Non-covalent Nanocomplexes of Folic Acid and Reducible Polyethylenimine for Survivin siRNA Delivery

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Abstract. *Background/Aim: Efficient delivery of siRNA is critical for its therapeutic applications. This study was aimed at the design, synthesis and evaluation of a novel delivery system based on non-covalent complexes of folic acid (FA) and a reducible polyethylenimine (PEI) derivative, PEI-SS. Materials and Methods: PEI-SS was synthesized by crosslinking low-molecular weight PEI using a reducible crosslinking agent. PEI-SS–siRNA complexes were synthesized and further combined with FA to form FA/PEI-SS–siRNA nanocomplexes. These were then evaluated in two tumor cell lines for survivin siRNA delivery. Results: FA/PEI-SS–siRNA complexes were taken-up by both HeLa and A549 cells efficiently. This was not affected by the expression level of the folate receptor on the tumor cell surface. Furthermore, FA/PEI-SS–siRNA complexes reduced the level of survivin expression in both cell lines. Conclusion: FA/PEI-SS is a potent siRNA carrier that warrants further evaluation.*

Antisense cDNA, antisense oligodeoxynucleotides, and small interfering RNA (siRNA) have been shown to impair tumor cell growth and sensitize tumor cells to apoptosis-inducing agents (1-3). siRNAs can induce gene silencing *via* RNA interference (RNAi) (4). However, *in vivo* delivery of siRNA remains a critical challenge. Cationic polymers have been shown to be effective in siRNA delivery and have advantages such as low immunostimulatory activity and easy chemical modification (5-7). Polyethylenimine (PEI), a cationic polymer, is particularly attractive due to its strong buffering capacity at the acidic endosomal pH (8, 9). However, low-molecular weight PEIs have limited efficiency for siRNA delivery whereas high molecular weight PEIs have high toxicity (10-12). A biodegradable PEI with reducible disulfide bonds has been shown to efficiently deliver an siRNA targeting human telomerase reverse transcriptase and to have reduced cytotoxicity (13).

Folic acid (FA) has been used as a ligand for specific targeting of cancer cells because folate receptor (FR) is frequently overexpressed in human tumors, such as ovarian carcinoma (14-16). Recently, we reported that by combining FA with PEI electrostatically in a formulation, much greater transfection activity could be obtained (17).

Survivin is a member of the inhibitor of apoptosis protein (IAP) family and a validated oncogene (18, 19). The expression of survivin is known to be correlated with shorter survival in the majority of cancers. Survivin is expressed in most cancers and considered an attractive therapeutic target. Inhibition of survivin expression by siRNA has been shown to induce apoptosis in cancer cells. However, therapeutic application of siRNA requires for development of an efficient delivery vehicle.

In the present study, we hypothesized that a bioreducible polyethylenimine, PEI-SS, is effective as a carrier for the delivery of survivin siRNA and the activity is further enhanced *via* complexation to FA. PEI-SS was synthesized and complexed to FA and siRNA electrostatically. The FA/PEI-SS–siRNA complexes were characterized and evaluated for *in vitro* transfection activity.

Materials and Methods

Materials. Branched PEI 2 kDa (PEI 2K), branched PEI 25 kDa (PEI 25 K), N-hydroxysuccinimide (NHS), 1-(3-dimethylamino-propyl)-3-ethylcarbodiimide (EDC), 3'-dithiobispropanoic acid (DTPA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and FA were obtained from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) and 4',6-diamidino-2-phenylindole (DAPI) were obtained from Gibco (Gibco BRL Co. Ltd, Gaithersburg, MD, USA). Survivin siRNA and 5'-Cy3 siRNA were obtained from Biomics Biotechnologies (Nantong, Jiangsu, China).

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Cell culture. HeLa cells were grown in a Dulbecco's modified Eagle's medium (DMEM) and A549 cells were grown in RPMI1640 medium, supplemented with 10% FBS and 1% antibiotics/antimycotics at 37˚C in a humidified atmosphere containing 5% CO_2.

Synthesis of PEI-SS. PEI-SS was synthesized as reported previously (20-21). Briefly, DTPA (2 mmol, 0.42 g), PEI 2K (2 mmol, 4 g), EDC (5 mmol, 0.96 g), and NHS (5 mmol, 0.57 g) were added to 2-(N-morpholino)ethanesulfonic acid buffer (pH 6.5, 5 ml) in a reaction flask and stirred. The reaction proceeded at room temperature under nitrogen atmosphere for 2 days. The resulting viscous solution was purified by exhaustive dialysis (3,500 g/mol cut-off) against 50 mM NaCl (3×5 l) and then deionized water. The PEI-SS polymer product was obtained by freeze drying (yield: 37%).

Preparation of FA/PEI-SS–siRNA complexes. Ten microliters of PEI-SS solution in ethanol (10 mg/ml) was injected into 90 μl HEPES buffer. The mixture was vortexed for 10 s, then sonicated for 20 s. Survivin siRNA (10 μmol/μl) was then added. FA was dissolved in phosphate buffer (pH 6.5) at 10 mg/ml. In the present study, we prepared FA/PEI-SS–siRNA complexes at different theoretical charge ratios, such as FA carboxylate:PEI nitrogen:siRNA phosphate=0:6:1 (PEI-SS–siRNA), 5:6:1 (FA:5), 10:5:1 (FA:10), 15:6:1 (FA:15), 20:6:1 (FA:20), 25:6:1 (FA:25), and 30:6:1 (FA:30).

Size and zeta potential measurement. The particle size and zeta potential of the PEI-SS–siRNA and FA/PEI-SS–siRNA complexes were measured on a Zetasizer Nano ZS 90 (Malvern Instruments, Ltd., Malvern, UK). PEI-SS–siRNA complexes at ratios of 1:1-10:1 and FA/PEI-SS–siRNA at ratios of 5:8:1-30:8:1 were measured. The results were calculated by averaging three measurements.

The size and surface morphology of the FA/PEI-SS–siRNA complexes (at ratio 20:8:1) were investigated by field emission scanning electron microscopy (SEM) (JSM-6700F; JEOL, Akishima, Tokyo, Japan). The sample was fixed onto a brass stub using double-sided adhesive tape and was coated with a thin layer of gold. Images were then taken at 3.0 kV accelerating voltage.

