

Comparative Evaluation of Target-Specific GFP Gene Silencing Efficiencies for Antisense ODN, Synthetic siRNA, and siRNA Plasmid Complexed with PEI–PEG–FOL Conjugate

Sun Hwa Kim,[†] Hyejung Mok,[†] Ji Hoon Jeong,[‡] Sung Wan Kim,[‡] and Tae Gwan Park^{*,†}

Department of Biological Sciences, Korea Advanced Institute of Science and Technology, Daejeon 305-701, South Korea, and Center for Controlled Chemical Delivery (CCCD)/Department of Pharmaceutics and Pharmaceutical Chemistry, University of Utah, Salt Lake City, Utah 84112. Received October 4, 2005; Revised Manuscript Received November 1, 2005

Cell specific gene silencing effects of antisense oligodeoxynucleotide (AS-ODN), synthetic small interfering RNA (siRNA-S), and siRNA expressing plasmid (siRNA-P) were comparatively evaluated. Poly(ethylenimine) (PEI) and PEI–*graft*-poly(ethylene glycol)–folate (PEI–PEG–FOL) conjugate were used to form nanosized polyelectrolyte complexes with the above three nucleic acids coding for inhibition of green fluorescent protein (GFP) expression. The three nucleic acid complexes formulated with either PEI or PEI–PEG–FOL had comparable sizes and surface ζ potential values. Among the three inhibitory nucleic acids, siRNA-S, when complexed with PEI–PEG–FOL, exhibited the most dose-effective and fastest gene silencing effect for FOL receptor overexpressing KB cells, because the siRNA-S could be directly delivered, via FOL receptor-mediated endocytosis, into the cytoplasm compartment where the degradation processing of target GFP mRNA occurred in a sequence-specific manner.

INTRODUCTION

Posttranscriptional gene silencing approaches, using various nucleic acid based molecules such as antisense oligodeoxynucleotides (AS-ODNs), ribozymes, and small interfering RNAs (siRNAs), have drawn much attention because of their potential therapeutic impact on treating various genetic disorder and infectious diseases including cancer. For the last two decades, AS-ODNs and ribozymes have been popularly used to knock down expression of specific genes. Quite recently, siRNA, which inhibits specific genes by double-stranded RNA molecules having 21–23 nucleotides, has emerged as a more powerful therapeutic genetic agent (1–3). Both AS-ODN and siRNA similarly inhibit posttranscriptional process of a target mRNA in a sequence-specific manner, but their action mechanisms in a cellular and molecular basis are vastly different. Although both of them exhibit considerable gene inhibition effects in vitro, their transfection efficiencies still remain in a relatively low level mainly due to poor intracellular uptake and severe enzymatic degradations in serum conditions (4, 5). To promote their gene inhibition effect, a wide range of cationic polymers and lipids have been employed to form nanoparticulate polyelectrolyte complexes with negatively charged AS-ODN and siRNA (6–8). The formation of nanosized complexes enhances the extent of intracellular uptake, increases stability against nucleases, and additionally confers cell specificity when conjugating a targeting moiety to the complexes.

Among the nonviral carriers, PEI has been popularly used for delivering plasmid DNA and AS-ODN for its superior transfection efficiency. A variety of chemically modified PEI derivatives such as PEGylated PEI and cross-linked PEI were also used to reduce its inherent cytotoxic problem and to enhance colloidal stability against aggregation under serum conditions

(9–11). In our previous works, PEI was grafted with PEG chains having a folate (FOL) moiety at its distal end to produce PEI–PEG–FOL conjugate (12). When the conjugate was complexed with plasmid DNA to form nanosized polyelectrolyte particles, greatly increased specific cellular uptake to FOL receptor overexpressing cancer cells was observed with concomitantly improved colloidal stability, compared to unmodified PEI. As a result, gene transfection efficiency with PEI–PEG–FOL was enhanced to a greater extent than that with unmodified PEI for FOL receptor overexpressing cells. This was attributed to folate receptor mediated endocytosis.

