safe cancer gene therapy.



INTRODUCTION

Oncolytic viruses, which can selectively infect and replicate in cancer cells, provide an attractive strategy for cancer treatment. Cancer gene therapy using oncolytic virus has shown great promise due to several advantages of the virus' ability to specifically replicate only in cancer cells, lyse this infected cancer cells, high production of viral progenies, and subsequent spreading of progenies to adjacent cancer cells.^{1–3} Due to these therapeutic potentials of oncolytic virus, it has been rapidly transitioned into human clinical trials for treatment of several types of cancers and its safety has been well-documented.^{4–7} Among the oncolytic virus, replication-competent oncolytic adenovirus (Ad) have a number of advantages, as Ad is well characterized, its genomes can be easily engineered, and Ad can be produced and purified at high titers.^{3,8,9} Moreover, therapeutic efficacy of oncolytic Ad can be further enhanced by inserting therapeutic genes. Combining oncolytic virotherapy and expression of anticancer therapeutic genes, such as

decorin, relaxin, cytokines and short hairpin RNA, has greatly increased the efficacy of cancer gene therapy.^{10–15} As a result, oncolytic Ad virotherapy has gained widespread popularity as a potential candidate for more efficient treatment of cancers. Despite these advantages, oncolytic Ad in clinical setting is limited by its dependence on the coxsackievirus and adenovirus receptor (CAR) for viral entry and host-immune response against Ad.^{16,17}

One way to overcome these limitations of Ad is through utilization of nonviral carriers, such as cationic polymers or liposomes, for delivery of Ad. The benefits of nonviral vectors are that they exhibit low immunogenicity, lower toxicity, and can carry unrestricted size gene. Many polymers have been developed to condense and protect nucleic acids inside the host

Received: April 22, 2015

Revised: June 17, 2015

Published: June 22, 2015

to enhance efficacy of gene therapy.¹⁸ However, these nonviral vectors alone have low transfection efficiency due to poor endosomal escape and nuclear import mechanisms.¹⁹ Therefore, by combining viral and nonviral vectors into a single hybrid vector, the limitations associated with each of these vectors as a single carrier can be overcome.¹⁷ In this regard, typical cationic polymers including poly(ethylenimine) (PEI), poly-L-lysine (PLL), and liposomes/lipids have been complexed with Ad resulting in enhanced transduction by charge-mediated internalization into host cells which consequently overcomes CAR dependency of adenoviral vectors.^{20–22} Among these polymers, 25 kDa PEI can easily be complexed with DNA and has shown high transfection efficiency due to the adept endosomal escape mechanism through proton sponge effect.²³ However, increase in transfection efficiency with increasing molecular weight of PEI is associated with increased cytotoxicity.²⁴ Low molecular weight PEI such as 1.8 kDa PEI shows less cytotoxicity, but it has limitations of its own in that it has diminished DNA condensing ability, which results in lower transfection efficiency than higher molecular weight PEIs.²⁵ To overcome these issues associated with PEI, reducible PEIs were synthesized through introduction of a cross-linker to low molecular weight PEI to optimize gene delivery efficacy with lower toxicity.^{26–28} Reductively degradable disulfide cross-linked with low molecular weight PEIs can have higher gene transfection efficiency as well as lower cytotoxicity in comparison to nondegradable high molecular weight PEI. An alternative strategy is conjugating cholesterol to PEI to enhance gene transduction activity and decrease cytotoxicity.^{29–32} The cholesterol-conjugated low molecular PEI has the property of water-soluble lipopolymer, which can form small micelles in aqueous solution through balance between hydrophilicity of PEI and hydrophobicity of cholesterol. Ram et al. reported that this cholesterol-conjugated low molecular PEI micelle complexed with interleukin-12 (IL-12) expressing pDNA showed great antitumor efficacy by enhancing both IL-12 and interferon gamma levels, which are key factors of antitumoral immune response.³³

In this study, we designed and synthesized a new type of bioreducible polymer (rPEI), which has a dendritic feature that allows Ad/polymer complex to form with more profound condensation with lower amount of polymer. This high condensing attribute can enhance adenoviral transduction efficiency as well as reduce the adverse effect of the polymer. When bioreducible PEI gets delivered to the cells, the disulfide bridge of the polymer backbone can easily be cleaved by intracellular glutathione and rapidly be removed from the host's body, implying minimized cytotoxicity. Furthermore, dendrimers have other advantages as a nonviral carrier for efficient gene delivery such as solubility and stability.³⁴

When the generation number of dendrimer increases, toxicity, surface charge, and hydrophobicity are also increased, which can limit the dendrimer's transfection efficacy and its application in clinical use. As increased branches resulting from increased generation count can be detrimental, we generated different molecular weights of dendrimer type rPEIs (16k, 32K), which was complexed with Ad, and evaluated polyplex's toxicity, transduction efficiency, and anticancer therapeutic effect. Here we first demonstrate the feasibility of dendrimers type rPEI, bioreducible polymers, carrying oncolytic Ad vector to a wide range of cancer cells, and identified the optimal molecular weight rPEI containing a disulfide bond for cancer gene therapy.

EXPERIMENTAL SECTION

2.1. Cell Lines and Adenoviruses. The human cancer (A549 (lung carcinoma), HT1080 (fibrosarcoma), MCF7 (breast adenocarcinoma), and human embryonic kidney (HEK) 293 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and maintained in Dulbecco's modified Eagle's Media (DMEM; Gibco-BRL, Grand Island, NY) containing 10% fetal bovine serum (FBS; Gibco-BRL) in 37 °C incubator with 5% CO₂. The Ad/GFP virus is an E1 region-deleted, replicating-incompetent Ad expressing green fluorescent protein (GFP) under the control of the cytomegalovirus promoter (Ad/GFP). RdB/shMet (oAd) is a shMet-expressing oncolytic Ad that replicates under the control of E1A, and E1B double mutated cancer-specific promoter.^{35,36} Ads were propagated in HEK 293 cells and purified by CsCl gradient ultracentrifugation. The number of viral particles (VP) was calculated from the optical density measurement at 260 nm, in which 1 absorbency unit is equivalent to 1.1 × 10¹² viral particles per mL.³⁷

2.2. Preparation of PEI(s-s). *First Cystamine Core.* One gram of cystamine dihydrochloride (4.44 mmol) was dissolved in 10 mL of MeOH, and 1.24 mL of tetraethylammonium (TEA; 0.009 mol) was added into the cystamine solution. The unsolved cystamine was removed by filtration. Methyl acrylate (9.5 mL, 0.107 mol) was mixed with 10 mL of MeOH, and then cystamine solution was slowly dropped to methyl acrylate solution over 30 min. The solution was reacted for 2 days at room temperature under a nitrogen atmosphere. After 2 days, to remove the MeOH and TEA, the solution was evaporated. The white precipitate with the interspersed viscous light yellow liquid was dissolved in diethyl ether and extracted with water. After extraction, the first cystamine core in diethyl ether was obtained by evaporation.

Second Cystamine Core. Cystamine dihydrochloride (10.9 g, 48.33 mmol) and 13.5 mL of TEA (0.097 mol) were dissolved in 100 mL of MeOH. Six hundred milligrams of first cystamine core (1.007 mmol) was dissolved in 5 mL and then slowly dropped to cystamine solution over 30 min. The solution was reacted for 4 days at 4 °C under a nitrogen atmosphere. After reaction, the mixture was evaporated to remove MeOH and TEA, and then dissolved in water. The sample was obtained by dialysis (MWCO = 500 Da, 1 day) and lyophilization. 500 mg of lyophilized sample (0.511 mmol) was dissolved in 5 mL of MeOH and 0.53 mL of TEA (0.004 mol) was added into the sample solution. Methyl acrylate (18.4 mL, 0.205 mol) was mixed with 10 mL of MeOH, and then the sample solution was slowly dropped to methyl acrylate solution over 30 min. The solution was reacted for 2 days at room temperature under a nitrogen atmosphere. After 2 days, to remove the MeOH and TEA, the solution was evaporated. The second cystamine core was obtained by extraction with diethyl ether and water, and evaporation.

Third Cystamine Core. Cystamine dihydrochloride (3.24 g, 14.40 mmol) and 4 mL of TEA (0.029 mol) were dissolved in 100 mL of MeOH. Three hundred milligrams of second cystamine core (0.180 mmol) was dissolved in 5 mL of MeOH and then slowly dropped to cystamine solution over 30 min. The solution was reacted for 4 days at 4 °C under a nitrogen atmosphere. After reaction, the mixture was evaporated to remove MeOH and TEA, then dissolved in water. The sample was obtained by dialysis (MWCO = 1000 Da, 1 day) and lyophilization. Three hundred milligrams of lyophilized sample (0.114 mmol) was dissolved in 5 mL of MeOH, and 0.256 mL of TEA (0.002 mol) was added into the sample solution. Methyl acrylate (16.4 mL, 0.183 mol) was mixed with 10 mL of MeOH, and then the sample solution was slowly dropped to methyl acrylate solution over 30 min. The solution was reacted for 2 days at room temperature under a nitrogen atmosphere. After 2 days, to remove the MeOH and TEA, the solution was evaporated. The third cystamine core was obtained by extraction with diethyl ether and water, and evaporation.