Analysis of PEI-SS–siRNA and FA/PEI-SS–siRNA complexes by gel retardation. PEI-SS and FA/PEI-SS were complexed with siRNA at various molar ratios and formation of the complexes was confirmed by a gel retardation assay. Two microliters of 6× sample loading buffer (50% glycerol, 1% bromophenol blue, and 1% xylene cyanol FF in Tris-borate EDTA) was added to each sample. The samples were loaded onto a 3% agarose gel containing 2 mg/ml ethidium bromide. Electrophoresis was run at 120 V for 15 min. The resulting gels were photographed under UV-illumination.

Cytotoxicity assay. HeLa cells and A549 cells, obtained from American Type Culture Collection (Rockville, MD, USA), were seeded at a density of 1×10^4 cells/well in a 96-well plate and grown for 24 h. The cells were then washed three times with PBS and incubated with PEI-SS or FA/PEI-SS with different concentrations of FA in serum-free media. After 4 h, the incubation media were replaced with fresh media and the cells were incubated at 37˚C for another 20 h. Cell viability was then determined by MTT assay. Ten microliters of MTT stock solution (0.5 mg/ml) was added to each well. After 2 h of incubation, media were removed. The crystals formed were dissolved by adding 100 μl of DMSO to each well. The results were converted into viability by measuring the optical density at 490 nm (OD490) on a microplate reader. The results are presented as the mean±SD of six replicates for each sample.

Flow cytometry. 5'-Cy3-labeled siRNA was formulated into the siRNA complexes and their uptake into HeLa and A549 cells was analyzed on an EPICS XL flow cytometer (Beckman Coulter Inc., Pasadena, CA, USA). Briefly, the cells were plated on 24-well plates at a density of 1×10^5 cells/well. After 24 h, the media were removed and cells were washed with PBS three times and placed in 1 ml of fresh media (FA-free). The cells were then incubated with 5'-Cy3-labeled siRNA complexes for 4 h at 37˚C. The cells were then collected, fixed in 4% para-formaldehyde and analyzed by flow cytometry. The data obtained were analyzed by the Cell Quest software (Beckman Coulter Inc., Pasadena, CA, USA).

Confocal microscopic analysis. Cells were cultured at a density of 1×10^4 cells/well in a glass-bottom cell culture dish for 24 h. Then the cells were treated with Cy3-siRNA, PEI–Cy3-siRNA, PEI-SS–Cy3-siRNA or FA/PEI-SS–Cy3-siRNA for 4 h at 37˚C. The cells were then washed three times and fixed with 4% formaldehyde for 15 min. Cellular nuclei were stained with DAPI (2.5 μg/ml) for 3 min at room temperature. Internalization of free and the complexed Cy3-siRNA were observed on a Zeiss 710 LSMNLO Confocal Microscope (Carl Zeiss, Jena, Germany).

Determination of survivin expression by western blot. Western blot was used to determine the effect of survivin siRNA delivered with different carriers on expression of survivin protein. HeLa and A549 cells were seeded into a 6-well plate at a density of 1×10^5 cells/well and were treated with different complexes after 24 h. Total protein was extracted from the cells after 48-h incubation using radio-immunoprecipitation assay buffer (RIPA Buffer; Sigma, St. Louis, MO, USA) with 1% protease inhibitor cocktail (Sigma, St. Louis, MO, USA) and 2% phenylmethanesulfonyl fluoride (PMSF; Sigma). Protein concentration was determined by using a Bicinchoninic acid Protein Assay kit (Bio-rad Laboratories Inc, USA). Protein samples (30 μg) were loaded onto a 12% polyacrylamide gel and electrophoresis was performed (90 V for spacer gel, 120 V for separation gel). The proteins were then transferred to a polyvinylidene fluoride (PVDF) membrane. Transferred blots were blocked with 5% bovine serum albumin (BSA) for 2 h and immunoblotted against the primary antibodies, survivin, or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Abcam Inc., Cambridge, MA, USA) at 4˚C overnight. Tris buffered saline tween (TBST) solution was used to rinse the membrane three times and the membrane was incubated with rabbit anti-goat IgG (Pierce, Rockford, IL, USA) as secondary antibody. Gel staining was performed using an enhanced chemiluminescence (ECL) kit (GE Healthcare, Buckinghamshire, UK).

Statistical analysis. The data were analyzed for statistical significance by using the Statistical Product and Service Solutions (IBM SPSS) for Windows (SPSS Inc., Chicago, IL, USA). Where indicated, the results are summarized as the mean±SD.

Results

Physicochemical properties of complexes. The zeta-potentials of PEI-SS–siRNA complexes with different N/P ratios and FA/PEI-SS–siRNA complexes with different FA concentrations are shown in Figure 1A and B. The zeta