It is of great interest to compare gene silencing efficiencies between AS-ODN and siRNA, using PEI–PEG–FOL conjugate as a cell-specific gene carrier, since their modes of action are fundamentally different. Herein we report comparative gene silencing effects of three types of nucleic acid based molecules coding for inhibiting green fluorescent protein (GFP) expression. AS-ODN, synthetic siRNA (siRNA-S), and siRNA plasmid (siRNA-P) were used to form polyelectrolyte complexes with PEI or PEI–PEG–FOL. The complexes were characterized in terms of diameter and surface charge value. Their gene inhibition extents of an exogenous GFP gene expressed in KB cells were quantitatively evaluated in terms of dose and incubation time.

EXPERIMENTAL PROCEDURES

1. Materials. Folate (FOL) was purchased from Sigma Chemical Co. (St. Louis, MO). Poly(ethylenimine) (branched PEI, MW 25 000) was obtained from Aldrich (Milwaukee, WI). Poly(ethylene glycol) (COOH–PEG–NH₂, MW 3400) was supplied by Nektar (Huntsville, AL). Dulbecco's phosphate buffered saline (PBS), fetal bovine serum (FBS), and Roswell Park Memorial Institute 1640 medium (RPMI 1640 w/o folate) were purchased from Gibco BRL (Grand Island, NY). A pEGFP-C1 vector was obtained from BD Bioscience Clontech (Palo Alto, CA). All other chemicals and reagents were of analytical grade.

2. Antisense ODN, Synthetic siRNA, and siRNA Plasmid. Antisense ODN, synthetic siRNA, and plasmid siRNA targeted

* Corresponding author. Tel: +82-42-869-2621. Fax: +82-42-869-2610. E-mail address: tgpark@kaist.ac.kr.

[†] Korea Advanced Institute of Science and Technology.

[‡] University of Utah.

Table 1. GFP Target Sequences for Antisense ODN, Synthetic siRNA, and siRNA Plasmid

nucleic acid type	sequence
antisense ODN	GAGCTGCACGCTGCCGTC (18-mer)
synthetic siRNA	
sense	AACUUCAGGGUCAGCUUGC (19-mer)
antisense	GCAAGCUGACCCUGAAGUU (19-mer)
siRNA plasmid	
forward	GATCCCCGCAAGCTGACCTGAAGTTCTTCAAGAG- AGAACTTCAGGGTCAGCTTGTCTTTTGGAAA
reverse	AGCTTTTCCAAAAAGCAAGCTGACCTGAAGTTCTC- TCTTGAAGAACTTCAGGGTCAGCTTGC

to the GFP gene were designed as shown in Table 1. HPLC-purified phosphorothioated antisense ODN was purchased from Bioneer (Daejeon, South Korea). Synthetic siRNA (antisense and sense strands) was obtained from Invitrogen (Carlsbad, CA). siRNA plasmid (pSUPER-siGFP), producing anti-GFP siRNA hairpin transcript in the cells, was constructed by using pSUPER-RNAi vector system (Oligoengine, Seattle, WA) and forward and reverse oligonucleotides (1). Forward and reverse oligonucleotides for cloning the pSUPER-siGFP were synthesized and purified by Bioneer (Daejeon, South Korea).

3. Preparation of PEI-PEG-FOL Conjugate. A cancer target ligand, folate, was introduced into the highly branched cationic polymer, PEI, to prepare a target-specific gene delivery vector. The PEI-PEG-FOL conjugate was synthesized according to the previous method (10, 12). The stoichiometric conjugation molar ratio between PEI and PEG-FOL was 1:4.46 as determined by ¹H NMR spectra. The calculated molecular weight of the resulting conjugate was about MW 42824.

4. Preparation and Characterization of Polyelectrolyte Complexes between PEI-PEG-FOL Conjugate and Various Nucleic Acids. Anionic nucleic acids and cationic polymeric conjugates produced self-assembled polyelectrolyte complexes in an aqueous phase due to their electrical interactions. AS-ODN, siRNA-S, and siRNA-P were separately complexed with unmodified PEI and PEI-PEG-FOL conjugate under various conditions. Three types of the nucleic acids (4 μg) dissolved in 200 μL of phosphate buffered saline (PBS, pH 7.2) were respectively mixed with 10.6 μg of PEI-PEG-FOL (nitrogen/phosphate ratio = 16) in 300 μL of PBS solution. The mixtures were incubated for 20 min at room temperature and diluted 5-fold into PBS solution. To determine the hydrodynamic diameter and ζ potential of the PEI-PEG-FOL (or PEI)/nucleic acid complexes, the resulting complex solutions were analyzed by a dynamic light scattering instrument (Zeta-Plus, Brookhaven, NY).

