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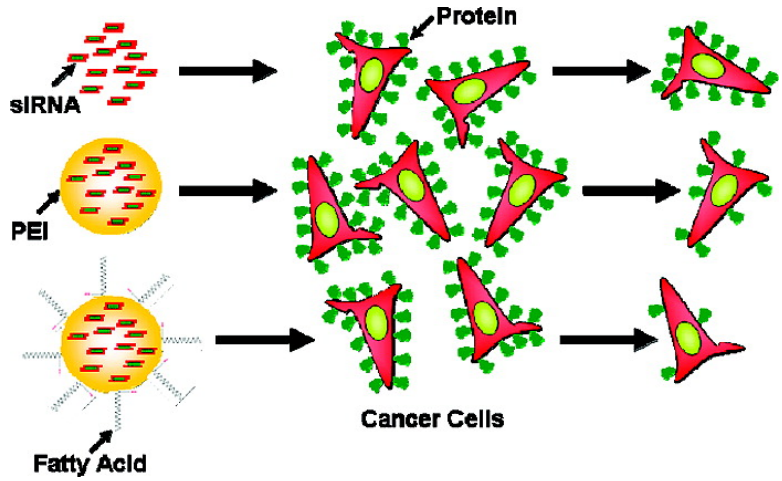

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Formulation and Delivery of siRNA by Oleic Acid and Stearic Acid Modified Polyethylenimine

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Abstract: This study was conducted to formulate a nonviral delivery system for the delivery of small interfering RNA (siRNA) to B16 melanoma cells *in vitro*. For this purpose, oleic and stearic acid modified derivatives of branched polyethylenimine (PEI) were prepared and evaluated. The hydrophobically modified polymers increased siRNA condensation up to 3 folds as compared to the parent PEI. The modified PEIs exhibited up to 3-fold higher siRNA protection from degradation in fetal bovine serum as compared to the parent PEI. The formulated complexes were shown to enter B16 cells in a time-dependent fashion, reaching over 90% of the cells after 24 h, as compared to only 5% of the cells displaying siRNA uptake in the absence of any carrier. A proportional reduction in siRNA cell uptake was observed with reduced polymeric content in the formulations. When used to deliver various doses of siRNA to B16 cells, the modified PEIs were superior or comparable to some of the commercially available transfection agents; the hydrophobically modified polymers gave 3-fold increased siRNA delivery than the parent PEI, ~5-fold higher delivery than jetPEI and Metafectene, a comparable delivery to Lipofectamine 2000, but a 1.6-fold decreased delivery compared to INTERFERin, which was the most efficient reagent in our hands. Using an siRNA specific for integrin α(v), a dose-dependent decrease in integrin α(v) levels was demonstrated in B16 cells by flow cytometry, revealing a more pronounced reduction of integrin α(v) levels for oleic- and stearic-acid modified PEIs. The overall results suggested that the hydrophobically modified PEIs provide a promising delivery strategy for siRNA therapeutic applications.

Keywords: Hydrophobic modification; polyethylenimine; small interfering RNA; RNA interference; cancer targeting

Introduction

RNA interference (RNAi) is a new technology that carries a promising therapeutic potential. In 2001, this post-transcriptional phenomenon was proven to exist as a defense mechanism in mammalian cells;[1] it was shown that a double-stranded RNA of 21−23 nucleotides, known as small-interfering RNA (siRNA), mediated RNAi and effectively silenced target genes.[2] Upon its introduction to cytosol, siRNA binds with specific proteins to conform the RNA-induced silencing complex (RISC).[3] RISC mediates the unwinding of siRNA duplex generating an oligonucleotide

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‡ This manuscript is dedicated to the memory of Dr. John Samuel, who passed away during the completion of this study.

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that binds to the target mRNA in a complementary manner. The resulting dsRNA gets cleaved by RISC and eventually destroyed by the intracellular machinery.[4−6] Since its discovery, siRNA has been developed as a screening tool for cancer studies,[7−9] and has been evaluated as a potential therapeutic agent for a variety of nucleic acid based diseases such as HIV,[10] hepatitis C,[11] and cancer.[12,13] siRNA has been employed to downregulate angiogenic and tumor-associated factors *in vitro* and *in vivo*.[14] It was shown to inhibit the expression of Ki-67 and proliferation in human renal carcinoma cells (HRCC).[15] Inhibition of proliferation and induction of apoptosis of HRCC was achieved by antitelomerase siRNA.[16]

However, developing a stable and efficient delivery system is a major challenge for therapeutic applications of siRNA.[17,18] Optimum delivery strategy aims to reduce off-target effects, to improve siRNA pharmacokinetic and biodistribution after administration, and to promote efficient gene silencing.[19] Viral vectors for siRNA delivery are associated with several drawbacks, such as the possibility of uncontrolled cell proliferation of transduced cells,[20] immune reactions to viral particles,[21] and inflammation of the transduced tissue.[22] Therefore, nonviral delivery systems are considered more favorable in therapy because of their reduced safety concerns and the relatively more convenient preparation techniques.[23−26]

In this study, we examined the potential of hydrophobically modified PEIs for stable condensation of siRNA in polyelectrolyte complexes and their ability to deliver siRNA to B16 melanoma cells *in vitro*. PEI is a cationic polymer that is used extensively in gene delivery studies;[27] it is an attractive carrier for intracellular gene delivery because of its well-established ability to condense nucleic acids via electrostatic interaction between the anionic phosphate in the nucleic acid backbone and the cationic primary, secondary, and tertiary amines of the polymer.[27,28] PEI was shown to be effective in condensing and delivering siRNA to target mRNA *in vitro* and *in vivo*.[29,30] It was able to transfer functionally active siRNA to a variety of cell types including cancer cells.[31] PEI-complexed siRNAs were shown to promote antitumoral effect in U87 orthotopic mouse glioblastoma model growing intracranially.[32] A significant reduction in tumor growth was observed after intraperitoneal administration of PEI-siRNA complexes in mouse model

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targeting the c-erbB2/neu (HER-2) receptor.[30] Additionally, PEI complexes of polyethylene glycol (PEG)-siRNA conjugates targeting vascular endothelial growth factor (VEGF) showed over 95% effective silencing of VEGF expression in PC-3 cells.[33] The efficacy of PEI-mediated siRNA delivery, however, was shown to be dependent on the structure and molecular weight of the PEI used in the formulation.[34]

The relative simplicity in modifying PEI backbone can generate delivery systems that are target specific and possibly less toxic than native PEI. In a recent study, grafting PEG-folate residues to PEI was shown to efficiently deliver functionally active siRNA into KB cells, a cell line originally derived from mouth epidermal carcinoma that highly express folate receptors.[35] In addition, targeting cancer neovasculature *in vivo* and efficient silencing of vascular endothelial growth factor receptor-2 (VEGFR-2) was achieved by PEI nanoparticles that were decorated with Arg-Gly-Asp (RGD) peptides.[36] Hydrophobic modification of PEI to improve cell membrane interactions is an alternative approach for siRNA delivery, as compared to receptor-specific modifications. One study has demonstrated that attaching cholesterol to PEI backbone promoted siRNA stability in water-soluble lipopolyplexes and inhibited VEGF expression in PC-3 cells *in vitro*, and ultimately induced tumor regression *in vivo*.[37] PEI−cholesterol conjugates were also shown to enhance intracellular uptake of DNA and improve transfection efficiency.[38] This system was effective for intratumoral delivery *in vivo* as well.[39,40] These results highlight the importance of lipid components in cationic polymers for efficient siRNA delivery. Based on this reasoning, we conducted this study to further investigate the beneficial effect of hydrophobic modification by grafting simpler aliphatic lipids to PEI. By using PEIs modified with endogenous lipids, we investigated the complexation of the chosen polymers with a model siRNA and assessed their capability to deliver siRNA intracellularly to B16 melanoma cells. An additional target-specific siRNA against integrin α(v) were used to evaluate functional siRNA delivery in this study. Our results indeed showed that PEIs modified with aliphatic lipids provide an improved model for siRNA delivery.