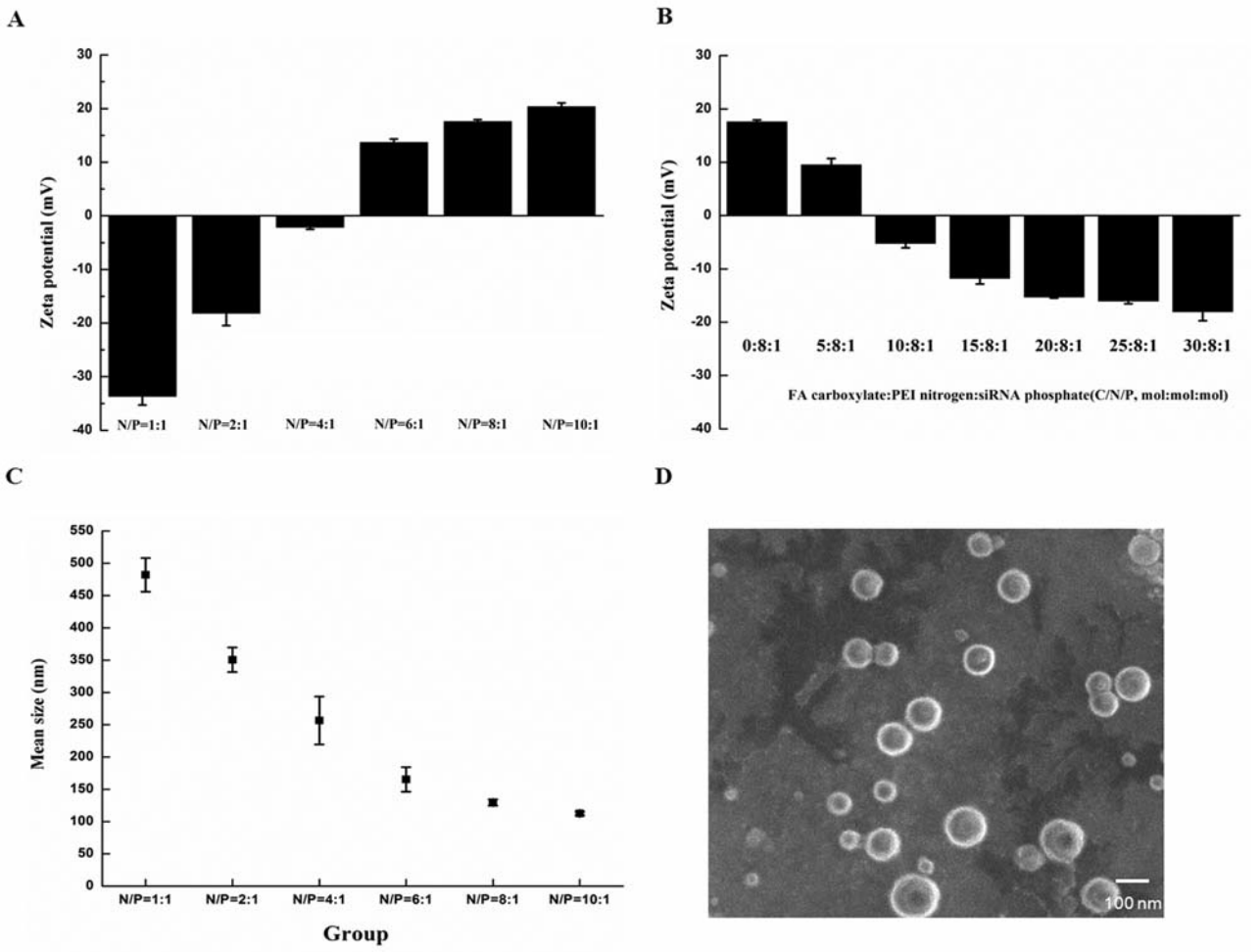

Figure 1. *Size and zeta potential of the siRNA complexes. A: Zeta potential of (a reducible polyethylenimine derivative, PEI-SS)–siRNA complexes at different N/P ratios. B: Zeta potential of FA/PEI-SS–siRNA complexes at different ratios. C: Size of PEI-SS–siRNA complexes at different N/P ratios. Error bar represents standard deviations (n=3). D: The scanning electron microscope (SEM) image of FA/PEI-SS–siRNA (at ratio 20:8:1) complexes.*

potential values were negative at N/P ratios of 1:1-4:1 but became positive when the N/P ratios increased to 6:1, 8:1 and 10:1 (Figure 1A). This indicates that PEI-SS induced a reversal of zeta potential from negative to positive. The addition of FA reduced the zeta potential of PEI-SS–siRNA complexes, which reached a minimum at the ratio of FA:PEI-SS:siRNA of 20:8:1 (Figure 1B). Increasing FA content induced a reversal of zeta potential from positive to negative.

Particle size measurement of PEI-SS–siRNA complexes with different N/P ratios are shown in Figure 1C. The data show that particle diameter was 129.5±4.9 nm at N/P of 8. The particle size and the shape of FA/PEI-SS–siRNA complexes were observed under SEM (Figure 1D). The shape of the complex (FA:PEI-SS:siRNA=20:8:1) was round and the mean particle size was under 200 nm.

Formation of complexes between siRNA and PEI-SS or FA/PEI-SS. Agarose gel retardation was used to evaluate the complexation of siRNA with different amounts of PEI-SS or FA/PEI-SS. A series of PEI-SS–siRNA complexes with different N/P ratios and FA/PEI-SS–siRNA with different FA concentrations were added to the agarose gel. As shown in Figure 2A, no fluorescence signals were observed when the ratios were above 6:1 for the PEI-SS–siRNA complexes. The complexes remained intact after adding different concentrations of FA and the FA/PEI-SS complexes were able to combine with siRNA at the tested ratios completely (Figure 2B).

Assessment of cytotoxicity. The cytotoxicity of PEI 2K (2 kDa), PEI 25K (25 kDa), PEI-SS and FA/PEI-SS was assessed on A549 cells and HeLa cells by the MTT assay. Cytotoxicity

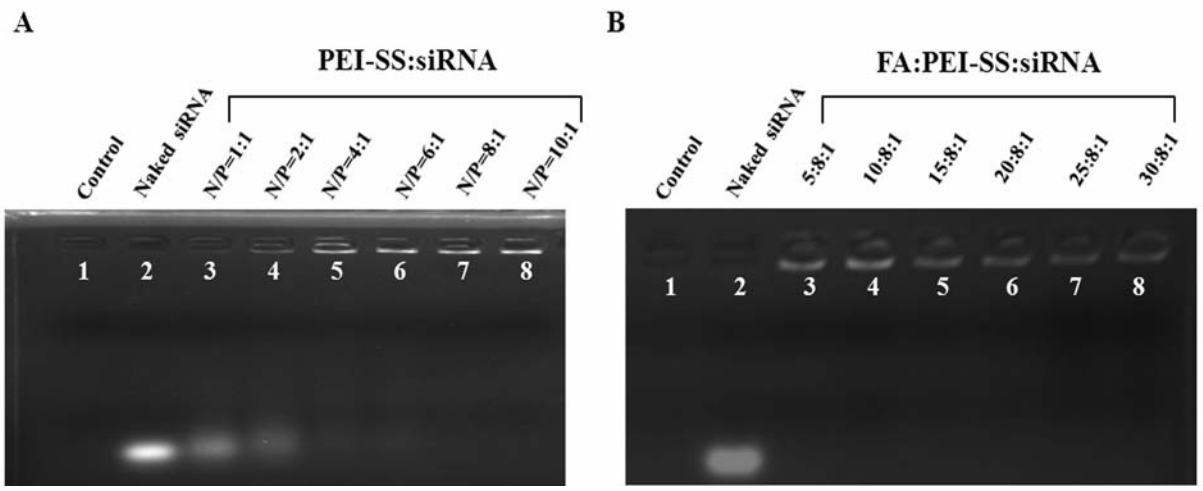

Figure 2. *Agarose gel analysis of the complexes. A: (A reducible polyethylenimine derivative, PEI-SS)–siRNA complexes at different N/P ratios. B: FA/PEI-SS–siRNA complexes with different amount of FA.*

studies were conducted to explore whether the complexes used for siRNA delivery had any toxicity and other indirect effects on the uptake. As shown in Figure 3, after 24 h of incubation, cell viability of HeLa cells and A549 cells was maintained at over 90% after treatment across all PEI-SS and FA/PEI-SS complexes. Conversely, PEI 2K exhibited much higher cytotoxicity than PEI-SS and FA/PEI-SS complexes.