2.3. PEI(s-s) 16 kDa and 32 kDa. Second (0.060 mmol) and third (0.025 mmol) cystamine cores (100 mg of each) were dissolved in 5 mL MeOH. Branched polyethylenimine (bPEI) (8.6 g, 4.8 mmol and 7.2 g, 4.0 mmol), and 1.8 kDa were mixed with 50 mL of each MeOH, then second and third cystamine core solutions were slowly dropped

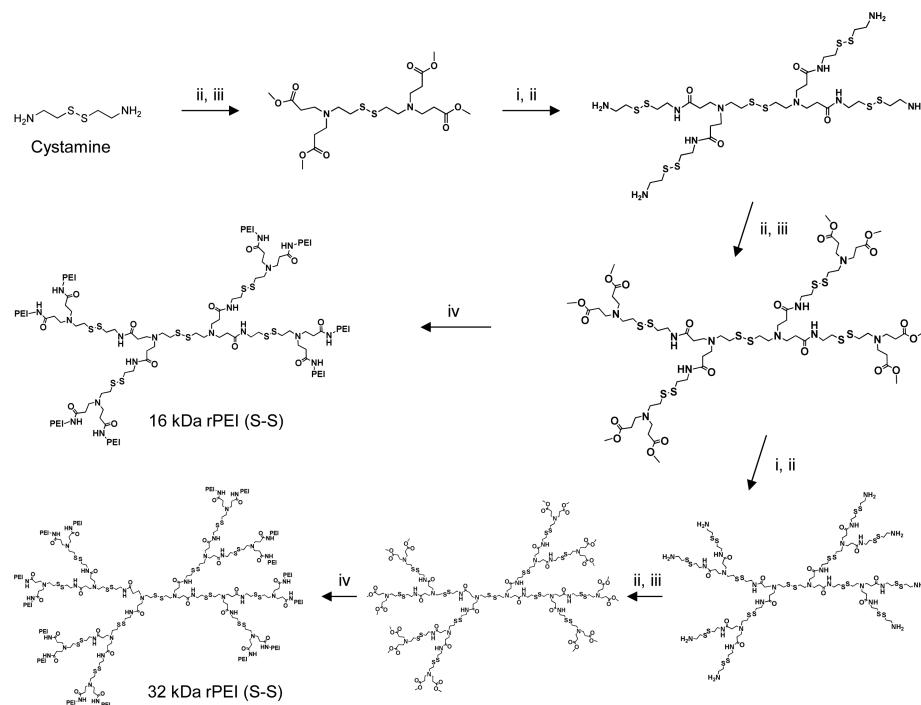


Figure 1. Scheme of bioreducible poly(ethylenimine) derivatives (PEI(s-s) 16 kDa and PEI(s-s) 32 kDa). (i) Cystamine dihydrochloride, (ii) triethylamine, (iii) methyl acrylate, and (iv) bPEI 1.8 kDa.

to 8.6 and 7.2 g bPEI solutions over 30 min, respectively. The solutions were reacted for 5 days at room temperature under a nitrogen atmosphere. After 5 days, MeOH was removed by evaporation. The PEI(s-s) 16 kDa (second cystamine core with 8.6 g bPEI) and 32 kDa (third cystamine core with 7.2 g bPEI) were obtained by dialysis (MWCO = 10 000 Da, 1 day), filtration (0.2 μ m), and lyophilization. Each reaction step was monitored by thin-layer chromatography and ^1H nuclear magnetic resonance (^1H NMR) (Bruker, 400 MHz). bPEI 25 kDa, Mw 25 000 Da was purchased from Sigma-Aldrich (St. Louis, MO). Branched polyethylenimine (bPEI 1.8 kDa, Mw 1800 Da, bPEI 10 kDa, Mw 10 000 Da,) was purchased from Polysciences (Warrington, PA).

2.4. Cytotoxicity of Bioreducible Polymers. The 25 kDa PEI, 16 kDa rPEI, or 32 kDa rPEI polymers were analyzed for cytotoxicity. The cell viability determination was performed by measuring the conversion of MTT to formazan as a function of time. A549, HT1080, and MCF7 cells were grown to 50% confluence in 96-well plates, then treated with varying polymer concentrations, up to 20 μ g/mL. After 48 h, 50 μ L of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT, 2 mg/mL in phosphate buffered saline (PBS); Sigma, St. Louis, MO) was added and incubated for 4 h at 37 °C. The supernatant was removed, and the precipitate was dissolved in 200 μ L dimethyl sulfoxide (DMSO; sigma). Plates were read on a microplate reader (Tecan Infinite M200; TecanDeutschland GmbH, Crailsheim, Germany) at 540 nm. The number of living cells in a PBS-treated cell group was analyzed for 100% cell viability.

2.5. Preparation of rPEI-Coated Adenovirus Complex and Size and Zeta-Potentials. Complexes between cationic polymers and Ad particles (1×10^{10} VP) were formed by prediluting the PEI or rPEI cationic components and the Ad components in an E-tube using PBS (pH 7.4). The molar ratios of cationic molecules to Ad particle is described in the figure legends or methods section. The diluted rPEI polymers were added dropwise to the solution of diluted Ad particles, mixed by inversion or tapping in a tube diluted to total volume of 100 μ L with PBS solution. The Ad/polymer was allowed to complex at room temperature for 30 min through electrostatic interaction. The average sizes and zeta-potentials of naked Ad, Ad/25 kDa PEI, Ad/16 kDa rPEI, and Ad/32 kDa rPEI were measured with a Zetasizer 3000HS (Malvern Instruments, Inc., Worcestershire, UK) with a He-

Ne laser beam (633 nm, fixed scattering angle of 90°) at 25 °C. Ad particles (1×10^{10}) were gently added to each polymer (1×10^4 polymer molecules/Ad particle) diluted in PBS for 30 min. After the formation of complexes, PBS (pH 7.4) was added to a final volume of 1 mL. The obtained sizes and potential values are presented as the average values from three measurements.

2.6. Cellular Uptake of Ad/Polymers. The naked Ad was conjugated with Fluorescein isothiocyanate (FITC, Sigma) in 1 mL PBS for 4 h, then Ad-FITC was dialysis (10K cut off, Slide-A-Lyzer Dialysis Cassettes, Life Technologies, Grand Island, NY) to remove unconjugated FITC at 4 °C in a cold room. A549, or HT1080 cells were plated onto 24-well plates at about 70–80% confluence. After 24 h, cells were treated with Ad-FITC, Ad-FITC/25 kDa PEI, Ad-FITC/16 kDa rPEI, and Ad-FITC/32 kDa rPEI at a multiplicity of infection (MOI) of 200 (A549) and 500 (HT1080) for 2 h, then washed with ice-cold PBS three times. Cellular uptake activity was quantified by measuring the fluorescence intensity with Tecan Infinite M200.

2.7. Transduction Efficiency Assay. Transduction efficiency of naked Ad/GFP, Ad/GFP/25 kDa PEI, Ad/16 kDa rPEI, or Ad/32 kDa rPEI was assessed by measuring GFP expression in both CAR-positive (A549) and CAR-low, negative (HT1080 and MCF7) cells. Cells were seeded at a density of 5×10^4 cells/well in 24-well plates. After 24 h, cells were treated with Ad/GFP, Ad/GFP/25 kDa PEI, Ad/16 kDa rPEI or Ad/GFP/32 kDa rPEI at 20 (A549), 100 (HT1080) or 300 (MCF7) MOI. After 48 h of incubation, cells were observed by fluorescence microscopy (Olympus IX81; Olympus Optical, Tokyo, Japan). For quantifying adenoviral transduction, GFP expression level was quantified by measuring the fluorescence at 485 nm for excitation and 535 nm for emission in a plate reader (Tecan Infinite M200), and were indicated as values of mean fluorescence intensity (MFI).^{38,39}

2.8. Competition Assay. A549 cells were seeded in 24-well plates at 5×10^4 cells per well. Twenty-four hours later, cells were pretreated with CAR antibody (20, 50 μ g/mL) in serum-free DMEM for 30 min. Naked Ad/GFP, Ad/GFP/25 kDa PEI, Ad/GFP/16 kDa rPEI, and Ad/GFP/32 kDa rPEI were added with a MOI of 50 to each well and incubated at 37 °C. Forty-eight hours later, cells were observed by fluorescence microscopy (Olympus BX51). GFP expression level was also quantified by fluorescence intensity (Tecan Infinite M200).

2.9. Cancer Cell Killing Activity. A549, HT1080, and MCF7 cells grown to 50% confluence in 24-well plates were transduced with naked oncolytic Rdb/shMet (oAd), oAd/25 kDa PEI, oAd/16 kDa rPEI, and oAd/32 kDa rPEI at an MOI of 5 (A549), 50 (HT1080), and 200 (MCF7), respectively. Two days after infection, 250 μ L MTT (Sigma, 2 mg/mL) was added to each well. The cells were incubated at 37 °C for 4 h, and the supernatant was then discarded. The precipitate was dissolved in 1 mL of DMSO (Sigma), and the plates were then read with a microplate reader at 540 nm. The number of living cells in a PBS-treated group was analyzed as 100% viability. For viral production assay, A549, HT1080, and MCF7 cells were transduced in the same conditions as above for 4 h. Then cells were washed with PBS and changed with 5% FBS-containing DMEM. After 4 days, infectious Ad viral particles were determined by limiting dilution assay.

2.10. Measurement of Met or VEGF Expression. A549 and HT1080 cells were seeded on 6-well plate with 1×10^5 cells per well. After 24 h, the plated cells were treated with oAd, oAd/25 kDa PEI, oAd/16 kDa rPEI, and oAd/32 kDa rPEI at an MOI of 2 (A549), and 20 (HT1080), respectively. Three days later, each conditioned medium was harvested, and the expression level of Met or VEGF was measured by Met (Invitrogen, Life technologies, Grand Island, NY) or VEGF (R&D Systems, Inc., Minneapolis, MN) ELISA assay kit. Measured value was normalized with cell lysate.

2.11. Statistical Analysis. The data are expressed as mean \pm standard deviation (SD). Statistical comparisons were performed with Stat View software (Abacus Concepts, Inc., Berkeley, CA) and Mann–Whitney tests (nonparametric method). The criterion for statistical significance was ${}^*P < 0.05$.

RESULTS AND DISCUSSION

Synthesis Scheme and Cytotoxicity of Bioreducible PEI. Newly designed and synthesized rPEI is presented in Figure 1. After synthesizing disulfide bond containing dendritic core, 1.8 kDa PEI was conjugated, and each reaction was analyzed by TLC and ^1H NMR. The occurrence of spectrum peaks of PEI(s-s)s were indicated as follows:

- **Cystamine dihydrochloride** (400 MHz, D_2O): δ 3.05 ($-\text{SCH}_2\text{CH}_2\text{NH}_3^+$), δ 3.42 ($-\text{SCH}_2\text{CH}_2\text{NH}_3^+$).
- **Core molecule** (400 MHz, MeOD): δ 2.48 ($-\text{NCH}_2\text{CH}_2\text{CO}-$), δ 2.80 ($-\text{SCH}_2\text{CH}_2\text{N}-$, $-\text{NCH}_2\text{CH}_2\text{CO}-$), δ 3.66 ($-\text{O}-\text{CH}_3$).
- **Bioreducible PEI (-s-s-)** (400 MHz, D_2O): δ 2.28 ($-\text{NCH}_2\text{CH}_2\text{CO}-$; core), δ 2.45–2.75 ($-\text{NCH}_2\text{CH}_2\text{N}-$; PEI), δ 2.98–3.38 ($-\text{SCH}_2\text{CH}_2\text{N}-$, $-\text{NCH}_2\text{CH}_2\text{CO}-$; core).

