articles

molecular pharmaceutics

VEGF siRNA Delivery System Using Arginine-Grafted Bioreducible Poly(disulfide amine)

Sun Hwa Kim,[†] Ji Hoon Jeong,[‡] Tae-il Kim,[†] Sung Wan Kim,[†] and David A. Bull*[,§]

Center for Controlled Chemical Delivery, Department of Pharmaceutics and Pharmaceutical Chemistry, University of Utah, Salt Lake City, Utah 84112, College of Pharmacy, Sungkyunkwan University, Suwon 440−746, South Korea, and Division of Cardiothoracic Surgery, School of Medicine, University of Utah Health Sciences Center, Salt Lake City, Utah 84132

Received September 10, 2008; Revised Manuscript Received November 17, 2008; Accepted November 18, 2008

Abstract: Small interfering RNAs (siRNAs) are able to silence their target genes when they are successfully delivered intact into the cytoplasm. Delivery systems that enhance siRNA localization to the cytoplasm can facilitate gene silencing by siRNA therapeutics. We describe an arginine-conjugated poly(cystaminebisacrylamide-diaminohexane) (poly(CBA-DAH-R)), a bioreducible cationic polymer, as an siRNA carrier for therapeutic gene silencing for cancer. After intracellular uptake of the siRNA/poly(CBA-DAH-R) polyplexes, the reductive environment of the cytoplasm cleaves the disulfide linkages in the polymeric backbone, resulting in decomplexing of the siRNA/poly(CBA-DAH-R) polyplexes and release of siRNA molecules throughout the cytoplasm. The siRNA/poly(CBA-DAH-R) polyplexes, which demonstrate increased membrane permeability with arginine modification, have a similar level of cellular uptake as siRNA/bPEI polyplexes. The VEGF siRNA/poly(CBA-DAH-R) polyplexes, however, inhibit VEGF expression to a greater degree than VEGF siRNA/bPEI in various human cancer cell lines. The improved RNAi activity demonstrated by the VEGF siRNA/poly(CBA-DAH-R) polyplexes is due to enhanced intracellular delivery and effective localization to the cytoplasm of the VEGF siRNAs. These results demonstrate that the VEGF siRNA/poly(CBA-DAH-R) polyplex delivery system may useful for siRNA-based approaches for cancer therapy.

Keywords: Cytoplasmic localization; siRNA; bioreducible cationic polymer; gene silencing; vascular endothelial growth factor; cancer therapy

Introduction

Synthetic siRNAs, 21−23 nucleotides in length, mediate target gene silencing by promoting mRNA degradation in the cytoplasm.[1] Since many diseases are caused by the inappropriate expression of disease-related genes, siRNA technology may provide new therapies for the treatment of diverse human diseases including cancer, obesity, heart disease, and diabetes.[2,3] siRNA therapeutics offer advantages over conventional pharmaceutics because of their powerful and specific gene silencing ability. The practical applications

* Corresponding author. Mailing address: Division of Cardiothoracic Surgery, School of Medicine, University of Utah Health Sciences Center, Salt Lake City, UT 84132. Phone: 801-581-5311. Fax: 801-585-3936. E-mail: david.bull@hsc.utah.edu.

[†] Department of Pharmaceutics and Pharmaceutical Chemistry, University of Utah.

[‡] Sungkyunkwan University.

[§] University of Utah Health Sciences Center.

(1) Hammond, S. M.; Caudy, A. A.; Hannon, G. J. Post-transcriptional gene silencing by double-stranded RNA. *Nat. Rev. Genet.* **2001**, *2*, 110–119.

(2) McManus, M. T.; Haines, B. B.; Dillon, C. P.; Whitehurst, C. E.; Van Parijs, L.; Chen, J.; Sharp, P. A. Gene silencing in mammals by small interfering RNAs. *Nat. Rev. Genet.* **2002**, *3*, 737–747.

(3) Dorsett, Y.; Tuschl, T. siRNAs: applications in functional genomics and potential as therapeutics. *Nat. Rev. Drug Discovery* **2004**, *3*, 318–329.

10.1021/mp800161e CCC: $40.75
Published on Web 12/04/2008

of siRNAs, however, have been limited by their instability and poor delivery to the necessary site of action.[4] The development of safe and effective delivery vehicles for siRNAs would greatly enhance the clinical utility of gene silencing siRNAs as a therapeutic modality for the treatment of cancer.

Various polycations have been developed as nonviral siRNA carriers to enhance the gene silencing activity of siRNA therapeutics.[5,6] Polycations can form highly stable polyelectrolyte complexes with siRNA molecules via electrostatic interactions between the positively charged polycations and the negatively charged phosphates of the nucleic acids. These polyelectrolyte formulations have been shown to improve the protection of siRNA against enzymatic degradation and increase the cellular uptake efficiency of siRNA.[7,8] The latter is important as sufficient intact, functional siRNA must be localized in the cytoplasm to achieve an effective intracellular concentration of siRNA to silence the target mRNA.[9]

Bioreducible cationic polymers containing disulfide linkages arranged regularly on the polymer backbone structure have been reported to be efficient gene delivery vehicles with minimal cellular toxicity.[10,11] The stable polyelectrolyte complexes composed of bioreducible cationic polymers and nucleic acids are readily degraded upon exposure to the reductive environment of the cytoplasm.[12,13] This unique characteristic of bioreducible cationic polymers leads to the release within the cytoplasm of nucleic acids (pDNA and siRNA). Unlike DNA transfection, delivery to the nucleus is not required for RNAi induction, due to the activity of siRNA in the cytoplasm. Therefore, polycations which are cleaved upon exposure to a reductive intracellular state offer the potential for controlled degradation in the cytoplasm, resulting in safe, high-efficiency siRNA delivery.

We have recently described several branched/linear bioreducible cationic polymers, composed of low molecular weight cationic monomers and polymerized with disulfide linkages, as promising gene delivery vehicles.[14−16] The bioreducible cationic polymers form stable, nanosized polyplexes with pDNA and siRNA and achieve higher transfection efficiencies with significantly less cytotoxicity compared to the conventional cationic polymer: branched polyethylenimine (bPEI).