5. Cell Culture and Transfection. KB cells (human epidermal carcinoma, Korean Cell Line Bank, Seoul, South Korea), were cultured in RPMI 1640 w/o folate medium supplemented with 10% FBS (the only source of folate) at 37 °C in a humidified atmosphere of 5% of CO₂. For gene silencing experiments, the nucleic acid complexes were cotransfected to KB cells along with the GFP plasmid according to the previously published method with minor modifications (13). Briefly, the cells were transfected with 2 μg of pEGFP-C1 vector using Lipofectamine 2000 reagent (2 mg/mL, Invitrogen, Carlsbad, CA) in 1 mL of RPMI1640 without folate containing 10% FBS for 3 h at 37 °C. The three nucleic acids were simultaneously cotransfected using 2 μg of AS-ODN, 0.5 μg of siRNA-S, or 2 μg of siRNA-P condensed with PEI or PEI-PEG-FOL

conjugates at various nitrogen/phosphate (N/P) ratios from 0 to 24. To determine a dose dependence of the nucleic acids on the inhibition of the target gene expression, cells were transfected with the complexes prepared at an N/P ratio of 16. The concentrations of the nucleic acids were as follows. AS-ODN and siRNA-P: 0.0, 0.1, 0.5, 1.0, 2.0, and 4.0 μg. siRNA-S: 0.00, 0.05, 0.10, 0.25, 0.50, and 1.00 μg. After replacing the transfection medium with 1 mL of serum containing medium, the transfected cells were incubated for a further 24 h and harvested by treatment of a cell lysis buffer solution (CellLytic M cell lysis reagent, Sigma-Aldrich, St. Louis, MO). For a time dependent GFP expression trend, PEI-PEG-FOL complexes were prepared with AS-ODN (2 μg), siRNA-S (0.5 μg), and siRNA-P (2 μg) at an N/P ratio of 16 and incubated for different time intervals after transfection. The level of GFP fluorescence was determined using a spectrofluorophotometer (SLM-AMINCO 8100, SLM Instruments Inc., Rochester, NY) with an excitation and emission wavelength at 488 and 507 nm, respectively. All values were normalized by cellular protein content, as determined by Micro-BCA protein assay (Pierce, Rockford, IL). The relative GFP expression level was then calculated as a relative percentage of GFP gene expression for the pEGFP-C1 transfected KB cells (negative control) compared to those cotransfected with nucleic acid complexes (AS-ODN, siRNA-S, or siRNA-P) [% GFP expression = (GFP intensity in sample/GFP intensity in negative control) × 100].

RESULTS AND DISCUSSION

Table 1 shows nucleotide sequences of anti-GFP AS-ODN, siRNA-S, and siRNA-P. Both siRNA-S and siRNA-P have the same target site in an mRNA sequence of GFP, but AS-ODN has a different one. For siRNA-P construction, forward and reverse ODN oligomers were cloned with a pSUPER-RNAi vector system as reported in our previous study (12). The three anti-GFP nucleic acid molecules, AS-ODN, siRNA-S, and siRNA-P, were complexed with PEI or PEI-PEG-FOL at an N/P ratio of 16. As listed in Table 2, the complexes of PEI-PEG-FOL with AS-ODN, siRNA-S, and siRNA-P were 94.4 ± 20.0, 120 ± 34.5, and 108.5 ± 19.0 nm in diameter with positive surface charge values of 1.0 ± 0.5, 2.5 ± 0.9, and 1.3 ± 0.7 mV, respectively. The complexes with PEI had slightly larger particle sizes with greater surface charge values than those with PEI-PEG-FOL, which was likely due to a charge shielding effect by PEGylation (14). At a fixed N/P ratio, the PEI and PEI-PEG-FOL complexes respectively showed comparable sizes and surface charge values regardless of nucleic acid molecules.