Materials and Methods

Materials. Branched PEI (25 kDa), triethylamine (TEA), octanoyl chloride (CA, 99%), stearoyl chloride (StA, 98.5%), oleoyl chloride (OA, 99%), and linoleoyl chloride (LA, 99%) were obtained from SIGMA (St. Louis, MO). Anhydrous ethyl ether and dichloromethane (DCM) were purchased from Fisher Scientific (Fairlawn, NJ). Deuterated chloroform ($CDCl_3$) and water (D_2O) used as ^1H NMR solvent were from Cambridge Isotope Laboratories (Andover, MA) and Aldrich (Milwaukee, WI), respectively. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from SIGMA (St. Louis, MO). Fetal bovine serum (FBS) was obtained from HyClone (Logan, UT). INTERFERin and jetPEI were purchased from Polyplus-Transfection (New York, NY). Lipofectamine 2000 was purchased from Invitrogen Corporation (Carlsbad, CA), and Metafectene was obtained from Biontex Laboratories (Munich, Germany). 4′,6-Diamidino-2-phenylindole (DAPI) was purchased from Invitrogen Molecular Probes (Oregon). Sequenced siRNA targeting mouse integrin α(v), was purchased from Ambion (sense: 5′-GGCCUUGAAGUGUACCCU-ATT-3′, and antisense: 5′-UAGGGUACACUUCAAGGC-CAG-3′. The scrambled siRNAs used as a model siRNA were *Silencer* Negative Control #1 siRNA (Catalogue #AM4635) and *Silencer* FAM labeled Negative Control #1 siRNA (Catalogue #AM4620), both purchased from Ambion (Austin, TX).

Cell Culture. B16.F10 cell line was grown and propagated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS at 37 °C and humidified 5% CO_2. The cell line was kindly provided by Dr. Mavanur Suresh,

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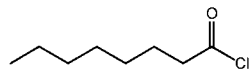
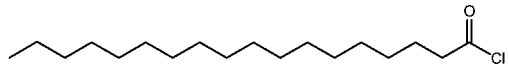
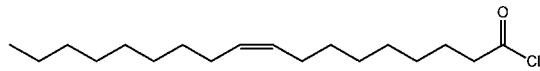
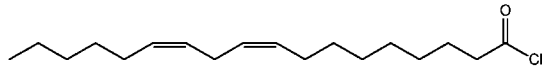
Table 1. PEI Substitution with Fatty Acid Chains

Polymer	Fatty-Acid Substitute	Level of Substitution (Lipid/PEI)
PEI-CA1	 Capryloyl Cloride	28.14
PEI-CA2		10.47
PEI-StA1	 Stearoyl Chloride	2.63
PEI-StA2		1.89
PEI-OA1	 Oleoyl Chloride	4.57
PEI-OA2		2.1
PEI-LA1	 Linoleoyl Chloride	6.04
PEI-LA2		5.55

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Synthesis and Characterization of Hydrophobically-Modified Polymers. A previously described procedure[41] was used to prepare the lipid-substituted PEIs (PEI-CA, PEI-StA, PEI-OA, PEI-LA) by N-acylation of the corresponding lipid chlorides with PEI (Table 1) at fatty acid:ethylenimine ratios of 1:15 and 1:86. Briefly, to obtain lipid-substituted PEI, 50 mg of PEI was dissolved in DCM 2.5 mL under N_2 at room temperature. After addition of 2 μL of TEA, the desired fatty acid was dissolved in 2.5 mL of DCM and gradually added to the PEI solution over a 30 min period. The solution was stirred for 12 h under N_2. Excess of ethyl ether was added to precipitate and wash ($\times$3) the polymer product, which was then dried under vacuum overnight at room temperature. The composition of the reaction products was determined by a 300 MHz ^{1}H NMR spectroscope (Bruker 300 AM; Billerica, MA). The proton shifts specific for fatty acids ($\sim$0.8 ppm; terminal $-CH_3$) and PEI ($\sim$2.5$-$2.8 ppm; $-HN-CH_2-CH_2-NH-$) were integrated, normalized for the number of protons in each peak, and used to obtain the lipid substitutions on polymers.

Determination of siRNA Condensation by Gel Retardation Assay. In sterile Eppendorf tubes, serially diluted polymers ranging from 62.5 ng to 2 μg were added to 2 μg of siRNA in RNase-free water and incubated for 30 min at 37 °C. Three μL of 6$\times$ sample buffer (50% glycerol, 1% bromophenol blue, and 1% xylene cyenol FF in Tris-borate-EDTA (TBE) buffer) was then added to each sample. The samples were loaded onto 2% agarose gel containing 0.2% mg/mL EtBr. Electrophoresis was performed at 130 V and $\sim$52 mA for 15 min. The resulting gels were photographed under UV-illumination. The pictures were digitized and analyzed with Scion image analysis software to determine the mean density of siRNA band. The binding percentage was calculated based on the relative intensity of siRNA in each well to reference wells of naked siRNA without any polymer. Each polymer was tested at least in 2 independent experiments.

Polyanion Competition Assay. The relative ability of complexes to release siRNA was measured after a challenge with the competing polyanion heparin.[42] Complexes were formed in 1:1 polymer:siRNA mass ratios after incubating 2 μg of polymer and siRNA for 30 min, and then were

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incubated with 3.12, 6.25, 12.5, 25, 50, and 100 μg of heparin sulfate at 37 °C for 1 h. The samples were run on agarose gel as described earlier. Results were presented as an average of at least 2 independent experiments.

Zeta Potential Measurement. Complexes of each polymer were formed at various polymer:siRNA mass ratios using 2 μg of siRNA. Zeta potential of each complex formulation ranging from 0.125:1 to 1:1 polymer:siRNA mass ratios was tested in water by 3 serial measurements using Zetasizer 3000 (Malvern, U.K.).

Serum Stability Studies. Naked siRNA (2 μg) was incubated with either 10% or 25% FBS at 37 °C. Samples were analyzed after 1, 4, and 24 h of serum incubation by agarose gel electrophoresis to determine the percentage of intact siRNA. To determine the protective effect of the polymers, complexes were prepared in several polymer: siRNA mass ratios, ranging from 0.03125:1 to 1:1, and incubated with 25% FBS for 24 h. Samples were then incubated for 1 h with 100 μg of heparin to ensure complete release of siRNA from the formulations, and then analyzed for intact-siRNA percentage by agarose gel electrophoresis as described earlier. The results represent an average of at least 3 independent experiments.

Uptake of siRNA by B16 Melanoma Cells. In these experiments, 6-carboxyfluorescein (FAM)-labeled siRNA was formulated in the complexes. 1.4 μg of siRNA was incubated with serially diluted amounts of the polymers ranging from 175 ng to 1.4 μg in PBS for 30 min at 37 °C. B16 murine melanoma cells (5×10^4) in 6-well plates were incubated with complexes containing 100 nM of siRNA in each formulation. In one study, to evaluate the complexes' ability to deliver several concentrations of siRNA, 24-well plates were used to incubate B16 cells with several concentrations of the complexes. When commercial transfection agents were used to deliver a dose range of siRNA, sterile Eppendorf tubes containing 1.4 μg of siRNA in RNase-free water were mixed with equal amounts of Lipofectamine 2000 or Metafectene for 20 min at room temperature. Moreover, 4 μL of jetPEI or INTERFERin were mixed with 1.4 μg siRNA for 20 min at room temperature. Thereafter, serial dilutions of the formulations were prepared in PBS and incubated with B16 cells in 24-well plates for 24 h at 37 °C. Percentage of siRNA-positive cells was determined by fluorescence activated cell sorting (FACS). For this, the samples were acquired on a Becton−Dickinson FACSort flow cytometer (Franklin Lakes, NJ) and the data was analyzed with CellQuest software. At least duplicates of each sample were tested.