Study of Cy3-siRNA uptake by flow cytometry. Flow cytometry was used to analyze the effect of delivery systems on siRNA uptake in order to evaluate the delivery efficiencies of the complexes. The uptake levels of naked siRNA, PEI 2K–siRNA, PEI-SS–siRNA and FA/PEI-SS–siRNA by FR-overexpressing (FR+, HeLa) and FR-negative (FR-, A549) cells were determined. As shown in Figure 4A and B, when treated with PEI-SS–siRNA at N/P=8:1, the cells exhibited markedly increased mean fluorescence intensity relative to those treated with naked siRNA and PEI 2K–siRNA. Moreover, the transfection efficiency of FA/PEI-SS–siRNA was significantly higher than that for PEI 2K/siRNA and PEI-SS–siRNA in both HeLa cells and A549 cells. Figure 4C and D show the cellular uptake of the FA/PEI-SS–siRNA complexes from the medium with various concentrations of free FA. Free FA (0.01 mM, 0.1 mM, and 1 mM) was added before the FA/PEI-SS–siRNA complexes. The complexes were taken up by FR^+ cells and FR- cells. The results show that free FA failed to inhibit transfection by FA/PEI-SS–siRNA complexes in both FR^+ or FR^- cells.

Confocal microscopy. Confocal microscopy was employed to investigate the uptake of the complexes by HeLa cells The cellular uptake efficiencies of naked siRNA, PEI 2K–siRNA, PEI-SS–siRNA and FA/PEI-SS–siRNA complexes were compared (Figure 5). Cellular nuclei were fluorescently labeled with DAPI, shown in blue; 5'-Cy3 siRNA fluorescence is shown in red. The results showed extensive internalization of Cy3-labeled FA/PEI-SS–siRNA. There was trafficking of the siRNA into the cytosol. FA/PEI-SS–siRNA complexes were more extensively internalized than PEI 2K–siRNA and PEI-SS–siRNA complexes.

Determination of survivin expression by western blot. The expression of survivin protein was measured by western blot. As shown in Figure 6, PEI-SS–siRNA at N/P=6:1-10:1 caused a decrease in survivin protein level relative to the control and naked siRNA-treated cells, both in HeLa cells and in A549 cells. The down-regulation of survivin by PEI-SS–siRNA was 65.07% and 64.30% in HeLa cells and A549 cells at N/P=8:1, respectively.

As shown in Figure 7, PEI-SS–siRNA caused a greater decrease in survivin protein expression compared to PEI 2K–siRNA and PEI 25K–siRNA. Moreover, the FA/PEI-SS–siRNA complexes also induced significant gene silencing in both FR^+ cells and FR^- cells. Survivin down-regulation by FA/PEI-SS–siRNA was 63.68% and 64.28% in HeLa cells and A549 cells, respectively.

Discussion

Efficient siRNA delivery remains a challenge. The development of high-efficiency and low-toxic siRNA delivery carriers is essential for successful siRNA-based cancer therapy (22-23). PEI is one of the most potent gene

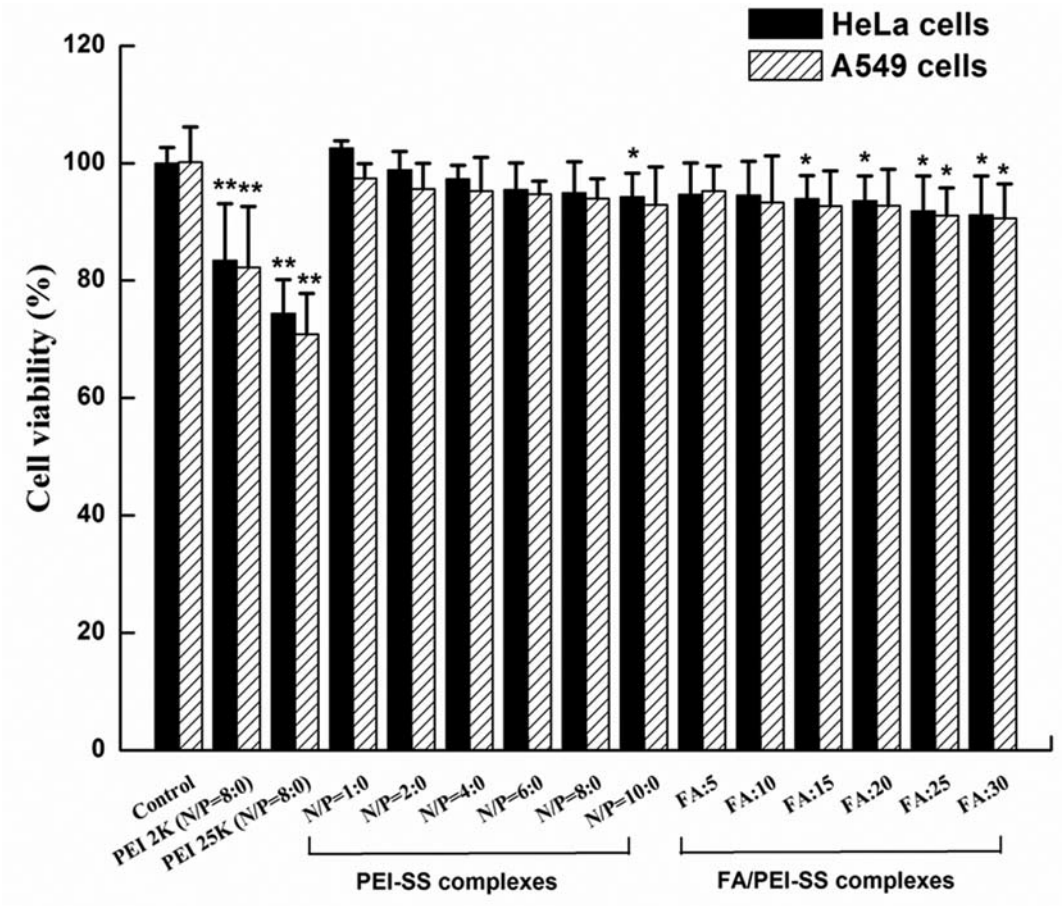

Figure 3. *Cytotoxicity of various siRNA complexes towards HeLa and A549 cells. Cells were incubated with siRNA complexes for 4 h, and cell viability was measured 20 h after treatment. Data shown are the percentage of viable cells relative to that of untreated cells. Each bar is the mean (and SD) of six experiments. *p<0.05 vs. control; **p<0.01 vs. control.*

delivery carriers because of its strong ability to bind nucleic acid and to facilitate endosomal escape by the 'proton sponge effect'. However, clinical use of PEI is hampered by its high cytotoxicity (24-26).