Target-specific GFP silencing activities of the three complexes were comparatively determined with folate receptor overexpressing KB cells. Anti-GFP nucleic acids (AS-ODN, siRNA-S, and siRNA-P) were complexed with PEI or PEI-PEG-FOL at different N/P ratios, and the resultant complexes were cotransfected with Lipofectamine/GFP plasmid (pEGFP-C1 vector) complexes. GFP gene silencing efficiencies of the complexes are shown in Figure 1. With increasing the N/P ratio, PEI-PEG-FOL complexes exhibited greater suppression of GFP expression than PEI complexes for the three nucleic acids. At an N/P ratio of 16, PEI-PEG-FOL complexes with AS-ODN, siRNA-S, and siRNA-P showed 68.9 ± 8.0%, 75.0 ± 15.3%, and 59.2 ± 3.4% inhibition of GFP expression,

Table 2. Particle Characterization of Antisense ODN, Synthetic siRNA, and siRNA Plasmid Condensed with PEI or PEI-PEG-FOL Conjugate

condensation agent	PEI			PEI-PEG-FOL		
	antisense ODN	synthetic siRNA	siRNA plasmid	antisense ODN	synthetic siRNA	siRNA plasmid
particle size (nm)	115.2 ± 15.3	164.2 ± 22.8	156.6 ± 26.3	94.4 ± 20.0	120 ± 34.5	108.5 ± 19.0
ζ potential (mV)	8.8 ± 1.3	10.3 ± 1.9	8.2 ± 2.0	1.0 ± 0.5	2.5 ± 0.9	1.3 ± 0.7

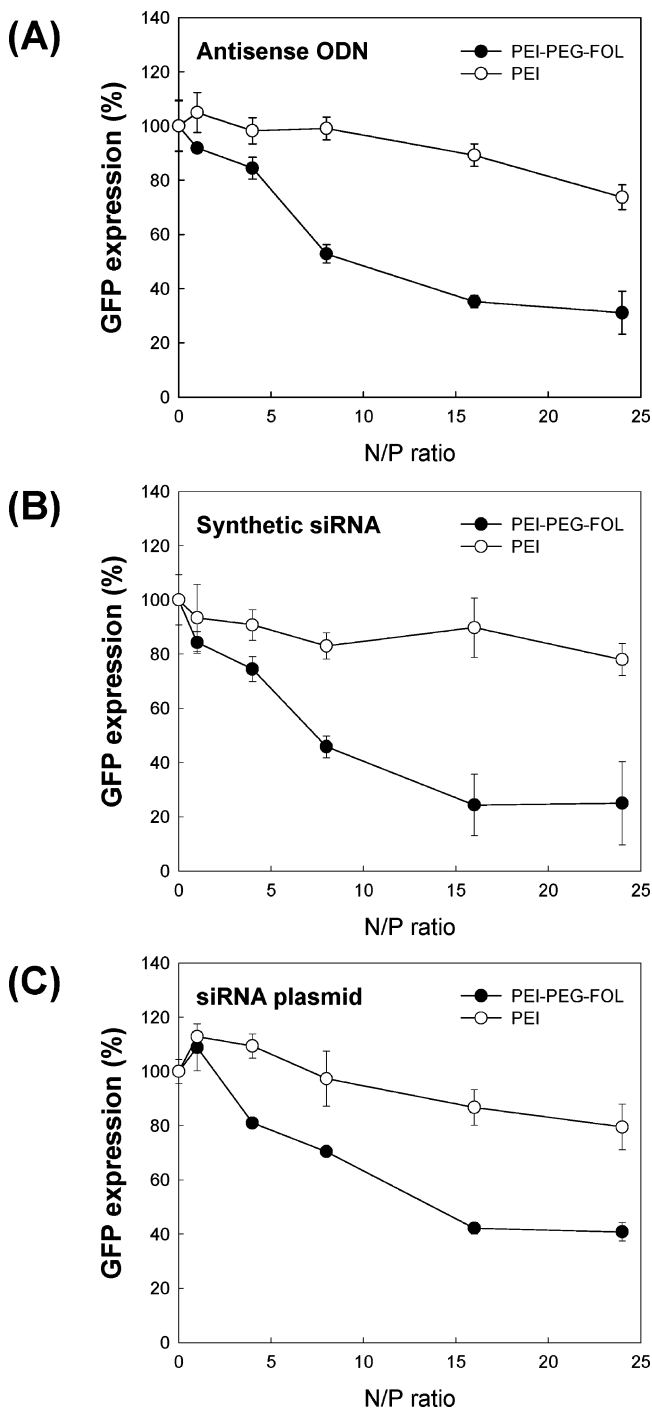


Figure 1. Cancer cell targeted GFP gene silencing effect of (A) antisense ODN, (B) synthetic siRNA, and (C) siRNA plasmid complexes with PEI (open circles) and PEI-PEG-FOL (filled circles) as a function of N/P ratio from 0 to 24 for KB cells.