To enhance the efficacy of therapeutic gene silencing, we synthesized an arginine-modified polydisulfide poly(CBA-DAH-R) as an siRNA carrier for cancer gene therapy. Arginine residues are reported to enhance the cellular membrane permeability of many biologically active molecules, including drugs, peptides, and plasmids.[17−19] The reductive degradation of the siRNA/poly(CBA-DAH-R) polyplexes was estimated from physicochemical measurements via dynamic light scattering and gel retardation analysis. The triggered release characteristics of the siRNA/poly(CBA-DAH-R) polyplexes in correlation with cytoplasmic siRNA localization and gene knockdown efficiency were investigated in human prostate carcinoma (PC-3 cells). To verify that poly(CBA-DAH-R) could be a potential siRNA

(4) Oishi, M.; Nagasaki, Y.; Itaka, K.; Nishiyama, N.; Kataoka, K. Lactosylated poly(ethylene glycol)-siRNA conjugate through acid-labile beta-thiopropionate linkage to construct pH-sensitive polyion complex micelles achieving enhanced gene silencing in hepatoma cells. *J. Am. Chem. Soc.* **2005**, *127*, 1624–1625.

(5) Li, S. D.; Huang, L. Targeted delivery of antisense oligodeoxynucleotide and small interference RNA into lung cancer cells. *Mol. Pharmaceutics* **2006**, *3*, 579–588.

(6) Lee, S. H.; Choi, S. H.; Kim, S. H.; Park, T. G. Thermally sensitive cationic polymer nanocapsules for specific cytosolic delivery and efficient gene silencing of siRNA: swelling induced physical disruption of endosome by cold shock. *J. Controlled Release* **2008**, *125*, 25–32.

(7) Zhang, S.; Zhao, B.; Jiang, H.; Wang, B.; Ma, B. Cationic lipids and polymers mediated vectors for delivery of siRNA. *J. Controlled Release* **2007**, *123*, 1–10.

(8) Kim, S. H.; Jeong, J. H.; Lee, S. H.; Kim, S. W.; Park, T. G. PEG conjugated VEGF siRNA for anti-angiogenic gene therapy. *J. Controlled Release* **2006**, *116*, 123–129.

(9) Lavigne, C.; Thierry, A. R. Specific subcellular localization of siRNAs delivered by lipoplex in MCR-7 breast cancer cells. *Biochimie* **2007**, *89*, 1245–1251.

(10) Gosselin, M. A.; Guo, W.; Lee, R. J. Efficient gene transfer using reversibly cross-linked low molecular weight polyethylenimine. *Bioconjugate Chem.* **2001**, *12*, 989–994.

(11) Yockman, J. W.; Kastenmeier, A.; Erickson, H. M.; Brumbach, J. G.; Whitten, M. G.; Albanil, A.; Li, D. Y.; Kim, S. W.; Bull, D. A. Novel polymer carriers and gene constructs for treatment of myocardial ischemia and infarction. *J. Controlled Release* (in press, available online 6 July 2008).

(12) Jeong, J. H.; Christensen, L. V.; Yockman, J. W.; Zhong, Z.; Engbersen, J. F.; Kim, W. J.; Feijen, J.; Kim, S. W. Reducible poly(amido ethylenimine) directed to enhance RNA interference. *Biomaterials* **2007**, *28*, 1912–1917.

(13) Kim, S. H.; Jeong, J. H.; Ou, M.; Yockman, J. W.; Kim, S. W.; Bull, D. A. Cardiomyocyte-targeted siRNA delivery by prostaglandin E2-Fas siRNA polyplexes formulated with reducible poly (amido amine) for preventing cardiomyocyte apoptosis. *Biomaterials* **2008**, *29*, 4439–4446.

(14) Christensen, L. V.; Chang, C. W.; Kim, W. J.; Kim, S. W.; Zhong, Z.; Lin, C.; Engberson, J. F.; Feijen, J. Reducible poly(amido ethylenimine)s designed for triggered intracellular gene delivery. *Bioconjugate Chem.* **2006**, *17*, 1233–1240.

(15) Christensen, L. V.; Chang, C. W.; Yockman, J. W.; Conners, R.; Jackson, H.; Zhong, Z.; Feijen, J.; Bull, D. A.; Kim, S. W. Reducible poly(amido ethylenediamine) for hypoxia-inducible VEGF delivery. *J. Controlled Release* **2007**, *118*, 254–261.

(16) Ou, M.; Wang, X. L.; Xu, R.; Chang, C. W.; Bull, D. A.; Kim, S. W. Novel biodegradable poly(disulfide amine)s for gene delivery with high efficiency and low cytotoxicity. *Bioconjugate Chem.* **2008**, *19*, 626–633.

(17) Futaki, S.; Goto, S.; Sugiura, Y. Membrane permeability commonly shared among arginine-rich peptides. *J. Mol. Recognit.* **2003**, *16*, 260–264.

(18) Choi, J. S.; Nam, K.; Park, J. Y.; Kim, J. B.; Lee, J. K.; Park, J. S. Enhanced transfection efficiency of PAMAM dendrimer by surface modification with L-arginine. *J. Controlled Release* **2004**, *99*, 445–456.

(19) Kim, W. J.; Christensen, L. V.; Jo, S.; Yockman, J. W.; Jeong, J. H.; Kim, Y. H.; Kim, S. W. Cholesteryl oligoarginine delivering vascular endothelial growth factor siRNA effectively inhibits tumor growth in colon adenocarcinoma. *Mol. Ther.* **2006**, *14*, 343–350.

delivery vehicle for target gene silencing in cancer cells, VEGF gene silencing by the poly(CBA-DAH-R) polyplexes with VEGF-targeted siRNAs was evaluated in four different human cancer cell lines (KB, HeLa, A2780, and A549).

Experimental Section

Materials. Branched polyethylenimine (bPEI, M_w 25,000), dithiothreitol (DTT), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT), and DL-buthionine-sulfoxamine (BSO) were purchased from Sigma-Aldrich (St. Louis, MO). Poly(CBA-DAH-R) was prepared as shown in the supplemental figure in the Supporting Information. Human VEGF siRNA (sense, 5′-GGAGUACCCUGAUGAGAUCdTdT-3′; antisense, 5′-GAUCUCAUCAGGGUACUCCdTdT-3′) and green fluorescent protein (GFP) siRNA (sense, 5′-GCACGACUUCUUCAAGUCCdTdT-3′; antisense, 5′-GGACUUGAAGAAGUCGUGCdTdT-3′) were synthesized, modified and purified by Dharmacon Co. (Lafayette, CO). For flow cytometry and confocal microscopy, siRNA was labeled with Cy3 dye at the 3′-terminal end of the sense strand. All cell culture products including fetal bovine serum (FBS), Roswell Park Memorial Institute 1640 medium (RPMI 1640), and Dulbecco′s phosphate buffered saline (PBS) were obtained from Invitrogen (Gibco BRL, Carlsbad, CA).