In addition, the GPC results are indicated in Table 1. The measured molecular weights of PEI 1.8 kDa, rPEI 16 kDa, and

Table 1

	molecular weight
PEI 1.8 kDa	1.8 kDa (P.D. = 1.14) ^a
rPEI 16 kDa	16 kDa (P.D. = 1.26) ^b
rPEI 32 kDa	32 kDa (P.D. = 1.24) ^b

^aMolecular weight as provided by the manufacturers. ^bMolecular weight was estimated by size-exclusion chromatography.

rPEI 32 kDa were 1.8, 16, and 32 kDa, respectively. These molecular weights of polymers were consistent with theoretical values, and it has a narrow polydispersity index (P.D.). These NMR and GPC results showed that bioreducible PEI(s-s)s were successfully synthesized.

Recently, disulfide bond cross-linking low molecular weight polymers have demonstrated improved and highly efficacious gene transduction efficacy with lower cytotoxicity^{40,41} com-

pared to 25 kDa PEI. The disulfide bond in the polymer has been shown to be stable in an extracellular environment *in vivo*, yet it can be rapidly degraded in the presence of reductive glutathione inside an intracellular environment. In this regard, our newly synthesized bioreducible PEI can offer high gene transduction efficiency as well as low toxicity due to its reductive function.

In order to evaluate the cytotoxicity of rPEIs, the cell viability of A549, HT1080, and MCF7 cells treated with rPEIs or 25 kDa PEI as a control were measured by MTT assays. As shown in Figure 2, 25 kDa PEI exhibited significant toxicity, and a

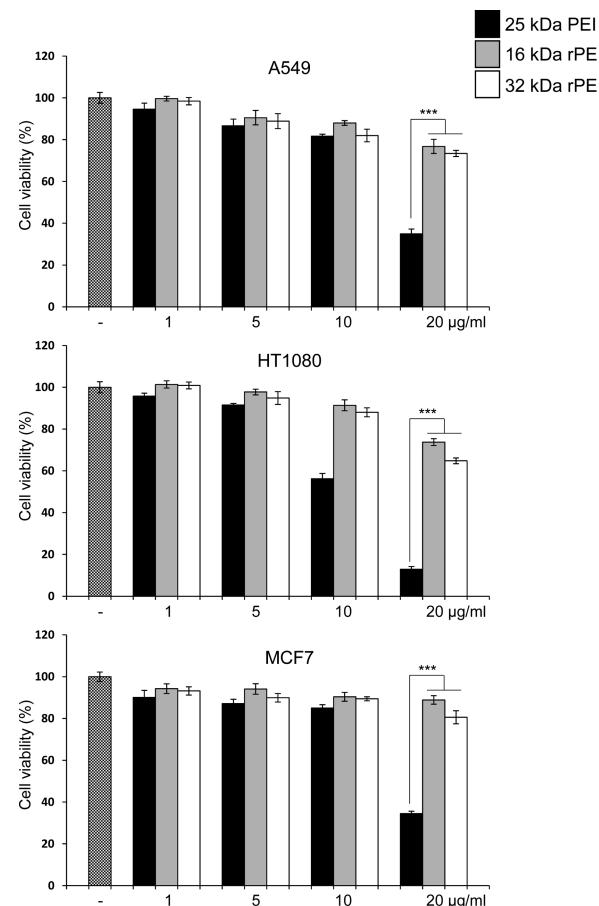


Figure 2. Cytotoxicity of rPEI. Cytotoxicity of polymers on A549, HT1080, and MCF7 cell viability. Cancer cells were treated with PBS, 25 kDa PEI, 16 kDa rPEI, or 32 kDa rPEI followed by an MTT cell viability assay. Cytotoxicity results were normalized against the PBS-treated group. The data represent the means \pm SD of triplicate experiments. *** $P < 0.001$ versus 25 kDa PEI.

much lower cytotoxicity was observed for rPEIs at concentrations below 20 $\mu\text{g}/\text{mL}$ (** $P < 0.001$). Meanwhile, 32 kDa rPEI exhibited more cytotoxicity than 16 kDa rPEI, but it still exhibited lower cytotoxicity than 25 kDa PEI at 20 $\mu\text{g}/\text{mL}$. These results may be attributed to the reducible property of rPEI, which is easily degraded in intracellular environment. As expected, we observed increased cytotoxicity of rPEI with increasing molecular weight. A previous report showed that the cationic polymers induce cytotoxicity by interacting with intracellular components and that higher molecular weight polymers have stronger interaction with these components, which results in higher toxicity.⁴² The viability of the cells treated with either rPEI (16 kDa, 32 kDa) was above 76.8%,

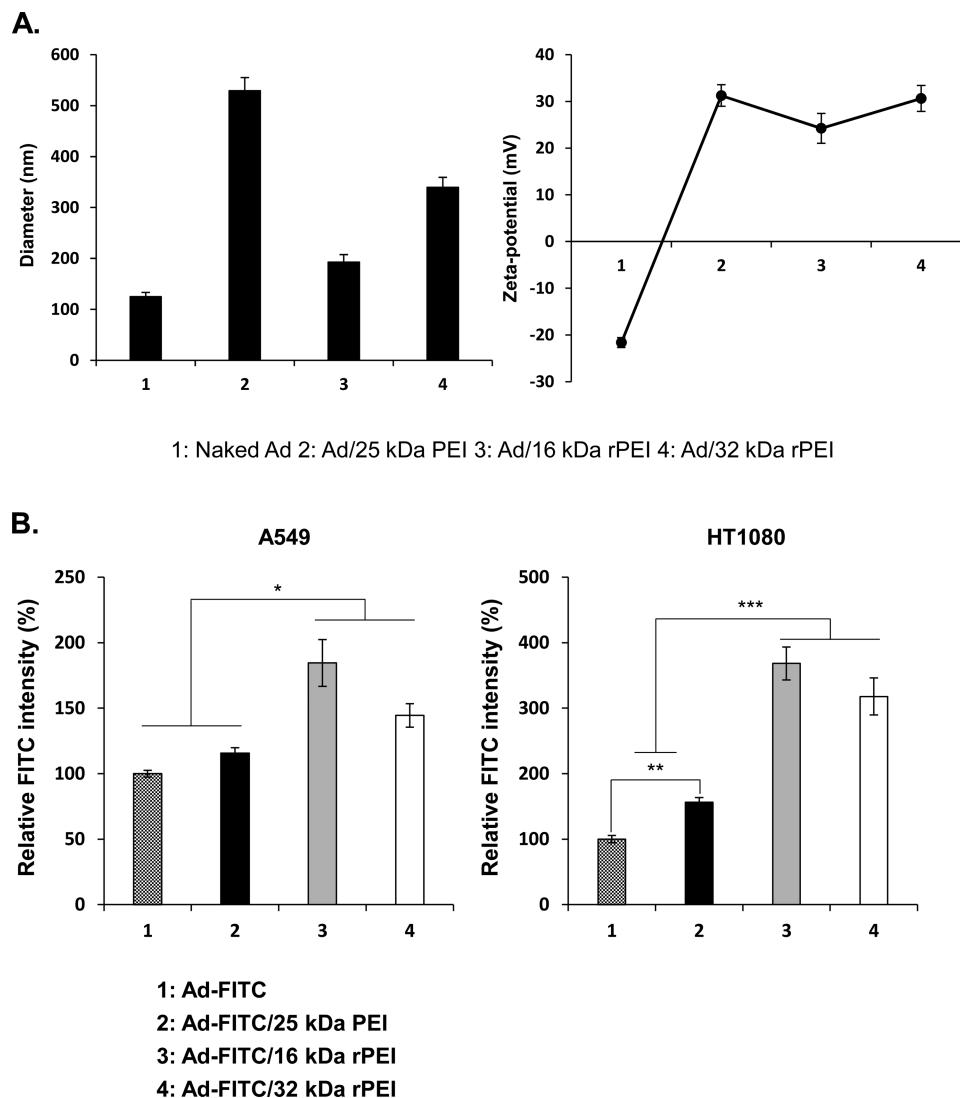


Figure 3. Characterization and cellular uptake activity of Ad/rPEIs. (A) Average size distribution and surface charge of naked Ad, Ad/25 kDa PEI, Ad/16 kDa rPEI, or Ad/32 kDa rPEI. The sizes and charges are the mean \pm SD of five independent experiments. (B) Cellular uptake assay of FITC-labeled Ad, Ad-FITC/25 kDa PEI, Ad-FITC/16 kDa rPEI, or Ad-FITC/32 kDa rPEI in A549 and HT1080 cells. The cellular uptake efficiency was measured by FITC intensity. Data describe mean \pm SD * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus indicated control.

73.4% for A549, 73.7%, 64.8% for HT1080, and 88.9%, 80.6% for MCF7 up to polymer concentrations of 20 μ g/mL, respectively. These findings were consistent with our expectations that the reducible polymers can easily be dissociated inside the cytoplasm, and the results demonstrated much lower cytotoxicity than 25 kDa PEI.