respectively, while PEI complexes showed an inhibition degree of $26.3 \pm 4.6\%$, $22.1 \pm 5.9\%$, and $20.5 \pm 8.4\%$, in the same order. These results evidently indicate that the PEI-PEG-FOL complexes were far more effective in GFP silencing than the PEI complexes due to their enhanced extents of cellular uptake (12). For the three PEI-PEG-FOL complexes, folate receptor mediated endocytosis certainly played a key role in facilitating them to transport within cells, resulting in effectively knocking down the expression of GFP gene. Since the gene transfection experiments were performed in the medium containing 10% serum, the gene silencing efficiencies of the PEI complexes were relatively lower compared to those of the PEI-PEG-FOL complexes. The enhanced gene silencing effects of the PEI-

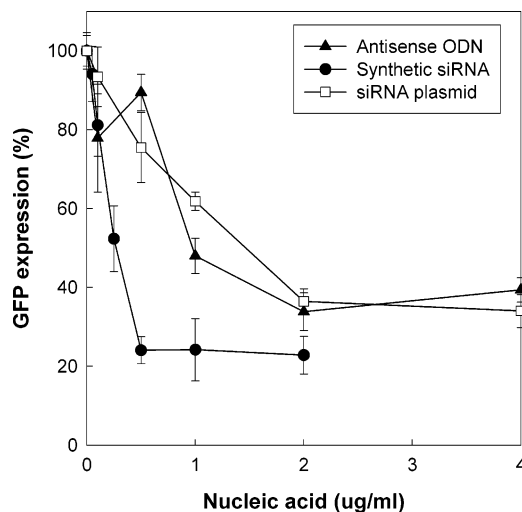


Figure 2. Comparative GFP gene suppression of antisense ODN (filled triangles), synthetic siRNA (filled circles), and siRNA plasmid (open squares) as a function of nucleic acid concentration. Antisense ODN and siRNA plasmid: 0.0–4.0 μg . Synthetic siRNA: 0.0–1.0 μg . Nucleic acids were complexed with PEI-PEG-FOL at an N/P ratio of 16.

PEG-FOL complexes could be additionally attributed to the presence of a PEG protective layer on the surface, preventing nonspecific adsorption of serum proteins and minimizing interparticular aggregation (15). It should be noted that, in our previous study, anti-GFP siRNA-P complexed with PEI-PEG-FOL inhibited GFP expression up to 89.5% for the KB cells that were pretransfected with GFP plasmid (12). In that study, the KB cells were pretransfected with GFP plasmid, and only GFP-positive cells were sorted for the transfection experiment. The reduced inhibition extent (59.2%) of GFP expression observed in the current study was likely caused by the fact that the KB cells were cotransfected with GFP plasmid and anti-GFP siRNA. The mixed population of GFP-positive and GFP-negative KB cells might result in the reduced extent of gene inhibition by siRNA.

Among the three nucleic acids, siRNA-S showed the most pronounced gene silencing effect. Although AS-ODN and siRNA-S have different GFP mRNA target sites, they must be delivered in the cytoplasm and inhibited translation of their respective target GFP mRNA sequences. siRNA-S exhibited a more effective gene inhibition effect than AS-ODN, which might be due to its greater specific gene suppression mechanism (16). It is also noted that siRNA-S showed greater gene silencing effects than siRNA-P with increasing N/P ratios. This was mostly likely caused by different intracellular gene trafficking and processing mechanisms between the two nucleic acids. siRNA-P must be located and transcribed in the nucleus prior to releasing several copies of siRNA transcripts to the cytoplasm. Thus it is reasonable to say that siRNA-S directly delivered in the cytoplasm exhibited more effective silencing of GFP expression compared to siRNA-P.