Dynamic Light Scattering (DLS) Assay. The siRNA polyplexes with poly(CBA-DAH-R) and bPEI were formed at various weight ratios (polymer/siRNA) ranging from 10:1 to 60:1 and from 0.1:1 to 60:1, respectively. A fixed amount of siRNA (3 μg) was complexed with different amounts of polymers (poly(CBA-DAH-R) or bPEI) in 0.4 mL HEPES buffer (HEPES 20 mM, 5% glucose, pH 7.4). After 30 min incubation at room temperature, the polyplex solution was 6-fold diluted with deionized water. To determine the degradation ability of the siRNA/poly(CBA-DAH-R) polyplexes, the siRNA polyplexes, which were formed with poly(CBA-DAH-R) and bPEI at a weight ratio of 40:1 and 1:1 respectively, were further incubated with 2.5 mM DTT for 2 h at 37 °C. Particle sizes and surface charges were measured using a Zetasizer 3000HS (Malvern Instrument, Inc., Worcestershire, U.K.) at a wavelength of 677 nm with a constant angle of 90 ° at 25 °C.

Electrophoretic Mobility Shift Assay. The siRNA/poly(CBA-DAH-R) polyplexes were prepared at different weight ratios ranging from 0:1 to 40:1. siRNA (0.3 μg) was condensed with varying amounts of poly(CBA-DAH-R) in an aqueous phase (PBS 30 μL, pH 7.4) and incubated at room temperature for 30 min. The siRNA/bPEI polyplexes were formed at a weight ratio of 1:1. Each sample solution was loaded onto a 2.0% agarose gel. Electrophoresis was carried out with a current of 120 V for 15 min in 1 × TAE buffer solution (10 mM Tris/HCl, 1% (v/v) acetic acid, 1 mM EDTA). The retardation of the polyplexes was visualized with an image analyzer equipped with a UV transilluminator (GelDoc, BioRad, Hercules, CA) after ethidium bromide staining. To characterize the cleavability of poly(CBA-DAH-R) under reductive conditions, the polyplexes were preincubated with 2.5 mM DTT for 2 h at 37 °C prior to electrophoresis.

Cells and Cell Culture Conditions. Human prostate carcinoma (PC-3), human oral cavity epidermal carcinoma (KB), human cervical carcinoma (HeLa), human ovarian carcinoma (A2780), and human lung carcinoma (A549) were obtained from American Type Culture Collection (Manassas, VA). Cells were routinely maintained in an RPMI 1640 medium supplemented with 10% fetal bovine serum. Cells were cultured as a monolayer in a humidified atmosphere containing 5% CO_2 at 37 °C. Cells were regularly passaged and reseeded 24 h before transfection experiments.

Cellular Toxicity Assay. Relative cell viability was determined using an MTT (Thiazolyl Blue Tetrazolium Bromide) assay. PC-3 cells were plated in 96-well plates at a density of 1×10^4 cells per well in 0.2 mL of culture medium and incubated for 24 h at 37 °C prior to polyplex treatment. The medium was replaced by a fresh serum-free transfection medium containing a desired amount of the siRNA polyplexes with poly(CBA-DAH), poly(CBA-DAH-R), and bPEI, which were formed at different weight ratios from 0:1 to 60:1. The polyplexes were prepared using 0.15 μg of siRNA and varying amounts of the polymers in 0.1 mL of PBS (pH 7.4). Cells were transfected by the polyplexes for 4 h at 37 °C and further incubated in a fresh serum-containing medium for 24 h at 37 °C. Fifty microliters of MTT solution (2 mg/mL) were added and incubated at 37 °C for 4 h. The produced formazan crystals were dissolved in 0.2 mL of DMSO followed by plate reading at 530 nm in a microplate reader (Bio-Rad 680, Hercules, CA). The cell viability was determined relative to the untreated control cells.

Flow Cytometry and Confocal Microscopy. The cellular uptake of the siRNA polyplexes was examined with flow cytometry and confocal microscopy by using Cy3 dye-labeled siRNA. The siRNA polyplexes with poly(CBA-DAH), poly(CBA-DAH-R), and bPEI were used at a weight ratio of 40:1, 40:1, and 1:1, respectively. At the chosen weight ratios of polymer to siRNA, the corresponding calculated charge ratios for poly(CBA-DAH), poly(CBA-DAH-R), and bPEI were 43:1, 25:1, and 8:1, respectively. PC-3 cells were plated in 6-well plates at an initial density of 2.5×10^5 cells per well for flow cytometry analysis. The siRNA polyplexes were formed with 0.75 μg of Cy3-siRNA and a desired amount of the polymers in an aqueous buffer solution (PBS 0.1 mL, pH 7.4) for 15 min at room temperature. The Cy3-siRNA formulations, including naked siRNA, siRNA/poly(CBA-DAH), siRNA/poly(CBA-DAH-R), and siRNA/bPEI polyplexes, were added to the wells containing a serum-free transfection medium. Prior studies in our laboratory had demonstrated that the transfection efficiencies for the Cy3-siRNA formulations did not differ between 4, 8, 12 and 24 h of incubation, and so incubation times of 4 h for the Cy3-siRNA formulations were used throughout the experiments. After 4 h incubation, cells were washed four times with cold PBS, harvested by trypsin digestion, and fixed in 75% EtOH solution for 30 min at 4

°C. Cells were analyzed on a flow cytometer (FACS Caliber, Becton-Dickinson, Mountain View, CA) using FL2 channel (Ex. 488 nm/Em. 575 nm). Data were processed using Windows Multiple Document interface software (WinMDI).

For confocal microscopy, PC-3 cells were seeded in confocal imaging dishes (Glass Bottom microwells, MatTek Corp., Ashland, MA) at a density of 1×10^4 cells per well. Cells were preincubated in the presence or absence of 10 mM DL-buthionine-sulfoxamine (BSO), which causes a significant decrease in the intracellular level of reduced glutathione (GSH), for 24 h prior to transfection. The siRNA polyplexes, which were prepared by the same method described above, were transfected for 2 h at 37 °C. The transfection medium was replaced with a fresh culture medium and the cells were incubated for a further 3 h at 37 °C. The cells were washed with cold PBS four times and fixed with 0.5% para-formaldehyde solution for 30 min at 4 °C. The localization of Cy3-labeled siRNA within the cells was visualized by a confocal laser scanning microscope (Olympus Fluoview FV300, Melville, NY) with a 100× oil-immersion objective lens using an argon/krypton mixed gas laser (Ex. 568 nm). Three-dimensional confocal images for the cells were constructed by using Velocity software (Improvision Inc., Lexington, MA).