In the present study, a bioreducible polyethylenimine, PEI-SS, was synthesized. Disulfide bonds in PEI can reduce cytotoxicity since they are cleavable in an intracellular reductive environment by the glutathione and thioredoxin reductases. The PEI-SS was further electrostatically coated with FA (FA/PEI-SS) for delivery of survivin siRNA. This was based on a previous study showing FA could enhance the transfection activity of PEI because of its unique structure (17). The properties of the complexes, including cytotoxicity, cellular uptake, survivin siRNA target silencing activity, were all investigated.

Particle sizes and zeta potentials of the PEI-SS–siRNA and FA/PEI-SS–siRNA complex showed that cationic charges of PEI-SS and PEI-SS induced a reversal of zeta potential of siRNA complexes from negative to positive. The addition of FA reduced the zeta-potential of PEI-SS–siRNA complexes (Figure 1A and B). We also evaluated the uptake levels of PEI-SS–siRNA and FA/PEI-SS–siRNA complexes in FR-overexpressing (HeLa) and FR-negative (A549) cells. The results suggested that FA in the FA/PEI-SS complex enhances the transfection of siRNA, but not *via* a FR-mediated pathway. FA tends to self-associate into multimers, which resemble anionic detergent micelles. Therefore, its presence might have facilitated endosomal escape of siRNA.

The uptake of the FA/PEI-SS–siRNA complex by HeLa cells was also investigated by confocal laser scanning microscopy. Fluorescently labeled FA/PEI-SS/Cy3-siRNA were more extensively internalized than other complexes (Figure 5). Survivin down-regulation by PEI-SS–siRNA and FA/PEI-SS–siRNA were measured in both HeLa and A549 cells by western blot (Figures 6 and 7). The FA/PEI-SS–siRNA complexes exhibited significant gene silencing activity.

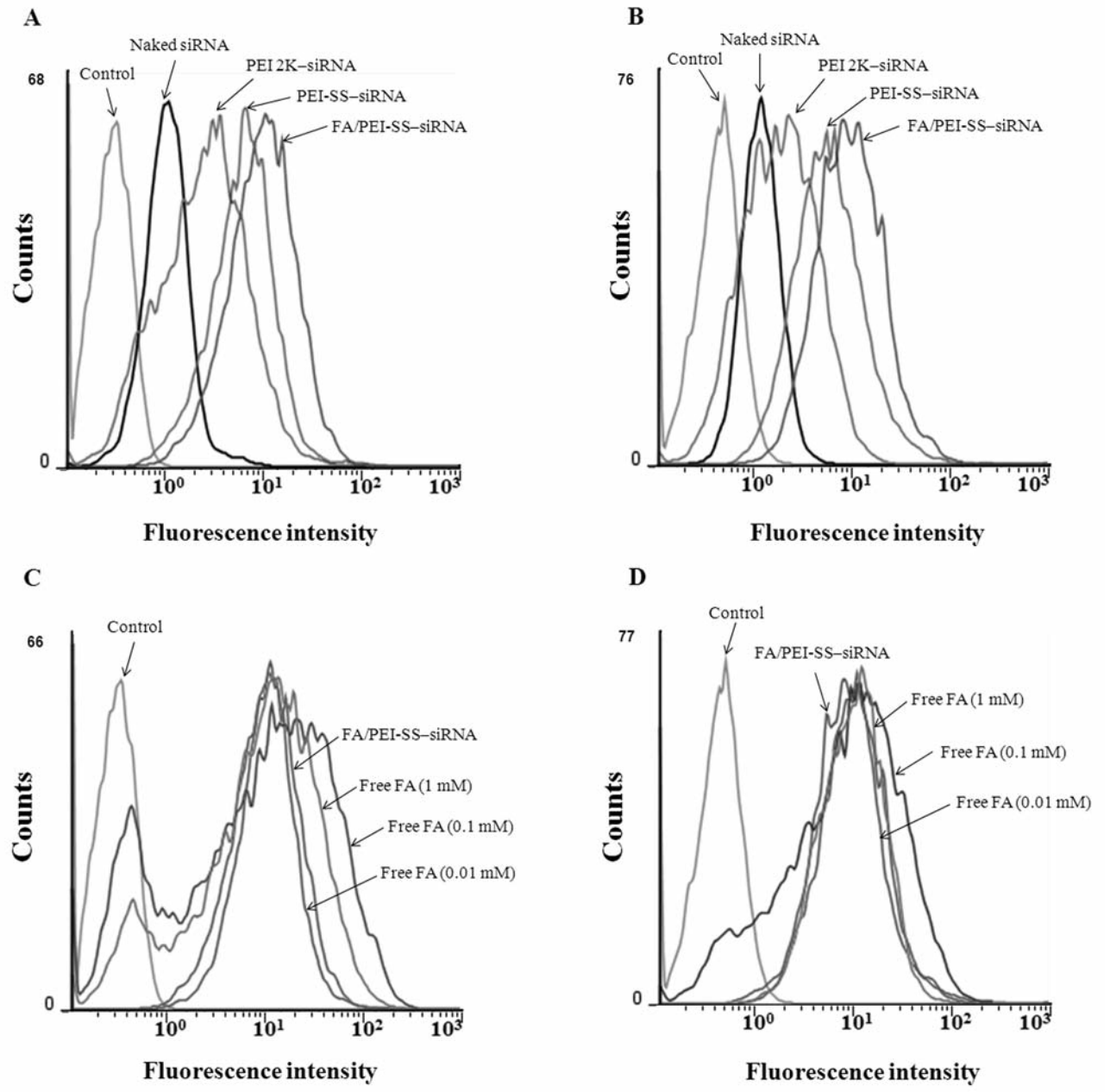

Figure 4. *Cellular uptake of Cy3-labeled siRNA complexes. Uptake by HeLa (A), and A549 (B) cells; and effect of free FA on uptake by HeLa (C) and A549 (D) cells.*

Conclusion

We developed an effective siRNA delivery system, FA/PEI-SS–siRNA. A disulfide-containing polyethylenimine, PEI-SS, was synthesized and shown to efficiently deliver siRNA. FA coated PEI-SS–siRNA complexes stably and further enhanced the delivery efficiency. siRNA was taken up by HeLa cells and A549 cells *via* FA/PEI-SS complexes, regardless of the FR expression status. There does not appear to be any limitation on the delivery system based on the type of tumor cell line. Our results suggest that FA/PEI-SS–siRNA nanocomplexes warrant further evaluation *in vivo*.