Dose-dependent anti-GFP activities of the three nucleic acids complexed with PEI-PEG-FOL (N/P ratio =16) are shown in Figure 2. With increasing the nucleic acid amount used in the transfection experiments, the three complexes inhibited GFP expression to a greater extent. However, siRNA-S complexes demonstrated the most powerful gene silencing effect. Nearly $76.0 \pm 3.4\%$ of GFP inhibition could be obtained at a dose of 0.5 $\mu\text{g}/\text{mL}$ for siRNA-S, whereas $66.2 \pm 4.8\%$ and $63.5 \pm 3.2\%$ of GFP inhibition were attained at a dose of 2 $\mu\text{g}/\text{mL}$ for AS-ODN and siRNA-P, respectively. These results reveal that target-specific gene silencing could be achieved by using much smaller amount of siRNA-S than AS-ODN and siRNA-P. This can be

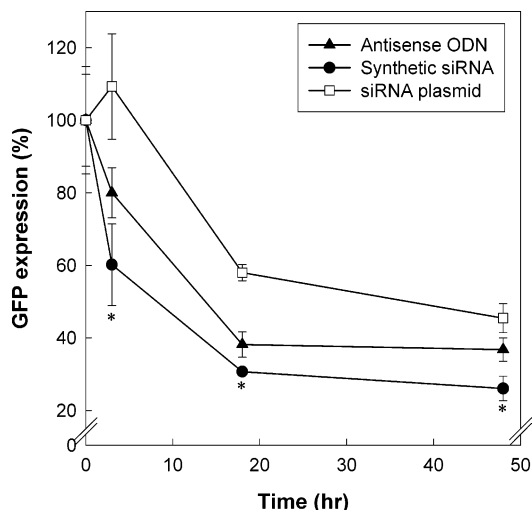


Figure 3. Time dependent inhibition of GFP gene expression by using antisense ODN (filled triangles), synthetic siRNA (filled circles), and siRNA plasmid (open squares). Nucleic acids were complexed with PEI-PEG-FOL at an N/P ratio of 16. Each dot represents the mean \pm SD from three replicate experiments; (*) $P < 0.05$ versus AS-ODN (Student's t -test).

attributed to the inherently specific and unique gene inhibition mode of siRNA-S, as described in the literature (16). Figure 3 shows GFP gene suppression profiles of the three nucleic acid complexes (N/P ratio = 16; nucleic acid amount: 2.0 μ g of AS-ODN, 0.5 μ g of siRNA-S, and 2.0 μ g of siRNA-P) as a function of time. siRNA-S and AS-ODN complexes inhibited GFP expression more rapidly than siRNA-P complexes. The siRNA-P complexes had a lag period before exerting its GFP gene silencing effect, presumably because they underwent a series of subcellular trafficking events, which hampers the production and localization of siRNA transcripts in the cytoplasm (17). Hence siRNA-P complexes exhibited more delayed gene silencing effect than siRNA-S and AS-ODN complexes.

In conclusion, three anti-GFP nucleic acids (AS-ODN, siRNA-S, and siRNA-P) were complexed with PEI-PEG-FOL conjugate and transfected to FOL receptor overexpressing cells to compare their FOL receptor-specific gene silencing efficiencies. All three complexes exhibited cancer cell specific gene inhibition behaviors, but siRNA-S was the most effective in gene silencing.

ACKNOWLEDGMENT

This work was supported by a grant (M10414030002-05N1403-00210) from the Ministry of Science and Technology, Republic of Korea.