Intracellular uptake of siRNA was observed by laser scanning confocal microscopy (LSCM). After growing to 50% confluence, B16 cells were incubated with naked or formulated siRNA for 3 h at 37 °C. The cells were then washed three times with PBS and fixed with 2% paraformaldehyde solution in PBS for 10 min. To stain the nuclei, fixed B16 cells were washed with PBS then DAPI was added for 5 min. The cells were examined using a confocal microscope Zeiss 510 LSMNLO (Carl Zeiss; Jena, Germany) with identical settings for each confocal analysis.

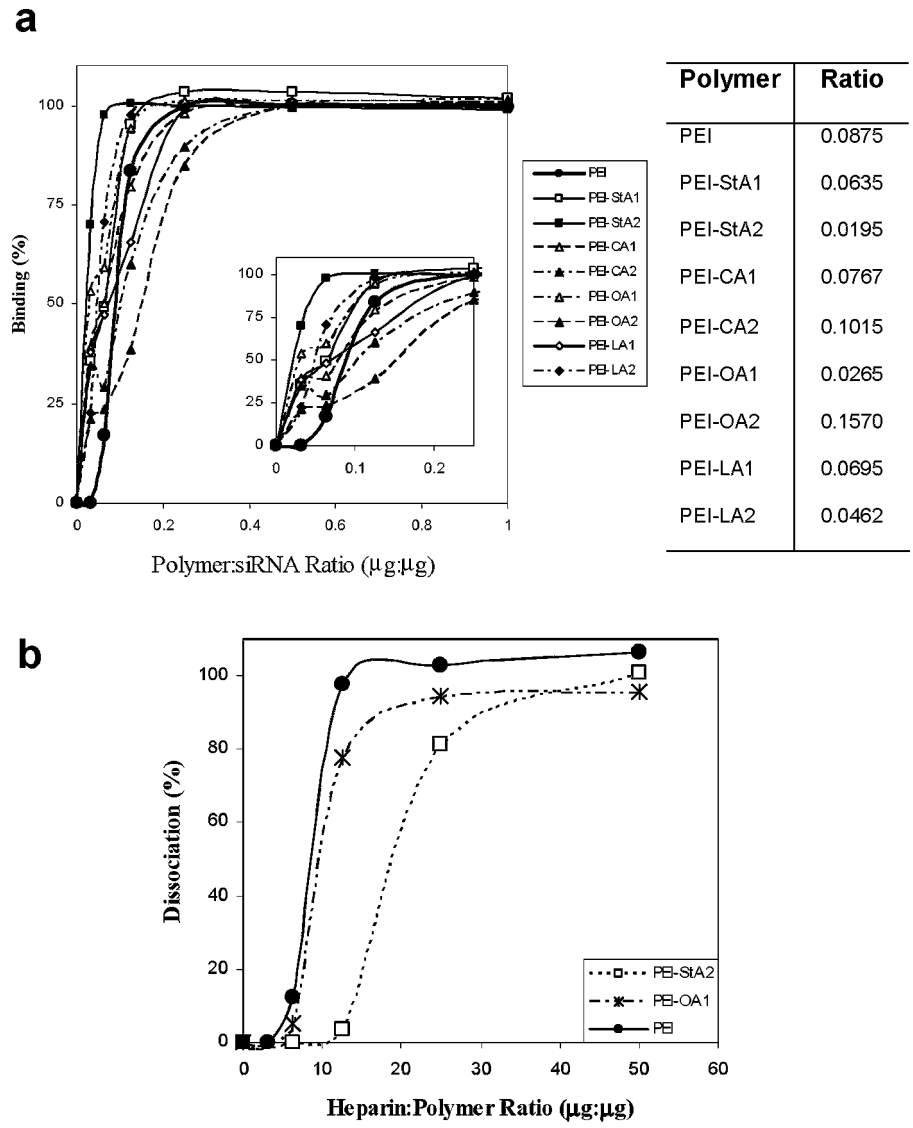
siRNA-Mediated Inhibition of Integrin α(v). To evaluate functional siRNA silencing, we used a validated siRNA against integrin α(v). In 12-well plates, 5×10^5 B16 melanoma cells were incubated with 50, 100, and 200 nM of siRNA either naked or in formulations with 1:1 polymer: siRNA mass ratios at 37 °C. Identical formulations with scrambled siRNA were used as controls. After 36 h, cells were washed and incubated for 30 min at 4 °C with monoclonal antibody against integrin α(v) (clone RMV-7; Santa Cruz Biotechnology). Cells were washed three times with FACS buffer (5% FBS in PBS) to remove excess antibody. For labeling, a secondary FITC-labeled antibody (Santa Cruz Biotechnology) was added and the samples were incubated for 30 min at 4 °C. Then, cells were washed three times with FACS buffer and the levels of protein expression on cell surface were determined by flow cytometry.

Cytotoxicity Study. Polymer cytotoxicity was tested on B16 cells grown in 96-well flat-bottomed microplates. Serial dilutions of each polymer were prepared in PBS and 5 μL of polymer solutions were added to 100 μL of culture medium in each well. Total polymer concentration in each well ranged from 0.35 to 2.8 μg/mL. The plates were incubated for 3, 12, 24, 48, and 72 h for assessment of viability. Each well was then incubated with 100 μL of MTT solution in culture medium (0.5 mg/mL) for 2 h. The formed crystals were dissolved by adding 300 μL of isopropyl alcohol to each well. Optical density was measured at 630 nm using a microplate reader. The results were converted into % viability by using the absorbance from untreated sample as a reference (100%), and expressing the absorbances obtained form the treatment groups as a percentage of the reference value. The results were summarized as mean ± SD of 7 replicates for each sample.

Statistical Analysis. The data were analyzed for statistical significance ($p < 0.05$) by one-way ANOVA. Where indicated, the results were summarized as mean ± SD.