Characterization of Ad/Polymer Complex. The formation of Ad/polymers nanoparticles was driven by electrostatic interaction between Ad and rPEIs. After generating Ad complexed with 25 kDa PEI or rPEIs, the average size and surface charge of complexes were assessed by dynamic light scattering (DLS) and a zeta potential analyzer (Figure 3A). The diameter of the naked Ad was 125 nm; however, the diameter of Ad/polymers was 529.1, 192.8, and 339.7 nm for 25 kDa PEI, 16 kDa rPEI, and 32 kDa rPEI, respectively. Additionally, the surface charge of the Ad/polymer complexes changed from a negative charge (-21.6 mV for naked Ad/GFP) to a positive with cationic polymers: 25 kDa PEI (31.2 mV), 16 kDa rPEI (24.3 mV), and 32 kDa rPEI (30.6 mV). Interestingly, Ad complexed with either rPEI exhibited a smaller diameter than

Ad/25 kDa PEI due to increased condensation between Ad and rPEIs, while the surface charge of Ad/rPEIs was comparable to Ad/25 kDa PEI. These results indicated that positively charged 16 kDa rPEI polymer to viral particles produced a particle diameter of approximately 200 nm with cationic surface, which suggests that Ad/rPEI can be efficiently transduced into cells. Figure 3B shows the cellular uptake efficiency of Ad conjugated with FITC complexed with 25 kDa PEI or rPEIs. The cellular uptake was markedly enhanced when Ad was complexed with either rPEIs compared to naked Ad or Ad/25 kDa PEI (* $P < 0.05$ for A549, *** $P < 0.001$ for HT1080). Sixteen kilodalton or 32 kDa rPEI-coated Ad enhanced the uptake by 1.6- or 1.2-fold in A549 with 2.4- or 2.0-fold higher uptake in HT1080 than Ad/25 kDa PEI, respectively. These results indicate that the size of the Ad/nanocomplex also contributes to the efficiency of cellular uptake. There are two key determining factors of gene transduction efficiency: (1) cellular uptake and (2) efficient endosomal escape. Internalization of Ad complexed with cationic polymer differs greatly from naked Ad, as the cellular uptake mechanism of Ad is CAR-mediated

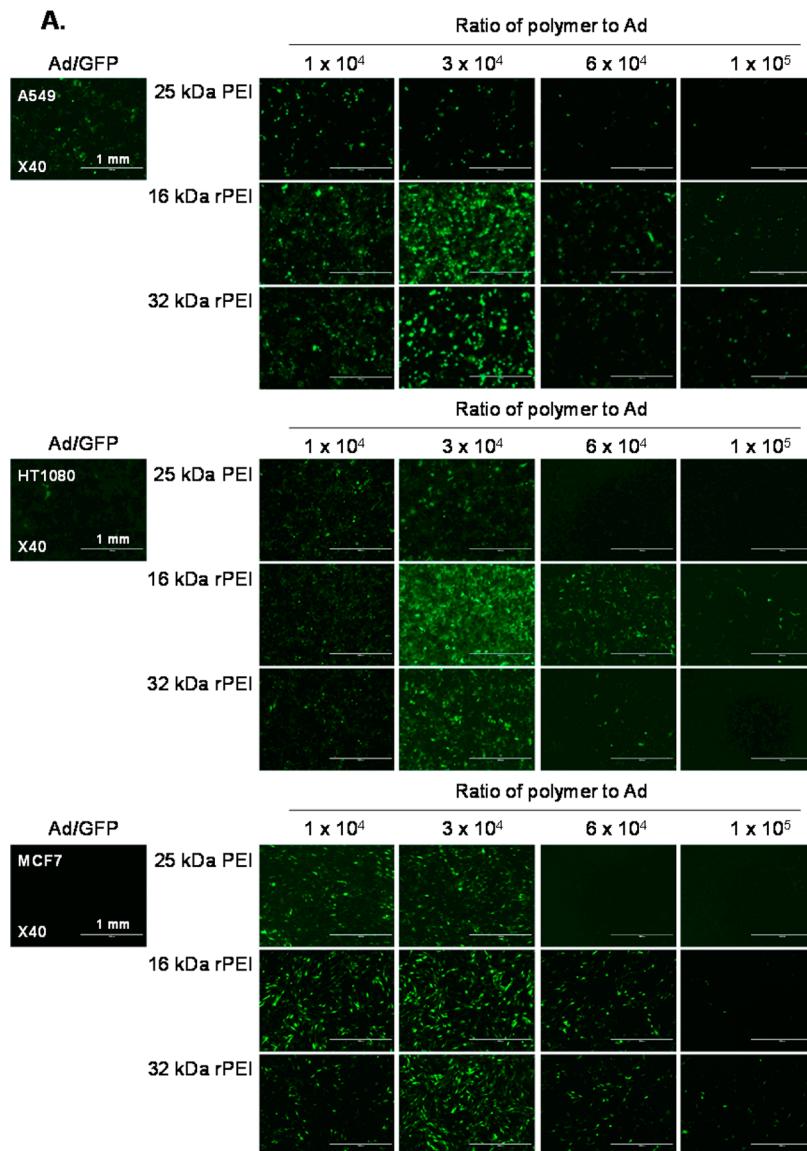


Figure 4. Transduction efficiency assay. Transduction efficiency of Ad/GFP, Ad/GFP/25 kDa PEI, Ad/GFP/16 kDa rPEI, or Ad/GFP/32 kDa rPEI in A549, HT1080, and MCF7 cancer cells. (A) Representative fluorescence microscopy images of transduced cells. Original magnification: $\times 40$. (B) GFP expression level was quantified by fluorescent analyzer. Data describe mean \pm SD. ** $P < 0.01$, *** $P < 0.001$ versus Ad/GFP/25 kDa PEI.

endocytosis, while Ad/polymer's uptake is dependent on the cationic surface charge of the polymer, and the polymer with stronger positive charge can be internalized more efficiently by macropinocytosis through interacting with a negatively charged cellular membrane.^{43,44} Here we clearly demonstrated that cellular uptake of Ad/rPEIs is more efficient than the conventional Ad uptake pathway. Of note, the size of Ad/cationic polymers is also integral for cellular uptake as polymers with similar cationic charge are heavily influenced by size, as rPEI, which has similar charge but smaller size than 25 kDa PEI, showed significantly enhanced cellular uptake. Furthermore, PEIs are well-known for high endosomal escape ability through the proton sponge effect, which contributes to high gene transduction efficiency, which must also contribute to the transduction efficacy of rPEIs. These results in conjunction strongly suggest that Ad complexed with either rPEI have high transduction activity due to the rPEIs' surface charge, size, and high endosome escape capacity.

Enhanced Transduction Efficiency of Ad/rPEIs. Naked Ad transduction is highly dependent on CAR expression level of cellular membrane. Malignant tumors often have diminished or ablated CAR expression, which nullifies Ad tumor infectivity, which contributes to low therapeutic efficacy.^{45,46} One strategy to overcome CAR dependency is to utilize hybrid vector of Ad and cationic polymer, as it can be internalized by CAR-independent macropinocytosis. To evaluate Ad/rPEI's CAR-independent transduction, CAR-positive A549, low CAR expressing HT1080, and CAR-negative MCF7 cells were transduced with Ad/rPEI or Ad/25 kDa PEI. The transduction efficiency of Ad/rPEIs was markedly increased compared to naked Ad in A549, HT1080, and MCF7 cells (Figure 4). This suggests that Ad/rPEIs can efficiently transduce cancer cells independent of CAR expression. Importantly, the beneficial effect of rPEI complexation were particularly pronounced in CAR-low (HT1080) and -negative (MCF7) cells, where the transduction efficiency was increased by 11.7- and 5.9-fold (3×10^4 16 kDa rPEI, ratio of rPEI to Ad) and 5.7- and 6.0-fold (1×10^5 32 kDa rPEI, ratio of rPEI to Ad).

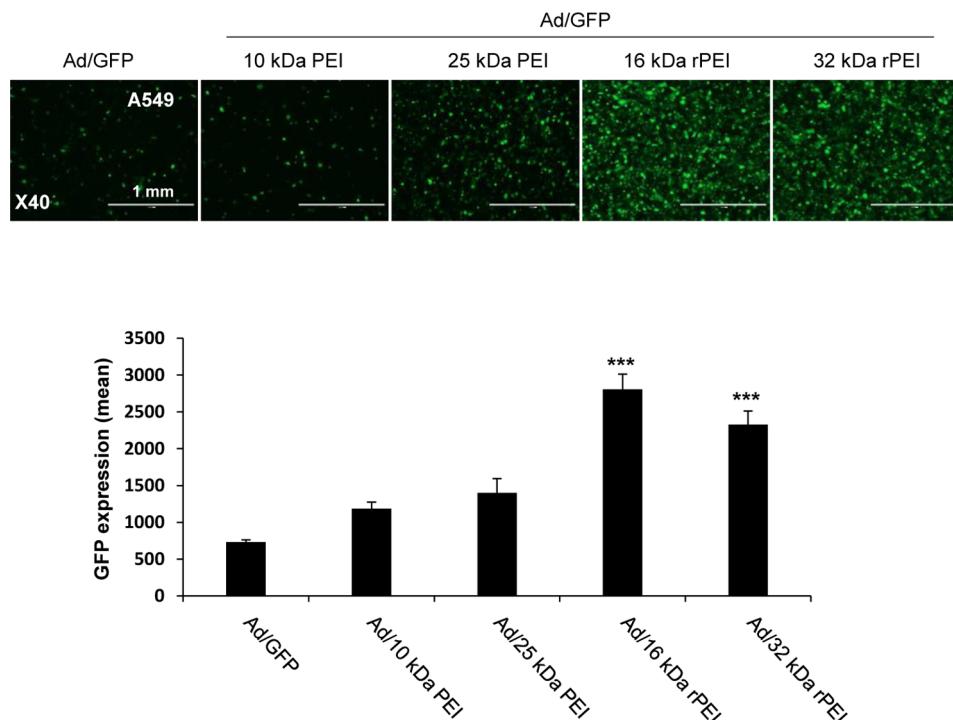


Figure 5. Transduction efficiency of Ad/GFP, Ad/GFP/10 kDa PEI, Ad/GFP/25 kDa PEI, Ad/GFP/16 kDa rPEI, or Ad/GFP/32 kDa rPEI in A549 cancer cells. Representative fluorescence microscopy images of transduced cells. Original magnification: $\times 40$. GFP expression level was quantified by fluorescent analyzer. Data describe mean \pm SD *** $P < 0.001$ versus Ad/GFP/25 kDa PEI.