LITERATURE CITED

- Jeong, J. H., Kim, S. W., and Park, T. G. (2003) A new antisense oligonucleotide delivery system based on self-assembled ODN-PEG hybrid conjugate micelles. *J. Controlled Release* 93, 183–191.
- Jeong, J. H., Kim, S. W., and Park, T. G. (2003) Novel intracellular delivery system of antisense oligonucleotide by self-assembled hybrid micelles composed of DNA/PEG conjugate and cationic fusogenic peptide. *Bioconjugate Chem.* 14, 473–479.
- Michael, T. M., and Phillip, A. S. (2002) Gene silencing in mammals by small interfering RNAs. *Nat. Rev. Genet.* 3, 737–747.
- Raymond, M. S., Aslam, A., Jun, X., Qin, Z., Qingquan, T., Gert, S., Grietje, M., Patrick, Y. L., Puthupattampil, V. S., and Martin, C. W. (2004) Cancer siRNA therapy by tumor selective delivery with ligand-targeted sterically stabilized nanoparticle. *Nucleic Acids Res.* 32, e149.
- Thazha, P. P., Charles, R. A., Prasad, D., Timothy, A. V., Namir, S., Russell, J., Brenda, F. B., Eric, E. S., Rechar, H. G., and Balkrishen, B. (2005) Positional effect of chemical modifications on short interference RNA activity in mammalian cells. *J. Med. Chem.* 48, 4247–4253.
- Oishi, M., Nagsaki, Y., Itaka, K., Nishiyama, N., and Kataoka, K. (2005) Lactosylated poly(ethylene glycol)-siRNA conjugate through acid-labile β -thiopropionate linkage to construct pH-sensitive polyion complex micelles achieving enhanced gene silencing in hepatoma cells. *J. Am. Chem. Soc.* 127, 1624–1625.
- Wilson, A., Zhou, W., Champion, H. C., Alber, S., Tang, Z. L., Kennel, S., Watkins, S., Huang, L., Pitt, B., and Li, S. (2005) Targeted delivery of oligodeoxynucleotides to mouse lung endothelial cells in vitro and in vivo. *Mol. Ther.* 12, 510–518.
- Jeong, J. H., Kim, S. H., Kim, S. W., and Park, T. G. (2005) Polyelectrolyte complex micelles composed of c-raf anti-sense oligodeoxynucleotide-poly(ethylene glycol) conjugate and poly(ethylenimine): Effect of systemic administration on tumor growth. *Bioconjugate Chem.* 16, 1034–1037.
- Merdan, T., Kunath, K., Petersen, H., Bakowsky, U., Voigt, K. H., Kopecek, J., and Kissel, T. (2005) PEGylation of poly(ethylenimine) affects stability of complexes with plasmid DNA under in vivo condition in a dose dependent manner after intravenous injection into mice. *Bioconjugate Chem.* 16, 785–792.
- Cho, K. C., Jeong, J. H., Chung, H. J., Joe, C. O., Kim, S. W., and Park, T. G. (2005) Folate receptor-mediated intracellular delivery of recombinant caspase-3 for inducing apoptosis. *J. Controlled Release* 108, 121–131.
- Kim, W. J., Yockman, J. W., Lee, M. H., Jeong, J. H., Kim, Y. H., and Kim, S. W. (2005) Soluble Flt-1 gene delivery using PEI-g-PEG-RGD conjugate for anti-angiogenesis. *J. Controlled Release* 106, 224–234.
- Kim, S. H., Jeong, J. H., Cho, K. C., Kim, S. W., and Park, T. G. (2005) Target-specific gene silencing by siRNA plasmid DNA complexed with folate-modified poly(ethylenimine). *J. Controlled Release* 104, 223–232.
- Takuro, H., Nobuhiko, Y., Fuminori, S., Tomoki, S., and Masaki, H. (2004) Evaluation of RNA interference in developing porcine granulose cells using fluorescence reporter genes. *J. Reprod. Dev.* 50, 599–603.
- Verbaan, F. J., Oussoren, C., Snel, C. J., Crommelin, D. J., Hennink, W. E., and Storm, G. (2004) Steric stabilization of poly-(2-(dimethylamino)ethylmethacrylate)-based polyplexes mediates prolonged circulation and tumor targeting in mice. *J. Gene Med.* 6, 64–75.
- Tang, G. P., Zeng, J. M., Gao, S. J., Maa, Y. X., Shi, L., Li, Y., Too, H.-P., and Wang, S. (2003) Polyethylene glycol modified polyethylenimine for improved CNS gene transfer: effects of PEGylation extent. *Biomaterials* 24, 2351–2362.
- Ryther, R. C., Flynt, A. S., Phillips, J. A., III, and Patton, J. G. (2005) siRNA therapeutics: big potential from small RNAs. *Gene Ther.* 12, 5–11.
- Christopher, W. A., Guilin, T., and Asher, Z. (2003) Vector systems for the delivery of small interfering RNAs: Managing the RISC. *ChemBioChem* 4, 1129–1136.

BC050289F