Results

siRNA Condensation by Oleic and Stearic Acid Modified PEI. The hydrophobically modified polymers, prepared by grafting lipid moieties on PEI backbone by N-acylation,[41] were expected to possess sufficient cationic charge to neutralize the anionic charge of an siRNA. To assess the polymers' ability to condense siRNA, gel retardation assay was used to analyze the complexes of siRNA with the native PEI or PEI derivatives (Table 1). As shown in Figure 1a, complete condensation of siRNA could be achieved with all polymers, including PEI, at ~0.4:1 polymer:siRNA ratio. The condensation ability of the derivatives PEI-OA1 and PEI-StA2 were shown to be more efficient than the parent PEI, as indicated by a left shift in binding vs concentration curves in Figure 1a. While ~0.087:1 mass ratio of PEI:siRNA was needed to achieve 50% siRNA binding, PEI-OA1 and PEI-StA2 required 0.026:1 and 0.019:1 polymer:siRNA ratios, respectively. Accordingly,



Polymer	Ratio
PEI	0.0875
PEI-StA1	0.0635
PEI-StA2	0.0195
PEI-CA1	0.0767
PEI-CA2	0.1015
PEI-OA1	0.0265
PEI-OA2	0.1570
PEI-LA1	0.0695
PEI-LA2	0.0462

Figure 1. Assessment of siRNA-polymer complexation by gel migration assay. (a) The indicated polymers at different concentrations were incubated with a fixed amount of siRNA for 30 min at 37 °C, and the complexes were run on an agarose gel. The amount of naked siRNA in each sample was calculated by densitometry and % binding was calculated accordingly. % binding as a function of polymer concentration was plotted and sigmoidal curve fits were added for each polymer. PEI-OA1 and PEI-StA2 showed higher binding efficiency than the parent PEI, given by lower concentrations required for 50% binding of the siRNA. The insert shows an expanded region of the original graph. Polymer ratios required for 50% binding of siRNA are listed in the table next to the graph. (b) Displacement of siRNA from complexes by heparin competition. Complexes of 1:1 polymer:siRNA ratios were incubated for 1 h at 37 °C with increasing concentrations of heparin sulfate, and the amount of free siRNA was determined by gel migration assay to obtain the extent of dissociation. PEI-StA2 complexes (open squares) showed maximum stability in the presence of heparin.

PEI-OA1 and PEI-StA2 were chosen for further analysis due to their better siRNA binding capability.

To evaluate the stability of the formulations, siRNA complexes of PEI, PEI-OA1, and PEI-StA2 were prepared at polymer:siRNA mass ratio of 1:1 to ensure complete condensation of siRNA by the polymers (Figure 1b). Upon addition of serially diluted heparin, 50% of siRNA was displaced from PEI complexes at heparin:polymer mass ratio of ~8.5. However, with PEI-OA1, a heparin:polymer mass ratio of ~9.45 was needed to reach the 50% siRNA displacement value. PEI-StA2 complexes were even more stable, since a ratio of ~18.7 was needed to displace 50% of siRNA from the formulation. Complete dissociation of siRNA from all complexes was observed when the ratio of heparin:polymer reached 50. These findings were in line with the relatively higher siRNA binding of the hydrophobically modified polymers compared to the unmodified PEI.

Zeta Potential of siRNA Complexes. Zeta potential analysis was carried out for the complexes at polymer:siRNA ratios of 1:1, 0.5:1, 0.25:1, and 0.125:1. The results (Figure 2) were consistent with the gel retardation assay where full complexation between siRNA and polymers was detected

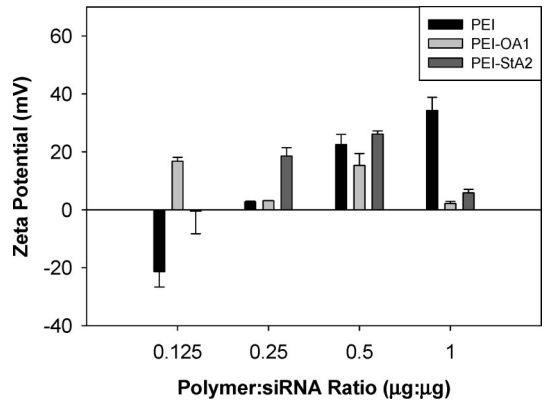


Figure 2. Determination of net surface charge by zeta potential analysis. The complexes were prepared at the indicated 4 different polymer:siRNA ratios, and their zeta potential was determined. The bars represent the averages of 3 different measurements (±SD).

in all formulations at 0.25:1, 0.5:1 and 1:1 ratios. With PEI, the complexes displayed increasing net surface charge proportional to the increasing polymer ratio in the formulations. Although the hydrophobically modified complexes did not show a uniform increase in surface charge, the polymers provided a sufficient net cationic charge on the particles at the polymer:siRNA mass ratios greater than 0.25:1. It was interesting to note that the modified polymers gave an increased cationic nature to the complexes at the lowest polymer:siRNA ratio, again indicating better binding of the polymers to siRNA after hydrophobic modification.

Protection of siRNA in Complexes from Degradation in Serum. Since siRNA is highly sensitive to degradation by nucleases,[43] the protective effect of the complexes against siRNA degradation was assessed in serum. We first investigated the kinetics of naked siRNA degradation in 10% and 25% FBS at 37 °C. As shown in Figure 3a, siRNA was completely degraded in 10% FBS after 24 h of incubation, while 4 h were sufficient for 25% FBS to completely degrade siRNA. When the complexes of different polymer:siRNA mass ratios were incubated in 25% FBS for 24 h, siRNA was fully recoverable and was protected from FBS degradation (Figure 3b) at polymer:siRNA ratios starting from 0.125:1. At the lower ratios 0.0625:1 and 0.03125:1, PEI-OA1 and PEI-StA2 demonstrated a significant protective effect for siRNA compared to parent PEI. The percentages of intact siRNA in PEI-OA1 and PEI-StA2 complexes were ~72 and ~97%, respectively, compared to only 29% in the case of PEI complexes at 0.0625:1 ratio.

Uptake of siRNA Complexes by B16 Melanoma Cells. Naked siRNA uptake by B16 cells was determined *in vitro*. B16 cells were treated with increasing concentrations of naked siRNA (1−100 nM) for 24 h. Our results indicate that the percentage of siRNA-positive cells increased in a

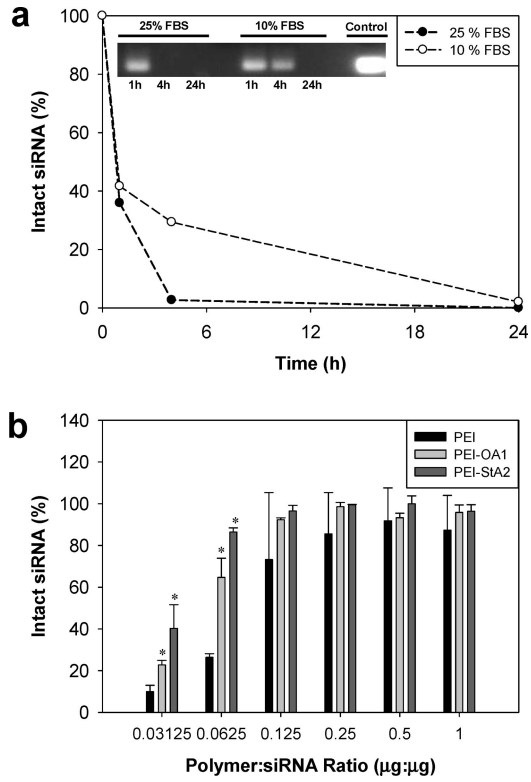


Figure 3. Determination of siRNA stability in presence of serum. (a) A fixed amount of naked siRNA was incubated with 10% and 25% FBS-containing medium for 1, 4 and 24 h, and the amount of intact siRNA was determined by gel migration assay. Control refers to siRNA incubated in the absence of serum. Densitometry was used to calculate the amount of intact siRNA remaining after the incubation period and the intact siRNA remaining was plotted as a function of time in 25% FBS (filled circles) and 10% FBS (open circles). (b) siRNA/polymer complexes, at various polymer:siRNA ratios, were incubated in 25% FBS for 24 h and the amount of intact siRNA was determined by gel migration assay and densitometry. Bars show the mean ± SD of intact siRNA obtained at different polymer:siRNA ratios for 3 different measurements (*; $p < 0.05$). The modified polymers gave a higher amount of intact siRNA at low polymer:siRNA ratios.

concentration-dependent fashion, but only ~5% of the cells displayed significant uptake when incubated with naked siRNA in the absence of any carriers (data not shown). However, when formulated at 1:1 siRNA:polymer ratios, over 98% of B16 cells were positive for siRNA with all polymers, PEI, PEI-OA1, and PEI-StA2 (data not shown). A time-course study of siRNA uptake was then investigated to better characterize the uptake pattern. As shown in Figure 4a, the percentage of siRNA-positive cells with naked siRNA was significant within 30 min of incubation and reached ~20%. It peaked after 1 h reaching 37% and then declined to less than 5% after 24 h of incubation. When cellular uptake of siRNA in 1:1 complexes was assessed, significant increase in the percentage of siRNA-positive cells was detected as compared to naked siRNA reaching over 50% within 30 min

(43) Haupenthal, J.; Baehr, C.; Zeuzem, S.; Piiper, A. RNAse A-like enzymes in serum inhibit the anti-neoplastic activity of siRNA targeting polo-like kinase 1. *Int. J. Cancer* **2007**, *121* (1), 206–10.