10^4 32 kDa, ratio of rPEI to Ad) compared to naked Ad, respectively (** $P < 0.001$). More importantly, at 3×10^4 16 kDa rPEI or 32 kDa rPEI ratio of rPEI to Ad, GFP expression was 7.7- and 7.1-fold higher in A549 cells, 2.9- and 1.5-fold higher in HT1080 cells, and 2.0- and 1.9-fold higher in MCF7 cells treated with Ad/rPEIs than Ad/25 kDa PEI, which demonstrated superior transduction efficiency of rPEIs in both CAR-positive and -negative cells. Moreover, we evaluated the transduction efficiency of adenovirus complexed with 10 kDa PEI; 10 kDa PEI had the closest molecular weight to our primary polymer of interest (rPEI 16 kDa). As shown in Figure 5, Ad/GFP/10 kDa PEI-treated cells showed higher GFP expression level than naked Ad/GFP treated cells, yet its GFP expression was lower than those complexed with 25, 32 kDa PEI, or 16 kDa rPEI. This result suggests that 16 kDa rPEI is a more efficient carrier of adenovirus than 10 kDa PEI (commercial PEI of similar kDa as rPEI used) and 25 kDa PEI, which is known as the “gold standard” of polymer-based gene delivery systems. According to our previous reports, gene transduction efficiency is decided by the optimal molar ratio of polymer to Ad.^{37,38,44,47} Lee et al. investigated cellular uptake of Ad/polymer complex and concluded that it is mediated by clathrin, caveolin-mediated endocytosis, or micropinocytosis.⁴⁴ This indicates that the cellular uptake mechanism of Ad/polymer differs from that of naked Ad. Size and surface charge of nanoparticles are crucial factors for passively targeted delivery, which is affected by polymer/Ad molar ratio. Furthermore, excess polymers can induce aggregation when complexed with adenovirus, which prevents efficient cellular uptake as well as endosomal escape, resulting in decreased transduction efficiency. Ad/polymer complex’s transduction efficiency is heavily dependent on the ratio of polymer to Ad.

Interestingly, the quantity of polymer used for optimal ratio of Ad/rPEIs (16 kDa: 1×10^4 ; 32 kDa: 3×10^4) identified in

this study was approximately 100-fold less than other polymers we have previously complexed with Ad such as ABP or PNLG.^{38,47} This suggests that a low amount of rPEI can efficiently encapsulate the Ad, resulting in high gene transduction with low toxicity.

Next, we performed a competition assay using the CAR specific antibody (Ab) to demonstrate that Ad/polymer is internalized independently of CAR-mediated endocytosis. CAR-positive A549 cells were preincubated with CAR-specific Ab prior to transduction with replication-incompetent GFP expressing Ad. GFP expression of naked Ad/GFP was substantially reduced in a dose-dependent manner for the amount of CAR-specific Ab treated to cells prior to transfection (35% and 47% decreases with 20 and 50 μ g/mL CAR Ab pretreatment, respectively; Figure 6). In contrast, GFP expression of Ad/GFP complexed with cationic polymers was not blocked by CAR Ab, suggesting that Ad/GFP/polymers’ uptake pathway was not mediated by interaction between adenoviral fiber and CAR. This data shows that uptake of Ad/rPEIs is internalized by an alternative pathway from the traditional CAR-mediated endocytosis pathway, which makes Ad/rPEIs feasible for treatment of malignant tumors regardless of their CAR expression. Several cationic polymers have been developed as gene delivery carriers over the past decades, and PEI is one of the most efficient and well-studied polymers for gene delivery.^{48,49} PEI is a synthetic polymer composed of ethylenimine monomers. The chemical structure of PEI is largely divided into linear and branched forms. The linear PEI consists of primary and secondary amines, whereas branched PEI contains three types of amines with both amine categories of linear PEI as well as tertiary amines. PEI facilitates condensation of nucleic acid such as DNA or siRNA and forms polyplexes at different N/P ratios. Both linear and branched PEI show high transfection efficacy in gene delivery

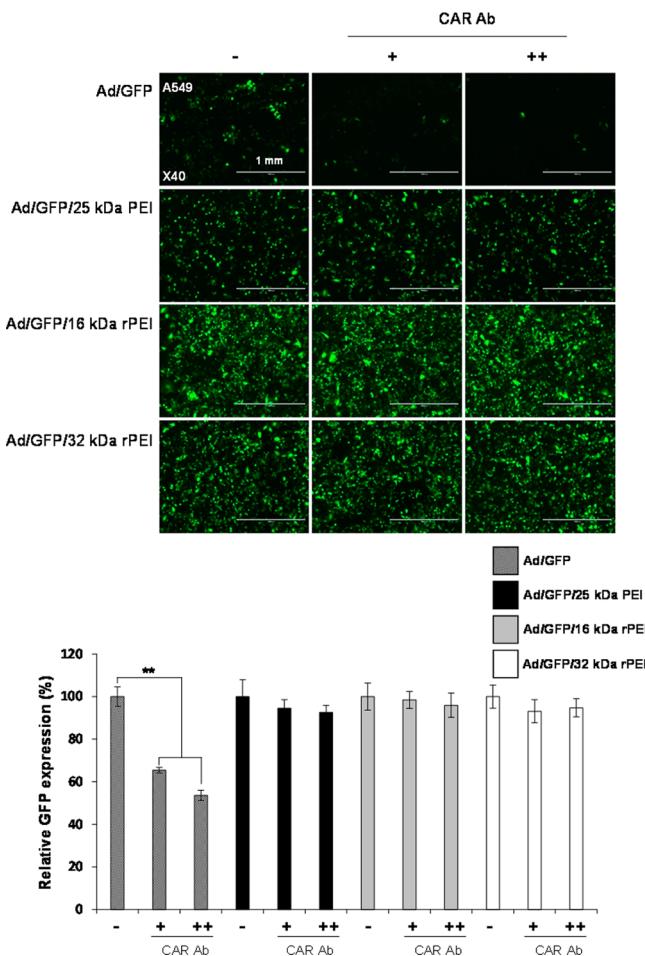


Figure 6. Competition assay of Ad/GFP, Ad/GFP/25 kDa PEI, Ad/GFP/16 kDa rPEI, or Ad/GFP/32 kDa rPEI with specific CAR. A549 cells were preincubated with CAR Ab (20, 50 μ g/mL), followed by treatment with naked Ad/GFP, Ad/GFP/25 kDa PEI, Ad/GFP/16 kDa rPEI, or Ad/GFP/32 kDa rPEI at 50 MOI. (A) GFP fluorescence microscopy images. (B) GFP expression levels were quantified by fluorescent analyzer. Original magnification: $\times 40$. Data describe mean \pm SD. **P < 0.01 versus presence of CAR Ab.

studies.⁵⁰ It has previously been reported that linear PEI is more efficient than branched PEI in vivo, as less hermetic complexation allows for more efficient dissociation of complexes.⁵¹ Branched PEI shows higher transfection efficacy in vitro due to stronger complexation than linear PEI.⁵² Of note, our reducible PEI (rPEI) forms an enhanced complex, which has advantageous attributes of both linear and branched PEI, as Ad/rPEI can easily dissociate by reducible linkage in vivo while still maintaining the stronger condensation property of branched PEI. This reducible dendritic feature of rPEI makes rPEI a strong candidate for a highly efficacious and effective carrier for Ad delivery in vitro and in vivo.

Enhanced Cancer Cell Killing Effect and Viral Production of oAd/rPEIs. The oncolytic Ad (RdB/shMet or oAd) was genetically designed and produced to selectively replicate in and kill cancer cells by tumor specific promoter. This E1A- and E1B- double mutated promoter inserted oncolytic Ad can tumor selectively produce the viral progeny and ultimately infect neighboring cancer cells.^{36,53} The signaling of the c-Met and its ligand, hepatocyte growth factor (HGF), is deregulated in many cancers, and is known to

critically play a role affecting both primary tumor growth and metastasis.⁵⁴ In this regard, there have been a number of therapeutic approaches evaluated such as small molecule inhibitors, peptide antagonists, and monoclonal antibodies for abrogate c-Met and HGF signaling.⁵⁵ Thus, Ad expressing shMet, which downregulates c-Met, can be a new targeted therapeutic approach for cancer gene therapy. To investigate whether the oncolytic effect of oAd coated with rPEIs could be improved, oAd was physically coated with rPEIs and treated to the cancer cells (Figure 7A). MTT assay revealed that naked oAd killed 30.3% (A549), 22.8% (HT1080) or 5.6% (MCF7), which is significantly less than the killing efficacy observed by Ad/polymers, as even Ad/25 kDa PEI, which showed the lowest cytotoxicity of Ad/polymers, exhibited 38% (A549), 33.4% (HT1080), or 53.5% (MCF7); Ad/16 kDa or 32 kDa rPEI exhibited greater cytotoxicity in all cell lines 64.3%, 50.3% (A549), 70.9%, 46.7% (HT1080) and 73.8%, 63.4% (MCF7). These results showed that the cancer cell killing effect of oncolytic Ad can be significantly improved by coating with rPEIs in both CAR-positive and -negative cancer cells. As shown in Figure 2, rPEI polymer toxicity did not significantly affect cell killing effect, so we performed viral production assay to evaluate virus' enhanced cancer cell killing efficacy (Figure 7B). A549, HT1080, and MCF7 cells were treated with oAd, oAd with 25 kDa PEI, 16 kDa rPEI, or 32 kDa rPEI for 4 h, and then media was changed to remove any polymers and measure quantity of viral progeny. As shown in Figure 7B, the viral production assay result of oAd or oAd/polymer complex was strongly correlated with that of cancer cell killing effect (Figure 7A). The mechanism of cancer cell killing effect by oncolytic Ad is highly dependable on its lytic cycle through production of viral progeny as well as expressing therapeutic gene. Previously, our group investigated CAR expression levels of various cell lines (A549: high, HT1080: intermediate, and MCF7: none).^{38,56} Since CAR expression level differs in each cell line, treatment of different MOI is needed for certain cell lines to show efficient transduction. The purpose of viral production assay is observation of viral progeny, which is decided by initial infection, and for this procedure treating with significant surplus of oncolytic Ad can induce apoptosis before viral progeny assembly that decreases viral production. As optimal condition and dose of oncolytic Ad varies significantly between each cell lines, different MOI was utilized in correlation with relative CAR expression. More specifically, oAd/16 kDa rPEI produced 8.9-, 5.5-, and 2.4-fold (A549), and 107-, 18-, and 6.3-fold higher viral production than oAd-, oAd/25 kDa PEI-, or oAd/32 kDa rPEI-treated cells, respectively. rPEIs did not affect viral production and oAd/16 kDa rPEI polymer showed the strongest cancer-killing effect with the highest viral production in the cells. These data showed that despite the polymer only helping with initial viral entry, it is important to note that more viral progenies are produced due to higher cellular uptake during initial entry as more cells are infected. After Ad infects the cells, 1000–10 000 viral progeny are produced and sequentially infect the surrounding cancer cells. Improved initial transduction can significantly enhance cancer treatment as lower initial dose of virus can be administered, and charge-mediated entry can show substantial difference in therapeutic outcomes in treatment of CAR-ablated or low-expressing cancers. For example, cancer cells that were infected with 50 MOI of oncolytic Ad/polymer can get similar therapeutic effects as naked oncolytic Ad administered at an MOI of 50 000–500 000. Moreover, reduced viral dose per admin-