In Vitro Transfection. For in vitro transfection studies, 2.5×10^5 cells (PC-3, KB, HeLa, A2780, and A549) per well were plated in a 6-well plate. After 24 h incubation, the culture medium was replaced by the transfection medium with or without 10% FBS prior to transfection. To decrease the intracellular reduction potential, cells were preincubated in the presence or absence of 10 mM BSO at 37 °C for 24 h prior to transfection. VEGF siRNA (1.5 μg) was condensed with a desired amount of the polymers in 0.2 mL of PBS. The VEGF siRNA polyplexes were left at an ambient temperature for 30 min and added into the transfection medium. After 4 h transfection at 37 °C, the transfection medium was replaced with a fresh medium containing 10% FBS and continuously incubated for a further 8 h. The medium was removed and supplemented with fresh culture medium containing heparin (20 μg/mL). After 18 h incubation, the medium was collected for VEGF ELISA assay. The amount of VEGF secreted from the cells was determined using a human VEGF immunoassay kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. All of the data are presented as the mean ± standard deviation (SD) of three independent measurements. Statistical analysis was carried out by a Student's t test. Statistical significance was assigned for p values <0.05.

Results and Discussion

Nonviral vector delivery of synthetic siRNAs can specifically silence target genes by inducing mRNA degradation within the cytoplasm.[20,21] Unlike DNA delivery systems, which require nuclear localization, synthetic siRNA delivery systems demand localization to the cytoplasm for RNAi induction. Localization to the cytoplasm of functional siRNA molecules is an important step to achieve efficient target gene silencing. Most cationic polymers for gene delivery, however, condense strongly with nucleic acids and form highly stable polyplexes in both the extra- and intracellular environment, even after endosomal escape to the cytoplasm.[12] Thus, we hypothesized that bioreducible cationic polymers which degrade in the reductive environment of the cytoplasm would be more likely to release siRNAs into the cytoplasm, thereby resulting in enhanced RNA silencing compared to the commercially available cationic polymer polyethylenimine, bPEI (25 kDa).

We chose to compare our bioreducible cationic polymer, arginine-conjugated poly (cystaminebisacrylamide-diaminohexane), to bPEI for several reasons. First, bPEI is considered one of the most successful nonviral gene carriers and is used extensively for in vitro as well as for in vivo gene delivery due to its high transfection efficiency and reproducibility. Second, bPEI possesses high endosomal buffering capacity, which facilitates the endosomal escape of the complexes via the hypothetical proton sponge effect. Third, while several cholesterol based cationic lipids have been generated as gene carriers and have demonstrated high transfection efficiency in mammalian cells, most have lacked an effective endosomal escape mechanism such as that found in bPEI. For all these reasons, as well as its wide commercial availability, bPEI is still accepted as the gold standard for nonviral, polymer based gene delivery.

siRNA/poly(CBA-DAH-R) Polyplexes Are Degraded under Reductive Conditions. To develop an efficient siRNA delivery system, we designed a new bioreducible cationic polymer poly(CBA-DAH-R) containing multiple disulfide linkages in the arginine-modified polymeric backbone. Poly(CBA-DAH-R) was prepared by arginine modification after the synthesis of poly(CBA-DAH) (supplemental figure in the Supporting Information). The polydisulfide poly(CBA-DAH) was synthesized from the copolymerization of amine donor 1,6-diaminohexane (DAH) to reducible cystaminebisacrylamide (CBA). Then the unprotected terminal amine group in the backbone of poly(CBA-DAH) was fully modified with L-arginine (R) to produce poly(CBA-DAH-R). The siRNA polyplex formation and the reductive degradation of poly(CBA-DAH-R) were assessed by dynamic light scattering (DLS) measurements (Figure 1). Poly(CBA-DAH-R) and bPEI were complexed with siRNA molecules at 200 nm in

(20) Kim, H. R.; Kim, I. K.; Bae, K. H.; Lee, S. H.; Lee, Y.; Park, T. G. Cationic solid lipid nanoparticles reconstituted from low density lipoprotein components for delivery of siRNA. *Mol. Pharmaceutics* **2008**, *5*, 622–631.

(21) Read, M. L.; Singh, S.; Ahmed, Z.; Stevenson, M.; Briggs, S. S.; Oupicky, D.; Barrett, L. B.; Spice, R.; Kendall, M.; Berry, M.; Preece, J. A.; Logan, A.; Seymour, L. W. A versatile reducible polycation-based system for efficient delivery of a broad range of nucleic acids. *Nucleic Acids Res.* **2005**, *33*, e86.