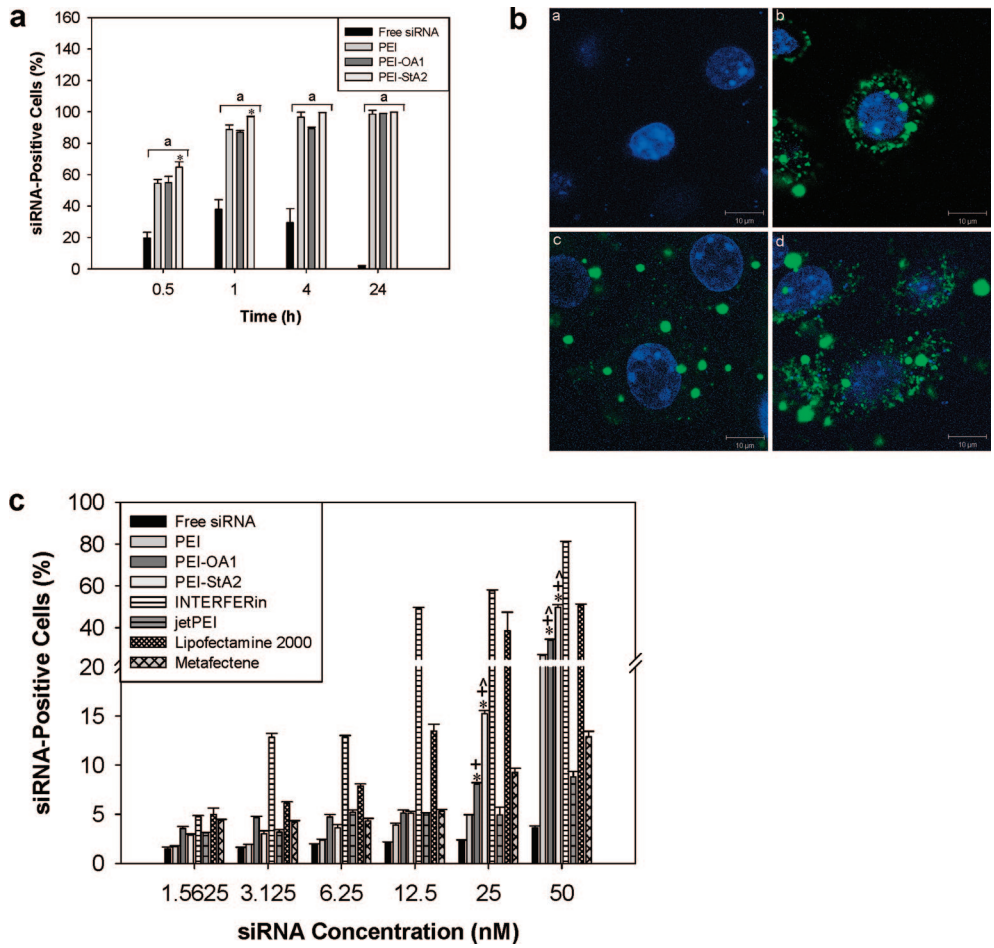


Figure 4. Cellular uptake of siRNA complexes by B16 cells. (a) Determination of siRNA-positive B16 cells over time by FACS. The study was conducted using 100 nM FAM-siRNA either naked or in 1:1 complexes with the polymers. Cells incubated with siRNA for indicated periods of time were harvested and analyzed in FACS. Percentage of siRNA-positive cells of complexed siRNA with the polymers was found to be significantly higher than the naked siRNA (a; $p < 0.05$). PEI-StA2 complexes showed significantly higher increase in siRNA-positive cells compared to the PEI complexes, at early time points (*; $p < 0.05$). Data are shown as the average ± SD of 3 experiments. (b) Confocal microscopy analysis of intracellular siRNA when B16 cells were incubated with 100 nM naked FAM-siRNA (a) or siRNA complexed with (b) PEI, (c) PEI-OA1, and (d) PEI-StA2. Nuclei (blue) are stained with 4′,6-diamidino-2-phenylindole (DAPI), and the scale bar for each image is 10 μm. Note the lack of siRNA for cells incubated with naked siRNA, unlike cells incubated with complexes that yielded distinct particles associated with the cells. (c) Delivery of siRNA by hydrophobically modified polymers and other commercially available carriers to B16 cells. siRNA complexes with the polymers were prepared at 1:1 polymer:siRNA ratios. Serially diluted complexes of 1:1 polymer:siRNA ratios were incubated in 24-well plates with B16 cells for 24 h. Significant increase in siRNA-positive cells was noticed with hydrophobically modified PEIs compared to PEI, (*; $p < 0.05$), jetPEI, (+; $p < 0.05$), and Metafectene (^; $p < 0.05$). Data are shown as an average ± SD of 3 experiments.

of incubation. After 1 h, PEI and PEI-OA1 complexes associated with ~90% of the cells, while PEI-StA2 complex was significantly higher reaching over 96% of the cells. Unlike naked siRNA, the percentage of siRNA-positive cells peaked after 4 h of incubation with the PEI and PEI-StA2 complexes while all complexes sustained this high level after 24 h of incubation.

Since flow cytometry cannot discriminate whether the siRNA was cell-surface bound or internalized, we used confocal microscopy to localize siRNA in B16 cells (Figure 4b). We chose a time point (3 h) where the percentage of siRNA-positive cells was submaximal based on flow cytometry data. The results of the confocal microscopy indicated the presence of the polymeric complexes of siRNA to enter B16 cells after 3 h of incubation, as evident by sequestration of complexes (green dots in Figure 4b, due to FAM-labeled siRNA) in the cytoplasm, possibly inside endosomes, and their localization around the nucleus (blue structures, due to DAPI staining). At this time point, there was no indication of naked siRNA inside the B16 cells. Confocal microscopy did not indicate any qualitative differences among the three polymers (PEI, PEI-OA1, and PEI-StA2) used for complex formation. These results were consistent with our flow cytometry results, showing strong

effect of the polymers to deliver the siRNA intracellularly. A discrepancy between the confocal microscopy results (indicated no uptake at 3 h) and flow cytometry results (indicated some uptake at 4 h), however, was present for naked siRNA (see Discussion on this issue).