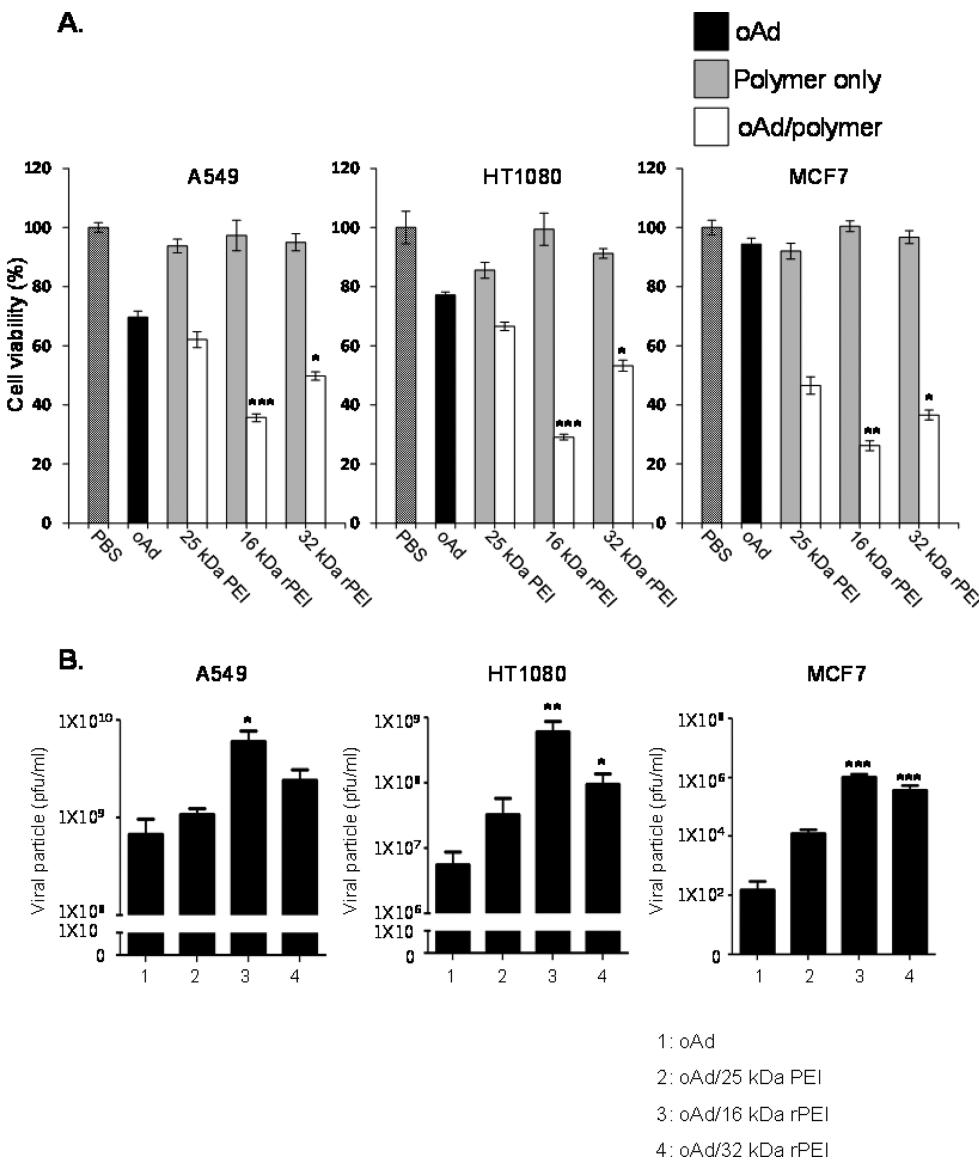


Figure 7. Cancer cell killing effect of naked oAd, oAd/25 kDa PEI, oAd/16 kDa rPEI, or oAd/32 kDa rPEI. (A) Cancer cells were infected with naked oAd, oAd/25 kDa PEI, oAd/16 kDa rPEI, or oAd/32 kDa rPEI at an MOI of 5 (A549), 50 (HT1080), and 200 (MCF7), respectively. At 2 days post infection, cell viability was determined by an MTT assay. Data describe mean \pm SD *P < 0.05, **P < 0.01, ***P < 0.001 versus oAd/25 kDa PEI. (B) The production of infectious Ad viral particles was also measured by limiting dilution assay at 3 days post infection. *P < 0.05, **P < 0.01 versus oAd/25 kDa PEI.

istration can be advantageous in terms of safety. Thus, a combination of oncolytic Ad vector and nonviral vector gives rise to increase therapeutic effect as well as safety.

Suppression of C-Met and VEGF Expression by oAd/rPEIs. Downregulation of Met signaling has high therapeutic potential against cancer, as it can suppress cancer cell migration and invasion as well as inhibiting both tumor growth and angiogenesis.⁵⁷ In theory, shMet which can downregulate Met signaling should be highly beneficial, but shMet as a single therapeutic agent has limitation due to short half-life of siRNA. In this study, we genetically inserted shMet into oncolytic Ad backbone to increase the quantity of shMet delivered and expressed in a tumor, as each replication and lytic cycle of Ad would amplify expression of shMet as Ad can produce approximately 1000–10 000 copies of viral progenies, which can sequentially infect neighboring cancer cells.⁵⁸ This resulted in both high and prolonged expression of shMet overcoming

the limitation of therapy based on siRNA as a single therapeutic agent. To further evaluate the effect of shMet to suppress the expression Met and VEGF, the secretion level of Met and VEGF were determined. As shown in Figure 8, the expression of Met and VEGF was significantly suppressed in the cells treated with oAd/16 kDa rPEI complex in comparison to oAd- or oAd/25 kDa PEI-treated cells. Furthermore, the secretion of Met from the oAd/16 kDa rPEI-treated A549 or HT1080 cells was inhibited by 60.7% or 54.2%, respectively, whereas the expression of Met or VEGF expression was decreased by a lesser extent by other treatment groups: 30.68% or 14.5% for naked oAd, 37.9% or 19% for oAd/25 kDa PEI, and 52.6% or 32.4% for oAd/32 kDa rPEI, respectively. Meanwhile, the secretion of VEGF from the oAd/16 kDa rPEI-treated A549 or HT1080 cells was inhibited by 79.2% or 70%, respectively, whereas the expression VEGF expression was decreased by 58.3% or 25% for naked oAd, 65% or 26% for oAd/25 kDa PEI,

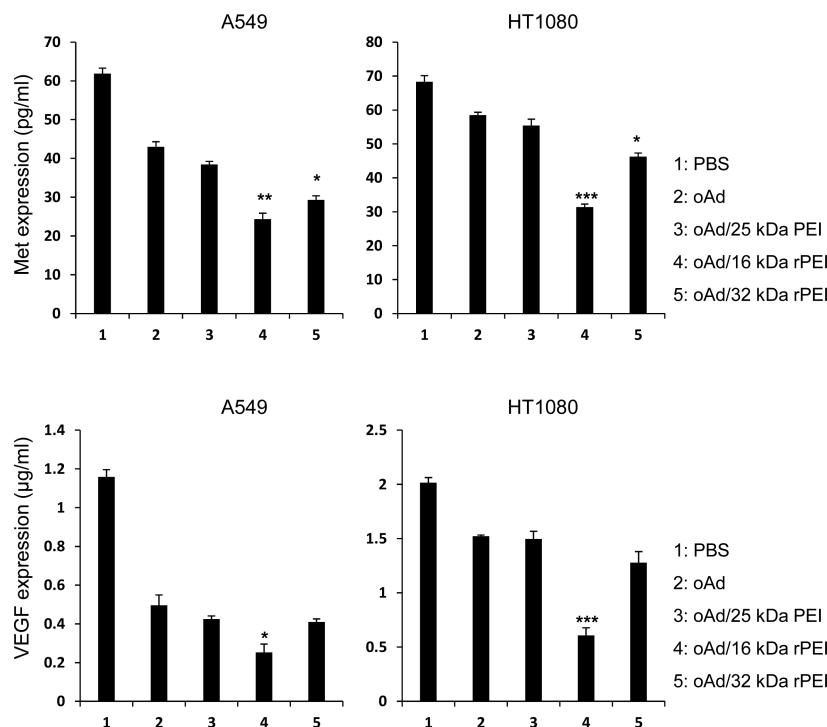


Figure 8. Measurement of MET or VEGF. Specific knock down of Met or VEGF expression by naked oAd, or oAd/polymers. A549 or HT1080 cells were treated with naked oAd, oAd/25 kDa PEI, oAd/16 kDa rPEI, or oAd/32 kDa rPEI at MOI of 2, 20, respectively. After incubation for 3 days, each conditioned medium was measured the expression of Met or VEGF by human c-Met or VEGF ELISA assay kit, respectively. Data describe mean \pm SD *P < 0.05, **P < 0.01, ***P < 0.001 versus oAd/25 kDa PEI.

and by 65.8% or 36.5% for oAd/32 kDa rPEI, respectively. Taken together, these data suggest that the oAd did not affect the functionality of Ad after complexation with any of the polymers used in this study. Conclusively, oAd/rPEI nanocomplex could maximize the cancer cell killing ability through both adenoviral oncolysis and suppression of Met and VEGF expression in broad range of cancer without significant polymer toxicity. However, previous studies have acknowledged poor tumor selectivity as a potential disadvantage for complexation of Ad with a cationic polymer, as strong cationic surface charge of the complex results in indiscriminate cellular uptake of the complex even by normal cells due to charge-mediated internalization of the complex into negatively charged membranes. In order to overcome such limitations, the outer shell or distal end of polymer needs to be conjugated with active targeting moieties such as antibodies, growth factors, small peptides, or ligands. This study has evaluated feasibility of Ad/rPEIs nanocomplex for gene delivery as well as oncolytic effect, and targeting moiety conjugated rPEIs will need to be evaluated separately for targeting and therapeutic efficacy both in vitro and in vivo.