Conflicts of Interest

No competing financial interests exist.

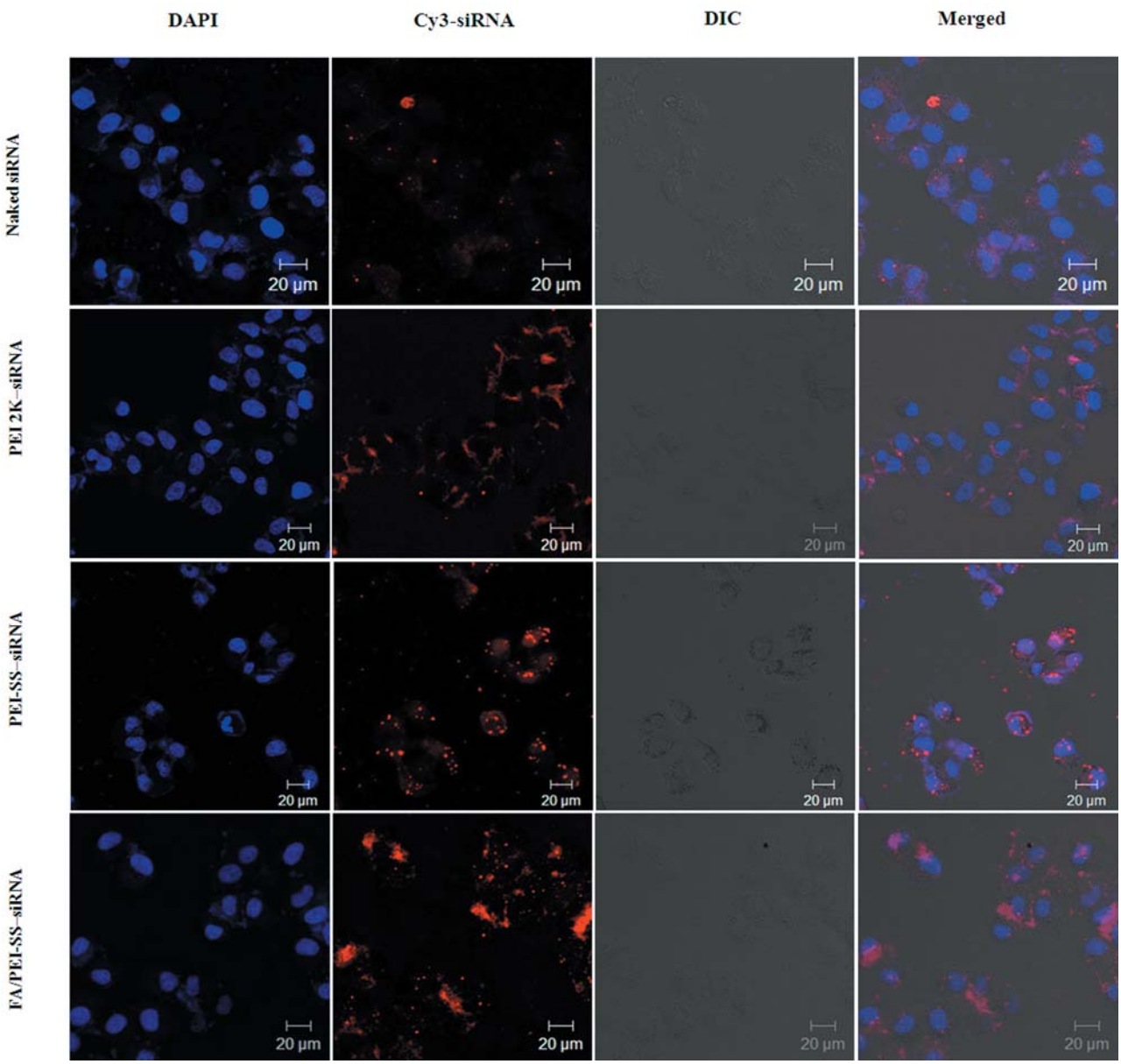

Figure 5. *Intracellular localization of naked siRNA, PEI 2-kDa (PEI 2K)–siRNA, (a reducible polyethylenimine derivative, PEI-SS)–siRNA and FA/PEI-SS–siRNA complexes. Cy3 fluorescence is shown in red, DAPI nuclear stain is shown in blue.*

References

1 Fuessel S, Herrmann J, Ning SL, Kotzsch M, Kraemer K, Schmidt U, Hakenberg OW, Wirth MP and Meye A: Chemosensitization of bladder cancer cells by survivin-directed antisense oligodeoxynucleotides and siRNA. Cancer Lett *232*: 243-254, 2006.

2 Kami K, Doi R, Koizumi M, Toyoda E, Mori T, Ito D, Kawaguchi Y, Fujimoto K, Wada M, Miyatake SI and Imamura M: Down-regulation of survivin by siRNA diminishes radioresistance of pancreatic cancer cells. Surgery *138*: 299-305, 2005.

3 Zhang Y, Chen ZD, Do CJ, Xu G and Luo W: siRNA targeting Survivin inhibits growth and induces apoptosis in human renal clear cell carcinoma 786-O cells. Pathol Res Pract *205*: 823-827, 2009.

4 Devi GR: siRNA-based approaches in cancer therapy. Cancer Gene Ther *13*: 819-829, 2006.

5 Qian XM, Long LX, Shi ZD, Liu CY, Qiu MZ, Sheng J, Pu PY, Yuan XB, Ren Y and Kang CS: Star-branched amphiphilic PLA-b-PDMAEMA copolymers for co-delivery of miR-21 inhibitor and doxorubicin to treat glioma. Biomaterials *35*: 2322-2335, 2014.