The ability of the hydrophobically modified PEIs to deliver a dose range of siRNA to B16 cells was compared to commercially available transfecting reagents including the following: INTERFERin, which was specifically designed for siRNA delivery, jetPEI, which was used to transfect HepG2 cells with antisense RNA,[44] Lipofectamine 2000, which demonstrated significant siRNA-mediated inhibition of tumor growth in human gastric carcinoma *in vitro*,[45] and Metafectene, which was used to mediate siRNA-silencing of PCNA gene in leukemic cell line.[46] All polymer formulations were prepared at mass ratios of 1:1; for INTERFERin and jetPEI, the manufacturer's recommendations were followed for the amount of polymer used in the formulation. B16 cells were pulsed with serially diluted complexes for 24 h where siRNA concentration in the formulations ranged from 1.56 to 50 nM. As shown in Figure 4c, when 25 nM of siRNA were delivered, PEI-OA1 and PEI-StA2 complexes demonstrated ~1.6-fold and ~3-fold increase in the percentage of siRNA-positive cells than the parent PEI, respectively. At 50 nM siRNA, ~1.3-fold and ~2-fold increase the percentage of siRNA-positive cells was observed with PEI-OA1 and PEI-StA2 compared to parent PEI. At this concentration, PEI-OA1 also showed significant increase in the percentage of siRNA-positive cells which was ~2.6-fold higher than Metafectene and ~4.3-fold higher than jetPEI. Similarly, PEI-StA2 demonstrated ~4-fold increase in the percentage of siRNA-positive cells than Metafectene and ~6-fold higher than jetPEI. PEI-StA2 was also found to be as efficient as Lipofectamine 2000 for siRNA delivery to B16 cell line. INTERFERin was the most effective delivery vehicle at all concentrations of siRNA. When compared to INTERFERin, PEI-StA2 was only ~1.6-fold less efficient in cellular uptake while PEI showed at least 3-fold reduction in cell uptake.

Effect of Polymer Ratio in Complexes on siRNA Delivery. In order to investigate the effect of polymer content on siRNA delivery, siRNA complexes were formulated with polymer:siRNA mass ratios of 1:1, 0.5:1, 0.25:1, and 0.125:1. As shown in Figure 5, reducing the polymer ratio in the formulation resulted in proportional reduction in the percentage of siRNA-positive cells. At 0.5:1 ratio, siRNA delivery by PEI-OA1 and PEI-StA2 complexes was shown to be 1.4-fold and 2.4-fold less than PEI, respectively. Further drop in polymeric content results in further reduction in the percentage of siRNA-positive cells. At ratios lower than 0.25:1, the percentage of siRNA-positive cells was comparable in all groups including naked siRNA. These results indicate that although complete siRNA condensation was achieved at 0.25:1 polymer:siRNA ratio with PEI, PEI-OA1, and PEI-StA2, higher polymer ratios are required in the formulation to achieve better siRNA delivery.

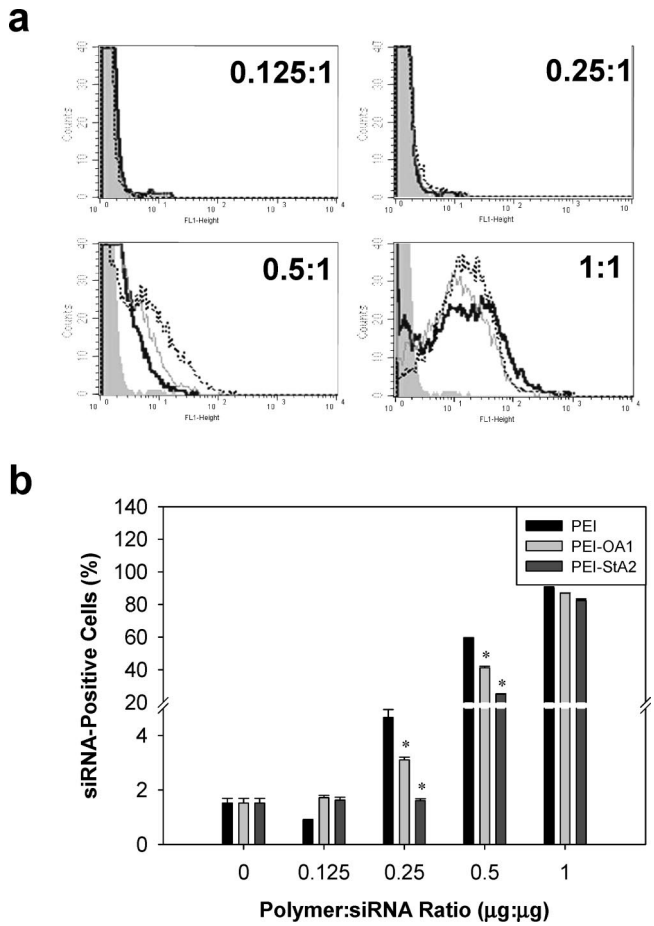


Figure 5. Effect of polymer:siRNA ratio on siRNA delivery. Complexes were prepared at the indicated polymer:siRNA ratios, incubated with the cells for 24 h, and siRNA uptake was subsequently determined by FACS. The study was done using 100 nM siRNA in each sample. (a) Changes in FACS histograms indicative of siRNA-positive cells as a function of polymer:siRNA ratios (indicated in the upper right corner of each histogram). Shaded areas represent background, dotted lines represent PEI complexed group, gray lines represent PEI-OA1 complexed group, and black lines represent PEI-StA2 complexed group. (b) Bars represent quantitative analysis of FACS histograms in (a) to obtain percentage of cells positive for the siRNA. Data are shown as an average ± SD of 3 experiments (*; $p < 0.05$).

Knockdown of Integrin α(v) by siRNA Using Modified PEIs. We examined the ability of hydrophobically modified PEIs to obtain functional siRNA silencing of

(44) Liang, S. J.; Xiao, W. L.; Mu, D. Z.; Wu, H. N.; Wang, X. J. [IL-1beta antisense RNA enhanced sensitivity of HepG2 cells to NK cell mediated cytotoxicity.]. *Xibao Yu Fenzi Mianyixue Zazhi* **2007**, *23* (8), 719–22.

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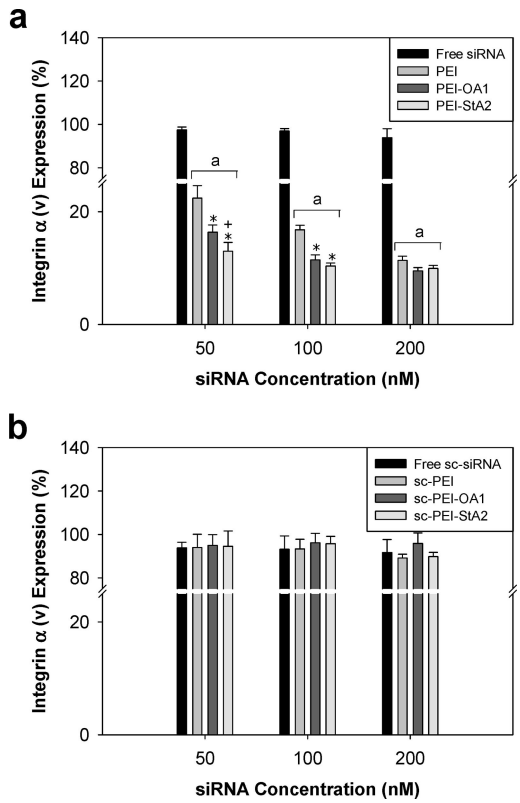

Figure 6. Inhibition of integrin α(v) expression by siRNA complexes in B16 cells exposed to three doses of siRNA. Increasing concentrations of (a) an siRNA targeting murine integrin α(v) or (b) a scrambled siRNA were incubated with B16 cells either naked or in complexes of 1:1 polymer:siRNA ratios for 36 h. Significant inhibition of integrin α(v) expression was noticed with all complexes compared to naked siRNA (a; $p < 0.05$). Significant difference was also present between the hydrophobically modified PEIs compared to PEI (*; $p < 0.05$), and between PEI-StA2 and PEI-OA1 at 50 nM siRNA (+; $p < 0.05$). Data are shown as an average of 3 experiments (±SD).