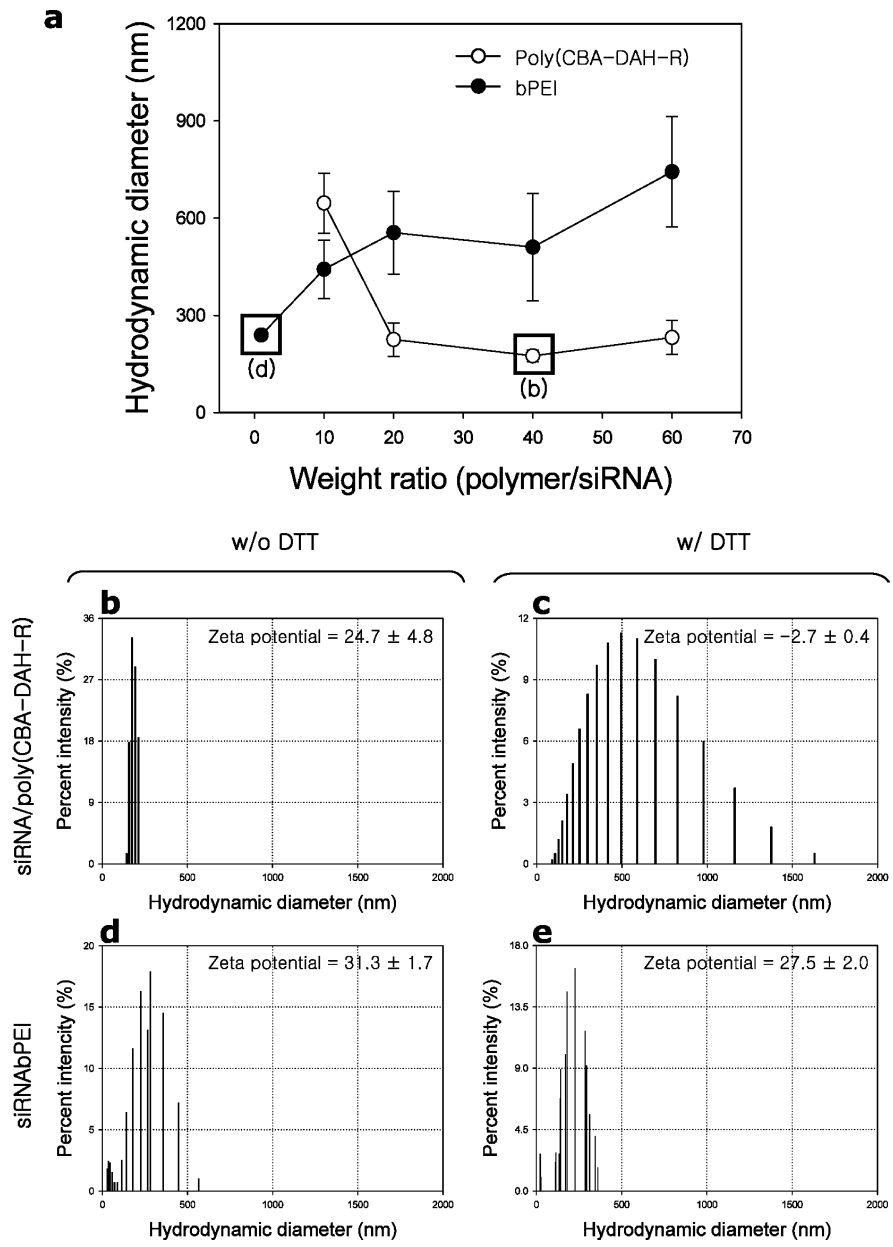

Figure 1. Particle sizes and surface charges of siRNA/poly(CBA-DAH-R) and siRNA/bPEI polyplexes. (a) Hydrodynamic diameters of the polyplexes at various weight ratios. Representative size distribution diagrams and zeta potentials of siRNA/poly(CBA-DAH-R) (b, c) and siRNA/bPEI (d, e) polyplexes at a weight ratio of 40:1 and 1:1, respectively. In panels (c, e) and (b, d), the polyplexes were treated with and without 2.5 mM DTT, respectively.

diameter with a weight ratio of 40:1 and 1:1, respectively. Poly(CBA-DAH-R) started to condense siRNA at a weight ratio of 10:1, though the polyplexes were not stabilized until a weight ratio of 20:1. The siRNA/poly(CBA-DAH-R) polyplexes were stably formed at a few hundred nanometers in size with weight ratios above 20:1. The bioreducible polycation poly(CBA-DAH-R) spontaneously formed stable nanosized polyplexes with siRNA molecules due to the electrostatic interactions between the positively charged side-chain arginine groups of the polymer and the negatively charged phosphate groups of the siRNAs. DLS analysis showed that the particle size of the siRNA/bPEI polyplexes was dramatically increased with an increase in the weight ratio, due to significant particle aggregation of highly positive charged polyplexes (zeta potential over +35 mV). It has been reported that strongly charged particles are rapidly aggregated under physiologic conditions.[22] To examine whether the siRNA/poly(CBA-DAH-R) polyplexes degrade and release free siRNAs under reductive conditions, the siRNA polyplexes with poly(CBA-DAH-R) and bPEI were preincubated in 2.5 mM DTT solution and the polyplexes were analyzed by DLS. There was no detectable difference in particle size and surface charge for the siRNA/bPEI polyplexes with and

(22) Wang, Y.; Chen, P.; Shen, J. A facile entrapment approach to construct PEGylated polyplexes for improving stability in physiological condition. *Colloids Surf., B* **2007**, *58*, 188–196.

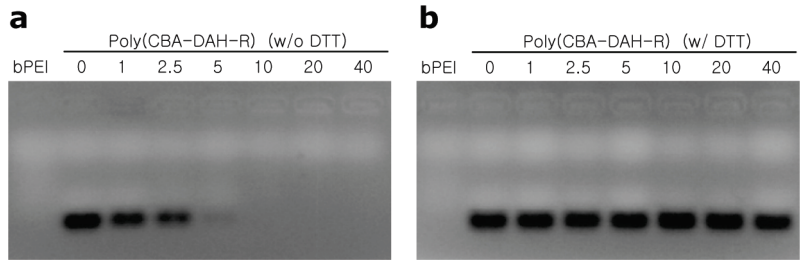

Figure 2. Gel retardation assay of siRNA/poly(CBA-DAH-R) polyplexes at various weight ratios without DTT treatment (a) and with 2.5 mM DTT treatment (b). siRNA/bPEI polyplexes were formulated at a weight ratio of 1:1.

without DTT treatment (Figure 1b, c). In a reductive environment, however, the size distribution peaks for the siRNA/poly(CBA-DAH-R) polyplexes at 200 nm particle size disappeared and the zeta potential was almost neutral (-2.7 ± 0.4 mV) (Figure 1d, e), suggesting the polyplexes were completely degraded due to the reductive cleavage of the multiple disulfide linkages in the poly(CBA-DAH-R).

The unique structural characteristics of poly(CBA-DAH-R) were further confirmed in the electrophoretic mobility shift assay (Figure 2). Poly(CBA-DAH-R) fully condensed siRNA molecules at weight ratios above 20:1, correlating with the particle formation data determined by DLS analysis (Figure 1). A reductive environment caused complete siRNA release from the siRNA/poly(CBA-DAH-R) polyplexes as a result of degradation of the polymers, while the structure of the siRNA/bPEI polyplexes was not affected by a reductive environment. Previous studies have shown that polydisulfide polycations exhibit efficient unpackaging of the polyplexes by the cleavage of disulfide bonds in reductive conditions, which is responsible for the release of nucleic acids.[16−18] The triggered degradation behavior of the siRNA/poly(CBA-DAH-R) polyplexes increases RNA silencing efficiency by improving the localization of the siRNA to the cytoplasm.

siRNA/poly(CBA-DAH-R) Polyplexes Decrease Cytotoxicity and Enhance Transfection Efficiency. To assess the efficacy of poly(CBA-DAH-R) as an siRNA carrier in vitro, the cytotoxic activity of the siRNA/poly(CBA-DAH-R) polyplexes against PC-3 cells was evaluated by MTT assay (Figure 3a). The siRNA/poly(CBA-DAH-R) polyplexes had much lower cytotoxicity than the bPEI formulations. Poly(CBA-DAH-R) exhibited nearly 100% relative cell viability at a weight ratio up to 40:1, while bPEI showed only 40% relative cell viability at a weight ratio near 10:1. There was no meaningful difference in cellular toxicity between the unmodified and the arginine-modified reducible polymers, suggesting that the arginine modification does not affect the biocompatibility of bioreducible cationic polymers. The cytotoxicity of polycations is caused by an increase in molecular weight as well as cationic charge density.[23] The cytotoxicity of higher molecular weight polycations, however, decreases with breakdown into lower molecular weight components.[24] Thus, the improved cell viability of poly(CBA-DAH-R) may be attributable to its biodegradability. Further in vitro studies conducted at nontoxic levels of bPEI, poly(CBA-DAH), and poly(CBA-DAH-R) formulations displayed over 90% cell viability with weight ratios under 1:1, 40:1, and 40:1, respectively.