CONCLUSION

In summary, we designed and synthesized a novel a multibiodegradable and bioreducible core-cross-linked PEI with high condensation capacity and surface charge that results in highly efficacious Ad delivery to cancer cells. This polymer can have high gene transduction ability with low cytotoxicity due to utilization of low molecular weight 1.8 kDa PEI and reductive function from disulfide linkage in the polymer backbone. Surface charge of Ad/rPEIs was sufficiently high (>20 mV) as it had similar surface charge as highly cationic 25 kDa PEI while having significantly smaller size than 25 kDa

PEI, which had significant contribution toward higher cellular uptake by Ad complexed with rPEIs. The importance of size over charge is further supported by Ad/16 kDa rPEI showing higher therapeutic efficacy than Ad/32 kDa rPEI as Ad/32 kDa rPEI had more cationic surface charge but still was less effective than Ad/16 kDa rPEI due to approximately 100 nm higher diameter. The rPEI can easily be complexed with Ad at higher condensation capacity which can significantly reduce polymer dose in clinical application. The Ad/rPEI complex exhibited increased transduction efficiency and is internalized by CAR-independent pathway with minimal toxicity. Ad complexed with either rPEI resulted in significantly augmented cancer cell killing effect and therapeutic gene expression by oncolytic Ad than naked Ad or Ad/25 kDa PEI. Taken together, these results demonstrate the feasibility and potential of multidegradable bioreducible PEI coated oncolytic Ad as a great therapeutic candidate in the treatment of malignant cancers.

AUTHOR INFORMATION

Corresponding Authors

*Address: Department of Bioengineering, College of Engineering, Hanyang University, 222 Wangsimni-ro, Seongdong-gu, Seoul, Republic of Korea. Tel: + 82 2 2220 0491; Fax: +82 2 2220 4850; E-mail address: chaeok@hanyang.ac.kr (C.-O.Y.).

*Address: Center for Controlled Chemical Delivery (CCCD), Department of Pharmaceutics and Pharmaceutical Chemistry, University of Utah, Salt Lake City, UT, USA. Tel: +1 801 581 6654; Fax: +1 801 581 7848; E-mail address: SW.Kim@pharm.utah.edu (S.W.K.).

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work was supported by grants from the National Research Foundation of Korea (2010-0029220, 2013M3A9D3045879), the Korea Food and Drug Administration (13172KFDA306), and the National Institutes of Health, USA (CA177932). This work was partially supported by the Brain Korea 21 plus Future Biopharmaceutical Human Resources Training and Research Team.

REFERENCES

- (1) Heise, C. C.; Williams, A. M.; Xue, S.; Propst, M.; Kirn, D. H. Intravenous administration of ONYX-015, a selectively replicating adenovirus, induces antitumoral efficacy. *Cancer Res.* **1999**, *59* (11), 2623–8.
- (2) Kirn, D. Clinical research results with dl1520 (Onyx-015), a replication-selective adenovirus for the treatment of cancer: what have we learned? *Gene Ther.* **2001**, *8* (2), 89–98.
- (3) Yamamoto, M.; Curiel, D. T. Current issues and future directions of oncolytic adenoviruses. *Mol. Ther.* **2010**, *18* (2), 243–50.
- (4) Hecht, J. R.; Bedford, R.; Abbruzzese, J. L.; Lahoti, S.; Reid, T. R.; Soetikno, R. M.; Kirn, D. H.; Freeman, S. M. A phase I/II trial of intratumoral endoscopic ultrasound injection of ONYX-015 with intravenous gemcitabine in unresectable pancreatic carcinoma. *Clin. Cancer Res.* **2003**, *9* (2), 555–61.
- (5) DeWeese, T. L.; van der Poel, H.; Li, S.; Mikhak, B.; Drew, R.; Goemann, M.; Hamper, U.; DeJong, R.; Detorie, N.; Rodriguez, R.; Hauk, T.; DeMarzo, A. M.; Piantadosi, S.; Yu, D. C.; Chen, Y.; Henderson, D. R.; Carducci, M. A.; Nelson, W. G.; Simons, J. W. A phase I trial of CV706, a replication-competent, PSA selective oncolytic adenovirus, for the treatment of locally recurrent prostate cancer following radiation therapy. *Cancer Res.* **2001**, *61* (20), 7464–72.
- (6) Liu, T. C.; Galanis, E.; Kirn, D. Clinical trial results with oncolytic virotherapy: A century of promise, a decade of progress. *Nat. Clin. Pract. Oncol.* **2007**, *4* (2), 101–17.
- (7) Reid, T.; Galanis, E.; Abbruzzese, J.; Sze, D.; Wein, L. M.; Andrews, J.; Randlev, B.; Heise, C.; Uprichard, M.; Hatfield, M.; Rome, L.; Rubin, J.; Kirn, D. Hepatic arterial infusion of a replication-selective oncolytic adenovirus (dl1520): Phase II viral, immunologic, and clinical endpoints. *Cancer Res.* **2002**, *62* (21), 6070–9.
- (8) Yamamoto, M. Conditionally replicative adenovirus for gastrointestinal cancers. *Expert Opin. Biol. Ther.* **2004**, *4* (8), 1241–50.
- (9) Yamamoto, M.; Curiel, D. T. Nonreplicating DNA viral vectors for suicide gene therapy: The adenoviral vectors. *Methods Mol. Med.* **2004**, *90*, 61–70.
- (10) Yun, C. O.; Kim, E.; Koo, T.; Kim, H.; Lee, Y. S.; Kim, J. H. ADP-overexpressing adenovirus elicits enhanced cytopathic effect by induction of apoptosis. *Cancer Gene Ther.* **2005**, *12* (1), 61–71.
- (11) Lee, Y. S.; Kim, J. H.; Choi, K. J.; Choi, I. K.; Kim, H.; Cho, S.; Cho, B. C.; Yun, C. O. Enhanced antitumor effect of oncolytic adenovirus expressing interleukin-12 and B7-1 in an immunocompetent murine model. *Clin. Cancer Res.* **2006**, *12* (19), 5859–68.
- (12) Kim, J. H.; Lee, Y. S.; Kim, H.; Huang, J. H.; Yoon, A. R.; Yun, C. O. Relaxin expression from tumor-targeting adenoviruses and its intratumoral spread, apoptosis induction, and efficacy. *J. Natl. Cancer Inst.* **2006**, *98* (20), 1482–93.
- (13) Yoo, J. Y.; Kim, J. H.; Kim, J.; Huang, J. H.; Zhang, S. N.; Kang, Y. A.; Kim, H.; Yun, C. O. Short hairpin RNA-expressing oncolytic adenovirus-mediated inhibition of IL-8: Effects on antiangiogenesis and tumor growth inhibition. *Gene Ther.* **2008**, *15* (9), 635–51.
- (14) Yoo, J. Y.; Kim, J. H.; Kwon, Y. G.; Kim, E. C.; Kim, N. K.; Choi, H. J.; Yun, C. O. VEGF-specific short hairpin RNA-expressing oncolytic adenovirus elicits potent inhibition of angiogenesis and tumor growth. *Mol. Ther.* **2007**, *15* (2), 295–302.
- (15) Choi, I. K.; Lee, Y. S.; Yoo, J. Y.; Yoon, A. R.; Kim, H.; Kim, D. S.; Seidler, D. G.; Kim, J. H.; Yun, C. O. Effect of decorin on overcoming the extracellular matrix barrier for oncolytic virotherapy. *Gene Ther.* **2010**, *17* (2), 190–201.
- (16) Roelvink, P. W.; Lizonova, A.; Lee, J. G.; Li, Y.; Bergelson, J. M.; Finberg, R. W.; Brough, D. E.; Kovacs, I.; Wickham, T. J. The coxsackievirus-adenovirus receptor protein can function as a cellular attachment protein for adenovirus serotypes from subgroups A, C, D, E, and F. *J. Virol.* **1998**, *72* (10), 7909–15.
- (17) Kasala, D.; Choi, J. W.; Kim, S. W.; Yun, C. O. Utilizing adenovirus vectors for gene delivery in cancer. *Expert Opin. Drug Delivery* **2014**, *11* (3), 379–92.
- (18) Park, T. G.; Jeong, J. H.; Kim, S. W. Current status of polymeric gene delivery systems. *Adv. Drug Delivery Rev.* **2006**, *58* (4), 467–86.
- (19) Parker, A. L.; Newman, C.; Briggs, S.; Seymour, L.; Sheridan, P. J. Nonviral gene delivery: Techniques and implications for molecular medicine. *Expert Rev. Mol. Med.* **2003**, *5* (22), 1–15.
- (20) Baker, A.; Saltik, M.; Lehrmann, H.; Killisch, I.; Mautner, V.; Lamm, G.; Christofori, G.; Cotten, M. Polyethylenimine (PEI) is a simple, inexpensive and effective reagent for condensing and linking plasmid DNA to adenovirus for gene delivery. *Gene Ther.* **1997**, *4* (8), 773–82.
- (21) Park, J. W.; Mok, H.; Park, T. G. Physical adsorption of PEG grafted and blocked poly-L-lysine copolymers on adenovirus surface for enhanced gene transduction. *J. Controlled Release* **2010**, *142* (2), 238–44.
- (22) Raja-Walia, R.; Webber, J.; Naftilan, J.; Chapman, G. D.; Naftilan, A. J. Enhancement of liposome-mediated gene transfer into vascular tissue by replication deficient adenovirus. *Gene Ther.* **1995**, *2* (8), 521–30.
- (23) Akinc, A.; Thomas, M.; Klibanov, A. M.; Langer, R. Exploring polyethylenimine-mediated DNA transfection and the proton sponge hypothesis. *J. Gene Med.* **2005**, *7* (5), 657–63.
- (24) Godbey, W. T.; Wu, K. K.; Mikos, A. G. Size matters: molecular weight affects the efficiency of poly(ethylenimine) as a gene delivery vehicle. *J. Biomed. Mater. Res.* **1999**, *45* (3), 268–75.
- (25) Kunath, K.; von Harpe, A.; Fischer, D.; Petersen, H.; Bickel, U.; Voigt, K.; Kissel, T. Low-molecular-weight polyethylenimine as a non-viral vector for DNA delivery: comparison of physicochemical properties, transfection efficiency and in vivo distribution with high-molecular-weight polyethylenimine. *J. Controlled Release* **2003**, *89* (1), 113–25.
- (26) Li, J.; Cheng, D.; Yin, T.; Chen, W.; Lin, Y.; Chen, J.; Li, R.; Shuai, X. Copolymer of poly(ethylene glycol) and poly(L-lysine) grafting polyethylenimine through a reducible disulfide linkage for siRNA delivery. *Nanoscale* **2014**, *6* (3), 1732–40.
- (27) Zhang, L.; Chen, Z.; Li, Y. Dual-degradable disulfide-containing PEI-Pluronic/DNA polyplexes: transfection efficiency and balancing protection and DNA release. *Int. J. Nanomed.* **2013**, *8*, 3689–701.
- (28) Liu, J.; Jiang, X.; Xu, L.; Wang, X.; Hennink, W. E.; Zhuo, R. Novel reduction-responsive cross-linked polyethylenimine derivatives by click chemistry for nonviral gene delivery. *Bioconjugate Chem.* **2010**, *21* (10), 1827–35.
- (29) Han, S.; Mahato, R. I.; Kim, S. W. Water-soluble lipopolymer for gene delivery. *Bioconjugate Chem.* **2001**, *12* (3), 337–45.
- (30) Kim, W. J.; Chang, C. W.; Lee, M.; Kim, S. W. Efficient siRNA delivery using water soluble lipopolymer for anti-angiogenic gene therapy. *J. Controlled Release* **2007**, *118* (3), 357–63.
- (31) Lee, M.; Rentz, J.; Han, S. O.; Bull, D. A.; Kim, S. W. Water-soluble lipopolymer as an efficient carrier for gene delivery to myocardium. *Gene Ther.* **2003**, *10* (7), 585–93.
- (32) Furgeson, D. Y.; Cohen, R. N.; Mahato, R. I.; Kim, S. W. Novel water insoluble lipoparticles for gene delivery. *Pharm. Res.* **2002**, *19* (4), 382–90.
- (33) Mahato, R. I.; Lee, M.; Han, S.; Maheshwari, A.; Kim, S. W. Intratumoral delivery of p2CMVmiL-12 using water-soluble lipopolymers. *Mol. Ther.* **2001**, *4* (2), 130–8.
- (34) Dufes, C.; Uchegbu, I. F.; Schatzlein, A. G. Dendrimers in gene delivery. *Adv. Drug Delivery Rev.* **2005**, *57* (15), 2177–202.
- (35) Lee, J. S.; Oh, E.; Yoo, J. Y.; Choi, K. S.; Yoon, M. J.; Yun, C. O. Adenovirus expressing dual c-Met-specific shRNA exhibits potent antitumor effect through autophagic cell death accompanied by