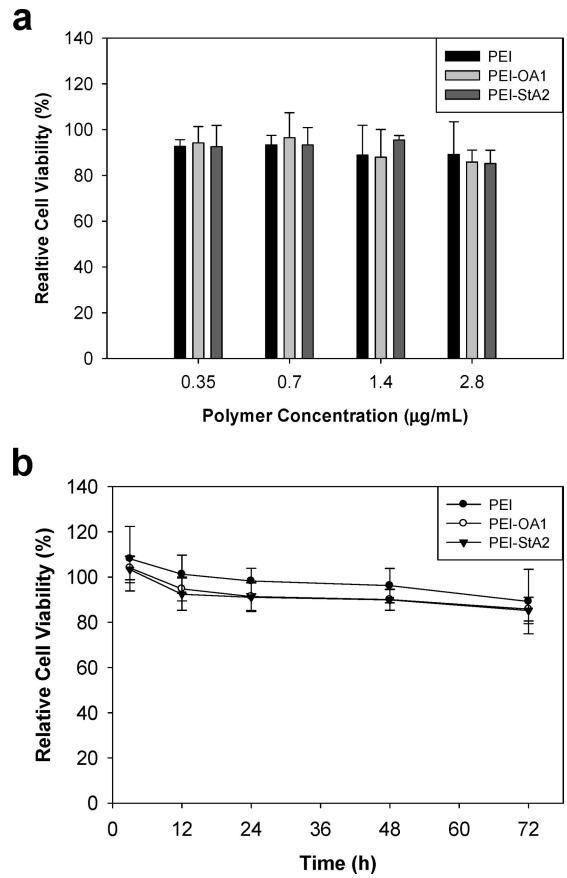

Figure 7. Cytotoxicity study for assessment of toxic effect of the siRNA complexes on B16 cells. The complexes were prepared at polymer:siRNA ratio of 1:1. (a) Complexes at polymer concentrations of 0.35 to 2.8 mg/mL were incubated with the cells for 72 h. (b) Complexes at polymer concentration of 2.8 μg/mL were incubated with the cells over a period of 72 h. This concentration is at least 2-time higher than the polymer concentration used in cellular uptake studies. Percentage of relative cell viability was determined by the MTT assay. Data are shown as mean (±SD) of 7 replicates for each sample.

integrin α(v) subunit on B16 cells. As shown in Figure 6a, all siRNA complexes significantly decreased surface expression of integrin α(v) compared to naked siRNA. Moreover, hydrophobic modification seemed to further enhance siRNA silencing over parent PEI; PEI-OA1 formulation mediated up to 27% reduction in surface expression of integrin α(v), while PEI-StA2 formulation mediated up to 45% reduction in integrin α(v) surface expression compared to parent PEI. At 50 nM siRNA, PEI-StA2 complexes gave marginal yet significant silencing of integrin α(v) as compared to PEI-OA1, but such a difference was not evident at the 100 and 200 nM siRNA doses. A scrambled siRNA, used as a control in this study, did not cause any reduction of surface integrin α(v) levels by naked siRNA or the corresponding siRNA complexes (Figure 6b).

Cytotoxicity Studies. The cytotoxic effect of the polymers on B16 cells was assessed by the MTT assay. Cytotoxicity studies were conducted to explore whether the polymer concentrations used for siRNA delivery had any indirect effect on siRNA uptake due to disruption of cell viability. After 72 h of incubation, the results revealed that PEI, PEI-OA1, and PEI-StA2 were not toxic to B16 cells at the polymer concentrations (<3 μg/mL) used in this study (Figure 7a). The cytotoxicity of the highest concentration was further examined by MTT assay over time and no significant changes in cell viability were noted over 72 h (Figure 7b).

Discussion

siRNA-based therapy is a promising approach for cancer treatment.[12,13] It has been demonstrated that targeting laryngeal cancer cells with siRNA induced early or late stage apoptosis.[47] Moreover, growth of laryngeal cancer has been inhibited *in vivo* when targeted with siRNA.[48] However, successful siRNA delivery has always been one of the major challenges to the therapeutic applications of siRNA in

clinic.[49] The therapeutic potential of siRNA is abrogated by low cellular uptake and poor stability profile; these negative consequences have affected the anticipated move of siRNA-based therapeutics from bench to bedside. Therefore, there has been an increasing interest in developing suitable systems for siRNA delivery.[30−33,35,36]

In our studies, we evaluated the ability of hydrophobically modified derivatives of branched PEI (25 kDa) to condense, protect, and successfully deliver siRNA to B16 melanoma cells *in vitro*. Our findings demonstrated that PEI-OA1 and PEI-StA2 were able to condense siRNA at lower concentrations as compared to PEI (Figure 1a), indicating better binding affinity. This has been confirmed by siRNA displacement using the polyanion heparin (Figure 1b). Yet, we expect that the electrostatic interaction might not be the only mechanism by which hydrophobically modified PEIs form complexes with siRNA. In fact, based on our zeta potential results (Figure 2), the expected increase in the net surface charge proportional to polymer ratio was observed only with the PEI complexes. With modified PEIs, we attributed the variability in surface charge, in spite of the increasing polymer ratio in the formulation, to the relatively flexible three-dimensional conformation of the grafted fatty acids. The flexibility of the fatty acids is able to create a nonuniform surface charge distribution on the particle, leading to unpredictable response in an electric field. Although we did not expect to see a reduction in zeta potential with increasing polymer content, this could be explained by the concomitant increment of the noncationic fatty acid content. Therefore, we suggest that the flexibility of the aliphatic fatty acids could also allow for physical encapsulation of siRNA, which may explain the superior condensing and protective effect of the modified polymers over PEI in spite of variable zeta potentials. The hydrophobically modified complexes, nevertheless, demonstrated a net cationic surface charge which was sufficient for successful cell uptake. This was confirmed by the efficient siRNA delivery by hydrophobically modified PEIs compared to naked siRNA, which was evident in our confocal microscopy study (Figure 4b). Confocal microscopy, more so than the flow cytometry, revealed the beneficial effect of polymers on siRNA; no uptake was visible for naked siRNA with confocal microscopy, whereas some uptake was evident from flow cytometry. Differences in sample preparation procedures, possible quenching of fluorescence in confocal microscopy (as observed by Li, SD et al. [50]), or nonspecific association of siRNA with cells under flow conditions, might have led to such a difference. Although exact reasons for such a difference are unknown in our studies, an independent study also noted some differences between the two methods using the analysis of viral binding and uptake.[51]