To investigate the influence of the arginine modification of poly(CBA-DAH-R) on transfection efficiency, the cellular uptake of fluorescently labeled siRNA formulations by PC-3 cells was monitored using flow cytometric analysis (Figure 3b). Most of the control and the naked siRNA treatment groups showed very low fluorescence intensity. However, the fluorescence peaks for the cells treated with various polyplex formulations were notably shifted to the right. The percentage of the cells gated from region M for the siRNA/poly(CBA-DAH), siRNA/poly(CBA-DAH-R), and siRNA/bPEI polyplexes was 57.0%, 81.0% and 88.2%, respectively. The extent of cellular uptake of the siRNA/poly(CBA-DAH-R) polyplexes was 1.4-fold greater than that of the siRNA/poly(CBA-DAH) polyplexes. There was no significant difference, however, between poly(CBA-DAH-R) and bPEI in the cellular uptake of the polyplexes. The enhanced delivery of the siRNA/poly(CBA-DAH-R) polyplexes is likely due to the arginine modification of the polydisulfide cationic polymers. Arginine residues, which are rich in membrane translocalization peptides, help enhance the cellular association and membrane permeability of many biologically active products.[19]

To assess the potential efficacy of siRNA therapy for cancer, the RNAi activity of the siRNA/poly(CBA-DAH-R) polyplexes was evaluated in human prostate carcinoma PC-3 cells using siRNA targeted against VEGF. VEGF has a predominant role in tumor angiogenesis (Figure 3c).[25,26] The VEGF siRNA polyplex formulations silenced VEGF gene expression in a sequence specific manner. Both the

(23) Fischer, D.; Li, Y.; Ahlemeyer, B.; Krieglstein, J.; Kissel, T. In vitro cytotoxicity testing of polycations: influence of polymer structure on cell viability and hemolysis. *Biomaterials* **2003**, *24*, 1121–1131.

(24) Kim, Y. H.; Park, J. H.; Lee, M.; Kim, Y. H.; Park, T. G.; Kim, S. W. Polyethylenimine with acid-labile linkages as a biodegradable gene carrier. *J. Controlled Release* **2005**, *103*, 209–219.

(25) Kim, S. H.; Jeong, J. H.; Lee, S. H.; Kim, S. W.; Park, T. G. Local and systemic delivery of VEGF siRNA using polyelectrolyte complex micelles for effective treatment of cancer. *J. Controlled Release* **2008**, *129*, 107–116.

(26) Hicklin, D. J.; Ellis, L. M. Role of the vascular endothelial growth factor pathway in tumor growth and angiogenesis. *J. Clin. Oncol.* **2005**, *23*, 1011–1027.

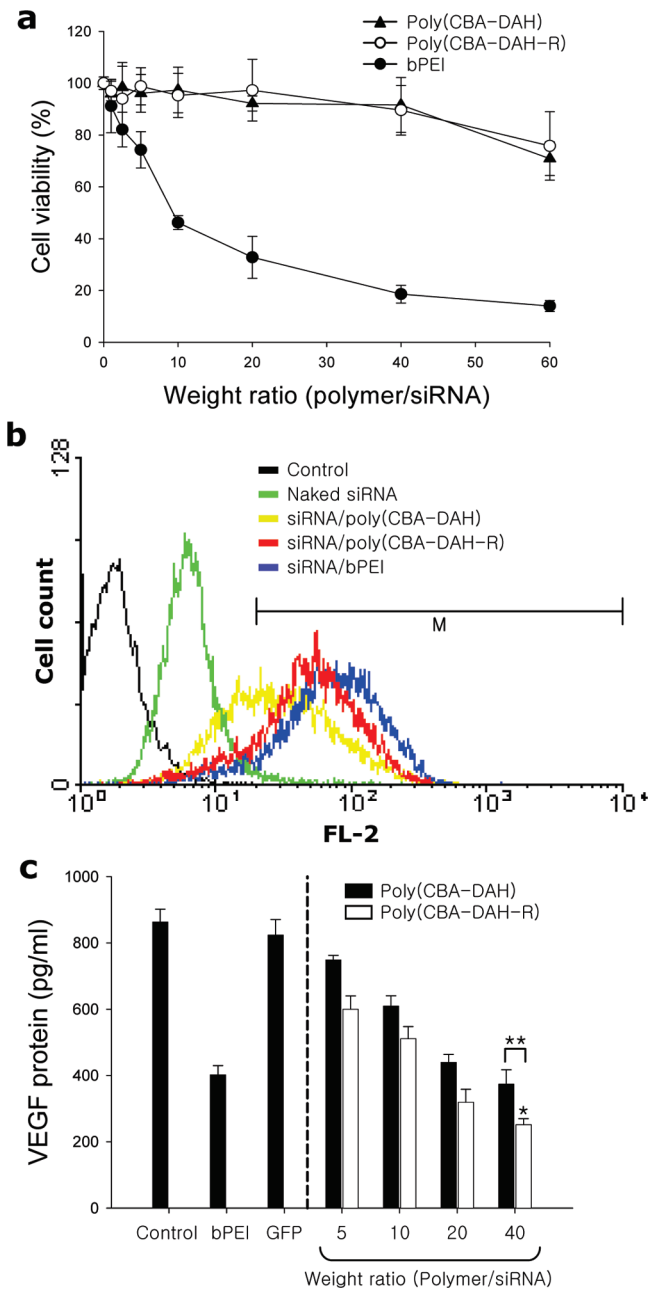

Figure 3. (a) Cellular toxicity assay of poly(CBA-DAH), poly(CBA-DAH-R), and bPEI polyplexes with siRNA as a function of weight ratio. (b) Representative flow cytometry histograms of PC-3 cells transfected with naked siRNA (green), siRNA/poly(CBA-DAH) (yellow), siRNA/poly(CBA-DAH-R) (red), and siRNA/bPEI (blue). Cy3-modified siRNA was used. M presents a gated region (fluorescence intensity (arbitrary unit): 350 – 10,000). (c) VEGF gene silencing of VEGF siRNA/poly(CBA-DAH-R) polyplexes at various weight ratios. PC-3 cells were transfected with bPEI (weight ratio 1:1), poly(CBA-DAH), and poly(CBA-DAH-R) formulations with VEGF siRNA. GFP siRNA/bPEI polyplexes were used as a control. Statistical significance, * = $p < 0.05$ versus bPEI, ** = $p < 0.05$ versus poly(CBA-DAH). The VEGF expression was analyzed using an ELISA for human VEGF.