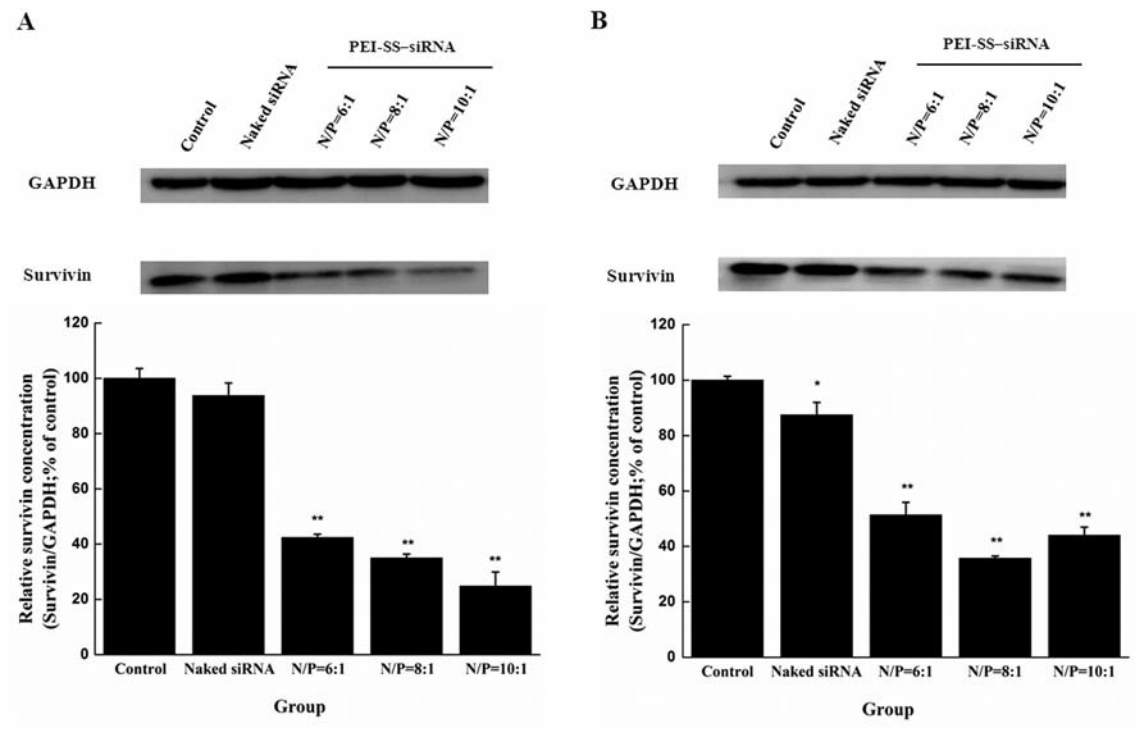

Figure 6. *Survivin expression in HeLa (A) and A549 (B) cells after treatment with a reducible polyethylenimine derivative (PEI-SS)–siRNA, as measured by western blot. Each bar is the mean of three experiments. *p<0.05 vs. control; **p<0.01 vs. control.*

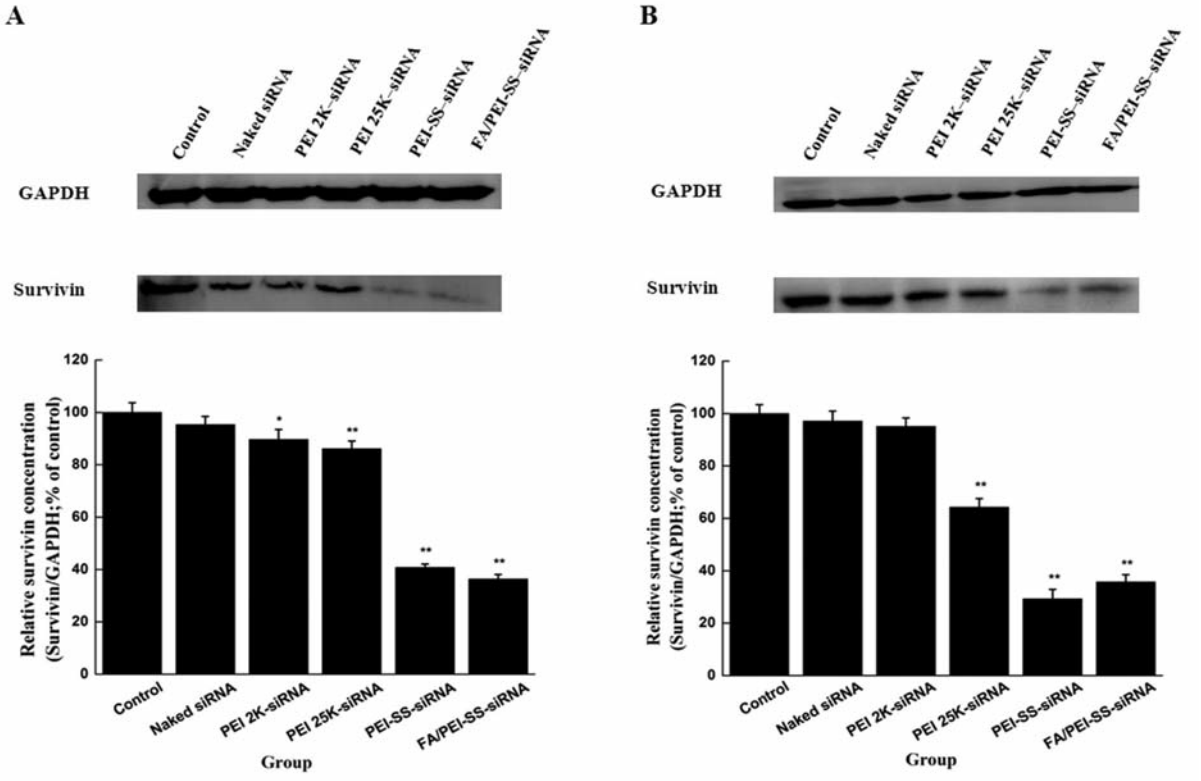

Figure 7. *Survivin expression in HeLa (A) and A549 (B) cells after treatment with various complexes, as measured by western blot. Each bar is the mean of three experiments. *p<0.05 vs. control; **p<0.01 vs. control.*