The time-dependent decline noted in the percentage of siRNA-positive cells after incubation with naked siRNA might be attributed in part to the instability of naked siRNA in culture medium (Figure 4a). This gradual reduction in the percentage of siRNA-positive cells from 37% after 1 h of incubation to ∼2% after 24 h of incubation was consistent with the serum degradation profile after siRNA incubation with 10% FBS where the levels of siRNA declined from 41% after 1 h of incubation to 2% after 24 h of incubation (Figure 3a). A recent study has related the loss of siRNA activity after incubation with serum to RNase A-like enzymes.[43] Haupenthal et al. clearly demonstrated that the antitumor activity of siRNA directed against polo-like kinase 1 was lost after 2 h of incubation with human serum at 37 °C; this effect was prevented by the addition of RNaseOUT, which is a potent inhibitor for RNase A.[43] On the other hand, the prolonged siRNA delivery and persistent percentage of siRNA-positive cells achieved with siRNA complexes could be also explained by the polymer-protective effect from serum degradation (Figure 4a). Others have addressed the protective effect of polymers on siRNA. It was reported that almost complete degradation of siRNA occurred after 8 h of incubation in 50% FBS, while micellar formulation of PEG-conjugated siRNA in PEI was able to protect the siRNA from degradation even after 48 h of incubation.[33] In addition, it was shown that siRNA degradation in 20% FBS could occur as soon as 30 min of incubation, while cholesterol-conjugated PEI efficiently protected siRNA from degradation.[37] The protective effect of polymer complexation against nucleases will be vital after systemic administration of siRNA. Cholesterol conjugation to branched PEI (1.8 kDa) also showed significant increase in siRNA uptake by PC-3 cells compared to unmodified PEI and promote antiangiogenic effect *in vitro* and *in vivo*.[37] Although a relatively higher polymeric ratio was required in that system to achieve successful siRNA delivery, these results strongly support our findings where hydrophobic modification of PEI improved siRNA delivery to target cells. Although PEI-StA2 complexes did not possess higher positive charge than PEI complexes at the experimental conditions used for cell uptake, they were found to demonstrate a significant increase in the percentage of siRNA-positive cells within the first hour of incubation compared to parent PEI (Figure 4a). Yet, when the ratio of PEI-StA2 was reduced in the formulation (Figure

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5), the percentage of siRNA-positive cells was significantly less than parent PEI. This indicated that excess polymer might be important for siRNA delivery, since it is shown from gel retardation assay, zeta potential analysis, and serum stability studies that polymer:siRNA mass ratio of 0.25:1 was enough for siRNA condensation to occur. This has been previously noticed with DNA, when Derouazi et al. reported that although complete DNA condensation by branched PEI (25 kDa) was detected at N/P ratio of 2, successful gene transfer was only observed at N/P of 6 or more, reaching optimum transfection level at N/P of 13.[52] Presumably, excess polymer enhances the plasma membrane permeability directly, and/or prevents undesirable binding of the complexes to anionic surfaces that might cause loss of the particles.

Consistent with our findings on siRNA condensation, PEI-OA1 and PEI-StA2 demonstrated a significant improvement in delivering a range of siRNA doses as compared to parent PEI. Others have also shown that lipid component in polymers positively influenced siRNA delivery, for example by the cholesterol-substituted PEI in PC-3 cells.[37] Behr and colleagues demonstrated that cationic lipoplexes can promote siRNA delivery to the brain at picomolar level which was significantly higher than linear PEI.[53] We also found that the lipid-containing commercially available transfecting agents, INTERFERin and Lipofectamine 2000 are more efficient in delivering siRNA than jetPEI and Metafectene. We presumed that the hydrophobic moieties could enhance complex−plasma membrane interactions, which may facilitate endocytosis process in turn. Furthermore, the superior ability to condense, protect, and deliver siRNA that was obtained with PEI-StA2 might be related to the chemical structure of stearic acid. The free-rotation property of the saturated carbon atoms in stearic acid was expected to give the molecule more flexibility to move inward or project outward the complex. Hence, it was not surprising to find PEI-StA2 showing the highest siRNA binding, protection, and delivery among the PEI derivatives.

Our results on integrin α(v) inhibition *in vitro* were consistent with the findings on siRNA delivery by flow cytometry. Integrin α(v) is an attractive target for cancer therapy since it forms a larger subunit of many integrins which are directly involved in tumor angiogenesis, growth, survival, proliferation, invasion, migration and metastasis.[54] Integrin α(v) was found to be involved in mediating melanoma tumorigenicity in human[55] and was also associated with higher metastatic ability of murine melanoma B16.F10 cells.[56] We found that the polymeric formulations mediate siRNA silencing of integrin α(v) *in vitro* in a dose-dependent manner compared to naked siRNA, while scrambled siRNA had no silencing effect either naked or formulated. Although both PEI-OA1 and PEI-StA2 showed higher silencing effect of siRNA compared to PEI, PEI-StA2 still provided a significant enhancement of siRNA silencing at a 50 nM concentration over PEI-OA1. This may be due to the higher stability of the formulation provided by PEI-StA2 (Figure 1b) and better protection from nuclease degradation (Figure 3b). Therefore, it is feasible that PEI-StA2 may exert this better efficiency through mediating siRNA protection and stability in the endosomal compartment, which allow for a higher amount of intact siRNA to reach the cytoplasm; the fact that both modified polymers were equipotent at higher siRNA concentrations (100 and 200 nM) may support this hypothesis. The reasons behind this issue, however, remain to be explored. Moreover, attempts to target integrin α(v) in melanoma using monoclonal antibodies have clearly demonstrated that blocking integrin α(v) on tumor cells directly mediated antitumor effects which was not due to the known antiangiogenic effect of integrin α(v) antagonists.[54,57−59] A recent study by Cao et al. used siRNA targeting integrin α(v) in combination with radiotherapy as a strategy for breast cancer therapy, since they noted an upregulation of integrin α(v)β(3) expression on MDA-MB-435 cells after irradiation, leading to radioresistance as compared to integrin α(v)β(3)-negative MCF-7 breast cancer cells. The authors found that siRNA treatment was able to effectively reduce integrin α(v) and integrin α(v)β(3) expression and increase the radiosensitivity of MDA-MB-435 cells.[60] This collective experience indicated that the functional reduction of cell surface integrin α(v) by hydrophobically modified PEIs might be an important target for clinical applications. Our future studies will

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focus on exploring this aspect of the siRNA delivery by hydrophobically modified PEIs.

We consider these polymers promising for therapeutic application, especially that they showed no signs of toxicity over prolonged time of incubation (Figure 7). Although it was reported that up to 50% reduction in cell viability occurred in mouse fibroblasts after 24 h of incubation with 10 μg/mL of PEI,[61] our systems did not display significant toxicity presumably due to (i) the intrinsic resistance of B16 melanoma cells, or (ii) lower concentrations used in our system. The concentrations used in our toxicity assessment were based on concentrations found to be effective for siRNA delivery in our hands. The toxicity issue is not critical for cancerous cells, since toxicity on the target cells will actually be a beneficial effect in addition to the specific siRNA therapy. The toxicity issue, however, is critical for normal cells since the latter will inevitably get exposed to siRNA/polymer complexes after systemic administration, and complexes that can provide good cellular internalization without directly affecting cell viability will lead to more tolerable formulations. Further studies are planned on this issue where the toxicity of the proposed hydrophobic PEIs will be evaluated at higher concentrations.

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Conclusions

Nontoxic polymeric systems were developed for siRNA delivery to B16 melanoma cells based on hydrophobic modification of PEI with oleic and stearic acids. Substituting these long-chain endogenous lipids onto PEI contributed to significantly better binding to siRNA, as well as better protection in a serum-containing medium. These two factors might have contributed to enhancement in siRNA delivery to the cells as compared to parent PEI. We found out that the presence of the hydrophobic moieties was indispensable to formulate stable and efficient delivery systems for siRNA, especially at low concentrations. The desired siRNAs were effectively delivered to almost all cells (>90%) in culture, and a resultant decrease in cell surface integrin $\alpha(v)$ levels was noted by using a functional siRNA against this target.

Abbreviations Used

PEI, polyethylenimine; siRNA, small interfering RNA; RNAi, RNA interference; RISC, RNA-induced silencing complex; HRCC, human renal carcinoma cells; PEG, polyethylene glycol; VEGF, vascular endothelial growth factor; FAM, 6-carboxyfluorescein; FACS, fluorescence activated cell sorting; LSCM, laser scanning confocal microscopy.

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