siRNA polyplexes with poly(CBA-DAH) and poly(CBA-DAH-R) decreased VEGF expression. As expected, the arginine-containing polyplexes showed consistently greater gene silencing efficiency than the unmodified polyplexes, presumably resulting from the enhanced cellular uptake of the siRNA/poly(CBA-DAH-R) polyplexes. The VEGF siRNA/poly(CBA-DAH-R) polyplexes formulated at a weight ratio of 40:1 exhibited prominent VEGF silencing activity, compared to the VEGF siRNA/poly(CBA-DAH) and VEGF siRNA/bPEI (a weight ratio of 1:1) polyplexes. Meanwhile, the VEGF siRNA/poly(CBA-DAH) polyplexes demonstrated a similar level of VEGF silencing as the bPEI formulations, though the siRNA/poly(CBA-DAH) polyplexes showed a lower level of cellular association than the siRNA/bPEI polyplexes (Figure 3b). These results imply that high-efficiency uptake of siRNA polyplexes does not necessarily guarantee the RNAi activity of siRNA therapeutics. In addition to enhancing the cellular delivery with arginine modification, the unique structural characteristic of poly(CBA-DAH-R) appears to exert considerably more influence in siRNA-mediated gene silencing compared to other polymer carriers.

Localization to the Cytoplasm of siRNA Molecules by Poly(CBA-DAH-R) Polyplexes Enables Strong RNAi Induction. To verify the hypothesis that reductive degradation of the siRNA/poly(CBA-DAH-R) polyplexes leads to improved RNA silencing, confocal microscopic observations on subcellular localization of fluorescein binding siRNA were carried out using PC-3 cells (Figure 4). In the cells transfected by the siRNA/poly(CBA-DAH-R) polyplexes, siRNA was evenly distributed throughout the cytoplasm. However, the cytoplasmic localization of siRNA was fully suppressed with the treatment of a glutathione depleting agent, BSO, which is known to inhibit intracellular reducing potential.[27] These findings demonstrate that intact siRNA molecules were successfully released from the destabilized polyplexes due to the degradation of the polymer backbones in the reductive environment of the cytoplasm. In contrast, small fluorescent spots were observed throughout the cells when transfected with the siRNA/bPEI polyplexes, irrespective of the presence or absence of BSO, suggesting that most of the siRNA molecules were still complexed in the large bPEI polyplex aggregates due to the limited degradation of the bPEI polyplexes and release of siRNA at the cellular level. These observations indicate that the triggered release of siRNA from the siRNA/poly(CBA-DAH-R) polyplexes could play a critical role in enhancing RNA silencing. To further confirm this hypothesis, the VEGF gene silencing experiments in the PC-3 cells were conducted under both reducing and nonreducing conditions (Figure 5a). When the VEGF siRNA/poly(CBA-DAH-R) polyplexes were transfected in the presence of 10 mM BSO, VEGF expression was significantly higher, while the bPEI polyplexes exhibited

(27) Shalini, S.; Bansal, M. P. Co-operative effect of glutathione depletion and selenium induced oxidative stress on API and NFkB expression in testicular cells in vitro: insights to regulation of spermatogenesis. *Biol. Res.* **2007**, *40*, 307–317.

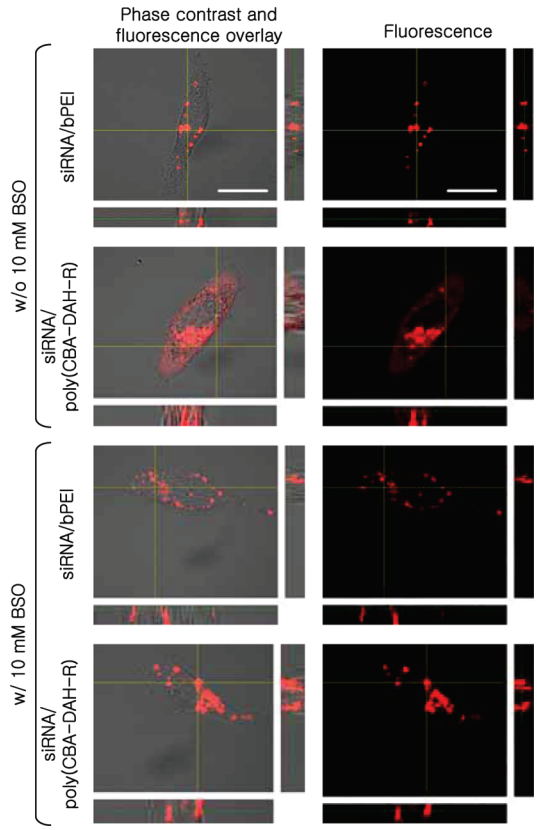

Figure 4. Subcellular confocal images of cytoplasmic siRNA release of siRNA/poly(CBA-DAH-R) and siRNA/bPEI polyplexes with and without glutathione-depleting agent BSO. Cy3-modified siRNA was used. Scale bars represent 10 μm.

the same level of RNAi activity against VEGF expression in both BSO treated and BSO untreated cells. These results indicate that the biodegradability of poly(CBA-DAH-R) increases the localization of siRNA to the cytoplasm.

VEGF siRNA/poly(CBA-DAH-R) Polyplex Delivery System for the Treatment of Various Human Carcinomas. The influence of serum proteins upon the VEGF gene silencing activity of the VEGF siRNA/poly(CBA-DAH-R) polyplexes was evaluated by conducting the transfection under 10% FBS conditions (Figure 5b). Although both poly(CBA-DAH-R) and bPEI showed approximately a 2-fold reduction in RNAi activity in the presence of serum proteins, the VEGF siRNA/poly(CBA-DAH-R) polyplexes still exhibited a higher level of VEGF inhibition compared to the VEGF siRNA/bPEI polyplexes. These results suggest that the VEGF siRNA/poly(CBA-DAH-R) polyplex delivery system could be a potential approach for siRNA-based cancer therapy.