6 Tobin LA, Xie YL, Tsokos M, Chung SI, Merz AA, Arnold MA, Li G, Malech HL and Kwong KF: Pegylated siRNA-loaded calcium phosphate nanoparticle-driven amplification of cancer cell internalization *in vivo*. Biomaterials *34*: 2980-2990, 2013.
7 Zhang Y, Zhou CG, Kwak KJ, Wang XM, Yung B, Lee LJ, Wang YM, Wang PG and Lee RJ: Efficient siRNA Delivery Using a Polyamidoamine Dendrimer with a Modified Pentaerythritol Core. Pharm Res *29*: 1627-1636, 2012.
8 Bahadur KCR and Uludag H: A Comparative evaluation of disulfide-Linked and hydrophobically-modified PEI for plasmid delivery. J Biomater Sci-Polym Ed *22*: 873-892, 2011.
9 Borgheti-Cardoso LN, Depieri LV, Kooijmans SAA, Diniz H, Calzzani RAJ, Vicentini FTMdC, van der Meel R, Fantini MCdA, Iyomasa MM, Schiffelers RM and Bentley MVLB: An *in situ* gelling liquid crystalline system based on monoglycerides and polyethylenimine for local delivery of siRNAs. European journal of pharmaceutical sciences: official journal of the European Federation for Pharmaceutical Sciences *74*: 103-117, 2015.
10 Kapilov-Buchman Y, Lellouche E, Michaeli S and Lellouche J-P: Unique surface modification of silica nanoparticles with polyethylenimine (PEI) for siRNA delivery using cerium cation coordination chemistry. Bioconjugate chemistry *26*: 880-889, 2015.
11 Nicoli E, Syga MI, Bosetti M and Shastri VP: Enhanced Gene Silencing through Human Serum Albumin-Mediated Delivery of Polyethylenimine-siRNA Polyplexes. PLoS One 102015.
12 Xie J, Teng L, Yang Z, Zhou C, Liu Y, Yung BC and Lee RJ: A polyethylenimine-linoleic acid conjugate for antisense oligonucleotide delivery. BioMed research international *2013*: 710502, 2013.
13 Kurosaki T, Morishita T, Kodama Y, Sato K, Nakagawa H, Higuchi N, Nakamura T, Hamamoto T, Sasaki H and Kitahara T: Nanoparticles Electrostatically Coated with Folic Acid for Effective Gene Therapy. Mol Pharm *8*: 913-919, 2011.
14 Kim SH, Jeong JH, Mok H, Lee SH, Kim SW and Park TG: Folate receptor targeted delivery of polyelectrolyte complex micelles prepared from ODN-PEG-Folate conjugate and cationic lipids. Biotechnol Prog *23*: 232-237, 2007.
15 Li HM, Miteva M, Kirkbride KC, Cheng MJ, Nelson CE, Simpson EM, Gupta MK, Duvall CL and Giorgio TD: Dual MMP7-proximity-activated and folate receptor-targeted nanoparticles for siRNA delivery. Biomacromolecules *16*: 192-201, 2015.
16 Qi R, Majoros I, Misra AC, Koch AE, Campbell P, Marotte H, Bergin IL, Cao ZY, Goonewardena S, Morry J, Zhang S, Beer M, Makidon P, Kotlyar A, Thomas TP and Baker JR: Folate receptor-targeted dendrimer-methotrexate conjugate for inflammatory arthritis. J Biomed Nanotechnol *11*: 1431-1441, 2015.
17 Guo WJ and Lee RJ: Efficient gene delivery *via* non-covalent complexes of folic acid and polyethylenimine. J Control Release *77*: 131-138, 2001.
18 Becker AL, Orlotti NI, Folini M, Cavalieri F, Zelikin AN, Johnston APR, Zaffaroni N, and Caruso F: Redox-active polymer microcapsules for the delivery of a survivin-specific siRNA in prostate cancer cells. ACS Nano 5: 1335-1344, 2011.
19 Uchida H, Tanaka T, Sasaki K, Kato K, Dehari H, Ito Y, Kobune M, Miyagishi M, Taira K, Tahara H and Hamada H: Adenovirus-mediated transfer of siRNA against survivin induced apoptosis and attenuated tumor cell growth *in vitro* and *in vivo*. Mol Ther *10*: 162-171, 2004.
20 Xia W, Li Y, Lou B, Wang PJ, Gao XL and Lin C: Bioreducible PEI-siRNA nanocomplex for liver cancer therapy: transfection, biodistribution, and tumor growth inhibition *in vivo*. J Nanomater *2013*: 1-11, 2013.
21 Xia W, Wang PJ, Lin C, Li ZQ, Gao XL, Wang GL, and Zhao XH: Bioreducible polyethylenimine-delivered siRNA targeting human telomerase reverse transcriptase inhibits HepG2 cell growth *in vitro* and *in vivo*. J Control Release *157*: 427-436, 2012.
22 Guo F, Tian J, Cui M, Fang M and Yang L: Down-regulation of matrix metalloproteinase9 by small interfering RNA inhibits the tumor growth of ovarian epithelial carcinoma *in vitro* and *in vivo*. Molecular medicine reports *12*: 753-759, 2015.
23 Salzano G, Navarro G, Trivedi MS, De Rosa G and Torchilin VP: Multifunctional Polymeric Micelles Co-loaded with Anti-Survivin siRNA and Paclitaxel Overcome Drug Resistance in an Animal Model of Ovarian Cancer. Molecular cancer therapeutics *14*: 1075-1084, 2015.
24 Chandrashekhar C, Pons B, Muller CD, Tounsi N, Mulherkar R and Zuber G: Oligobenzylethylenimine enriches linear polyethylenimine with a pH-sensitive membrane-disruptive property and leads to enhanced gene delivery activity. Acta Biomater *9*: 4985-4993, 2013.
25 Richard I, Thibault M, De Crescenzo G, Buschmann MD and Lavertu M: Ionization behavior of chitosan and chitosan-DNA polyplexes indicate that chitosan has a similar capability to induce a proton-sponge effect as PEI. Biomacromolecules *14*: 1732-1740, 2013.
26 Xie QL, Guo XY, Xianjin J and Wang YY: PEI/DNA formation affects transient gene expression in suspension Chinese hamster ovary cells *via* a one-step transfection process. Cytotechnology *65*: 263-271, 2013.

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