- senescence-like phenotypes in glioblastoma cells. *Oncotarget* **2015**, *6* (6), 4051–65.
- (36) Kim, J.; Kim, J. H.; Choi, K. J.; Kim, P. H.; Yun, C. O. E1A- and E1B-Double mutant replicating adenovirus elicits enhanced oncolytic and antitumor effects. *Hum. Gene Ther.* **2007**, *18* (9), 773–86.
- (37) Choi, J. W.; Jung, S. J.; Kasala, D.; Hwang, J. K.; Hu, J.; Bae, Y. H.; Yun, C. O. pH-sensitive oncolytic adenovirus hybrid targeting acidic tumor microenvironment and angiogenesis. *J. Controlled Release* **2015**, *205*, 134–43.
- (38) Kim, J.; Li, Y.; Kim, S. W.; Lee, D. S.; Yun, C. O. Therapeutic efficacy of a systemically delivered oncolytic adenovirus–biodegradable polymer complex. *Biomaterials* **2013**, *34* (19), 4622–31.
- (39) Kim, J.; Nam, H. Y.; Choi, J. W.; Yun, C. O.; Kim, S. W. Efficient lung orthotopic tumor-growth suppression of oncolytic adenovirus complexed with RGD-targeted bioreducible polymer. *Gene Ther.* **2014**, *21* (5), 476–83.
- (40) Lee, Y. S.; Kim, S. W. Bioreducible polymers for therapeutic gene delivery. *J. Controlled Release* **2014**, *190*, 424–39.
- (41) Ryu, K.; Kim, T. I. Therapeutic gene delivery using bioreducible polymers. *Arch. Pharmacal Res.* **2014**, *37* (1), 31–42.
- (42) Tian, L.; Kang, H. C.; Bae, Y. H. Endosomolytic reducible polymeric electrolytes for cytosolic protein delivery. *Biomacromolecules* **2013**, *14* (8), 2570–81.
- (43) Nam, H. Y.; Nam, K.; Lee, M.; Kim, S. W.; Bull, D. A. Dendrimer type bio-reducible polymer for efficient gene delivery. *J. Controlled Release* **2012**, *160* (3), 592–600.
- (44) Lee, C. H.; Kasala, D.; Na, Y.; Lee, M. S.; Kim, S. W.; Jeong, J. H.; Yun, C. O. Enhanced therapeutic efficacy of an adenovirus-PEI-bile-acid complex in tumors with low coxsackie and adenovirus receptor expression. *Biomaterials* **2014**, *35* (21), 5505–16.
- (45) Matsumoto, K.; Shariat, S. F.; Ayala, G. E.; Rauen, K. A.; Lerner, S. P. Loss of coxsackie and adenovirus receptor expression is associated with features of aggressive bladder cancer. *Urology* **2005**, *66* (2), 441–6.
- (46) Sachs, M. D.; Rauen, K. A.; Ramamurthy, M.; Dodson, J. L.; De Marzo, A. M.; Putzi, M. J.; Schoenberg, M. P.; Rodriguez, R. Integrin alpha(v) and coxsackie adenovirus receptor expression in clinical bladder cancer. *Urology* **2002**, *60* (3), 531–6.
- (47) Kim, P. H.; Kim, T. I.; Yockman, J. W.; Kim, S. W.; Yun, C. O. The effect of surface modification of adenovirus with an arginine-grafted bioreducible polymer on transduction efficiency and immunogenicity in cancer gene therapy. *Biomaterials* **2010**, *31* (7), 1865–74.
- (48) Abdallah, B.; Hassan, A.; Benoist, C.; Goula, D.; Behr, J. P.; Demeneix, B. A. A powerful nonviral vector for in vivo gene transfer into the adult mammalian brain: Polyethylenimine. *Hum. Gene Ther.* **1996**, *7* (16), 1947–54.
- (49) Tang, M. X.; Redemann, C. T.; Szoka, F. C., Jr. In vitro gene delivery by degraded polyamidoamine dendrimers. *Bioconjugate Chem.* **1996**, *7* (6), 703–14.
- (50) Intra, J.; Salem, A. K. Characterization of the transgene expression generated by branched and linear polyethylenimine-plasmid DNA nanoparticles in vitro and after intraperitoneal injection in vivo. *J. Controlled Release* **2008**, *130* (2), 129–38.
- (51) Wightman, L.; Kircheis, R.; Rossler, V.; Carotta, S.; Ruzicka, R.; Kursa, M.; Wagner, E. Different behavior of branched and linear polyethylenimine for gene delivery in vitro and in vivo. *J. Gene Med.* **2001**, *3* (4), 362–72.
- (52) Godbey, W. T.; Wu, K. K.; Mikos, A. G. Poly(ethylenimine) and its role in gene delivery. *J. Controlled Release* **1999**, *60* (2–3), 149–60.
- (53) Kim, J.; Cho, J. Y.; Kim, J. H.; Jung, K. C.; Yun, C. O. Evaluation of E1B gene-attenuated replicating adenoviruses for cancer gene therapy. *Cancer Gene Ther.* **2002**, *9* (9), 725–36.
- (54) ter Brake, O.; Konstantinova, P.; Ceylan, M.; Berkhout, B. Silencing of HIV-1 with RNA interference: A multiple shRNA approach. *Mol. Ther.* **2006**, *14* (6), 883–92.
- (55) Abounader, R.; Lal, B.; Luddy, C.; Koe, G.; Davidson, B.; Rosen, E. M.; Laterra, J. In vivo targeting of SF/HGF and c-met expression via U1snRNA/ribozymes inhibits glioma growth and angiogenesis and promotes apoptosis. *FASEB J.* **2002**, *16* (1), 108–10.
- (56) Kim, J.; Nam, H. Y.; Kim, T. I.; Kim, P. H.; Ryu, J.; Yun, C. O.; Kim, S. W. Active targeting of RGD-conjugated bioreducible polymer for delivery of oncolytic adenovirus expressing shRNA against IL-8 mRNA. *Biomaterials* **2011**, *32* (22), 5158–66.
- (57) Cantelmo, A. R.; Cammarota, R.; Noonan, D. M.; Focaccetti, C.; Comoglio, P. M.; Prat, M.; Albini, A. Cell delivery of Met docking site peptides inhibit angiogenesis and vascular tumor growth. *Oncogene* **2010**, *29* (38), 5286–98.
- (58) Choi, J. W.; Lee, J. S.; Kim, S. W.; Yun, C. O. Evolution of oncolytic adenovirus for cancer treatment. *Adv. Drug Delivery Rev.* **2012**, *64* (8), 720–9.