To make the VEGF siRNA/poly(CBA-DAH-R) formulation applicable to a wide range of tumor types, the VEGF gene silencing activity of the VEGF siRNA/poly(CBA-DAH-R) polyplexes was further assessed in various human cancer cells including oral (KB), cervical (HeLa), ovarian (A2780), and lung (A549) in addition to the prostate carcinoma PC-3 cells (Figure 6). The cancer cell lines manifested different levels of VEGF production and VEGF inhibition by the

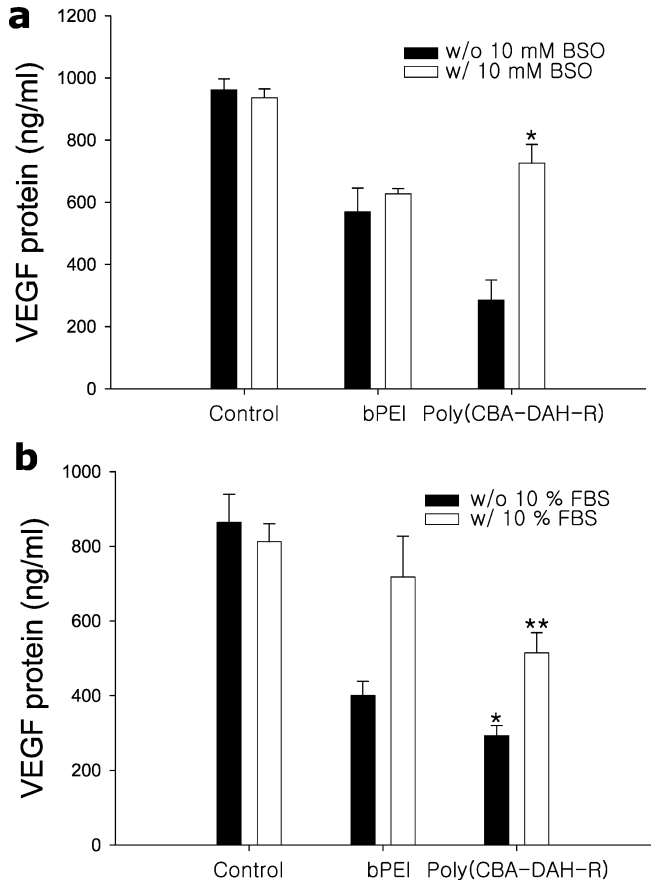

Figure 5. (a) Effect of glutathione-depleting agent BSO on VEGF gene silencing of VEGF siRNA/poly-(CBA-DAH-R) polyplexes in PC-3 cells. Cells were pretreated with or without 10 mM BSO for 24 h prior to transfection. Statistical significance, * = $p < 0.01$ versus w/o 10 mM BSO. (b) Effect of serum proteins on VEGF gene silencing of VEGF siRNA/poly(CBA-DAH-R) polyplexes in PC-3 cells. Cells were transfected in the media with or without 10% FBS. Statistical significance, * = $p < 0.05$ versus bPEI (w/o 10% FBS), ** = $p < 0.01$ versus bPEI (w/ 10% FBS). The amount of VEGF secreted from the cells was determined using an ELISA for human VEGF.

VEGF siRNA formulations. It is known that cancer cells upregulate VEGF expression, but the degree of upregulation varies, depending on the cancer cell type.[26,28] The differences in VEGF gene silencing of the VEGF siRNA/poly(CBA-DAH-R) polyplexes in varied cancer cell types are probably determined by differences in the levels of intracellular reducing potential, since different cells have different capacities for glutathione synthesis to maintain the reductive environment of the cytoplasm.[29] Poly(CBA-DAH-R) invari-

(28) Roberts, W. G.; Delaat, J.; Nagane, M.; Huang, S.; Cavenee, W. K.; Palade, G. E. Host microvasculature influence on tumor vascular morphology and endothelial gene expression. *Am. J. Pathol.* **1998**, *153*, 1239–1248.

(29) Wang, W.; Ballatori, N. Endogenous glutathione conjugates: occurrence and biological functions. *Pharmacol. Rev.* **1998**, *50*, 335–356.

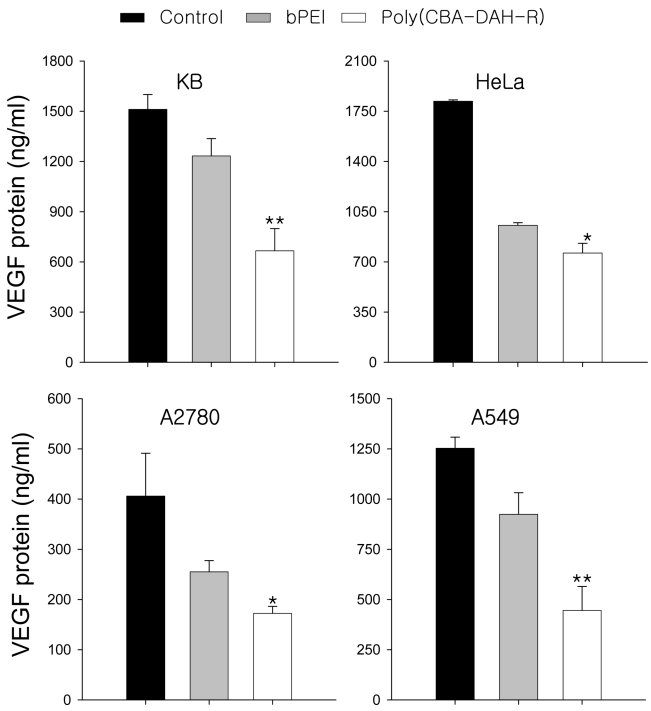

Figure 6. VEGF gene silencing of VEGF siRNA/poly(CBA-DAH-R) polyplexes in various human cancer cells. Statistically significant difference from bPEI, * = $p < 0.05$, ** = $p < 0.01$. The VEGF expression level was determined using an ELISA for human VEGF.

ably exhibited higher RNAi activity than bPEI in all cancer cell lines tested, suggesting that the VEGF siRNA/poly(CBA-DAH-R) polyplex delivery system could be useful in the treatment of various types of human cancers. To validate the current in vitro study, we are currently conducting an in vivo study using a human tumor xenograft model, which we anticipate reporting separately in the near future.

In conclusion, we have demonstrated a new approach for RNAi gene silencing using a VEGF siRNA/poly(CBA-DAH-R) polyplex delivery system. The siRNA/poly(CBA-DAH-R) polyplexes successfully localize siRNA to the cytoplasm due to the reductive degradation of the polymers. In addition to the feature of triggered release, arginine modification of poly(CBA-DAH-R) enhances cellular permeability, leading to effective down regulation of VEGF expression in various human cancer cells. Since the siRNA/poly(CBA-DAH-R) polyplex formulation has low cytotoxicity, high efficiency in target gene silencing, and broad efficacy in various cell types, the siRNA/poly(CBA-DAH-R) polyplex offers a broad range of potential applications for delivering therapeutic siRNAs, including cancer, obesity, heart disease, and diabetes.

Acknowledgment. This work was supported by grants from the National Institutes of Health HL071541 (D.A.B.) and CA107070 (S.W.K.) and the Korea Research Foundation (KRF-2007-357-D00074).

Supporting Information Available: Description of supplementary experimental methods and figures. This material is available free of charge via the Internet at http://pubs.acs.org.

MP800161E