Impact of Lipid Substitution on Assembly and Delivery of siRNA by Cationic Polymers

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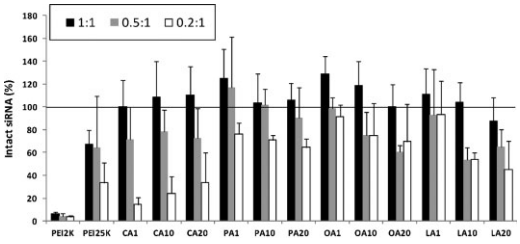
Characterization of a polymer library engineered to enhance their ability to protect and deliver their nucleotide cargo to the cells is reported. The ζ-potential continuously increased with higher polymer:siRNA weight ratio, and the ζ-potential of lipid-modified polymers:siRNA complexes were higher than PEI2 at all ratios. At polymer:siRNA ratio of 1:1, all lipid-substituted polymers showed complete protection against degradation. Lipid-modified polymers significantly increased the cellular uptake of siRNA complexes and down-regulation of GAPDH and P-gp (max. 66% and 67%, respectively). The results indicate that hydrophobic modification of low molecular PEI could render this otherwise ineffective polymer to a safe effective delivery system for intracellular siRNA delivery and protein silencing.



Introduction

Post-transcriptional gene silencing mediated by RNA molecules is currently explored as a unique and promising therapeutic strategy. RNA interference is an evolutionary conserved gene silencing mechanism triggered by small interfering RNAs (siRNAs), which mediate sequence-specific mRNA degradation.[1] In the cytoplasm, siRNAs are incorporated into the RNA-induced silencing complex (RISC) protein complex that contains the Argonaute 2 endonuclease.[2] Only antisense, or guide, strand of the siRNA duplex is retained inside the RISC. Subsequently, the activated RISC uses the guide strand to bind to the complementary region on the target mRNA, followed by cleavage (also called 'slicing') of the complementary mRNA at a discrete position between bases 10 and 11 with respect to the 5' end of the guide strand.[3] The cleavage fragments are then further degraded by cellular RNases.[4] Delivering siRNA against intracellular targets in an effective way, however, has been challenging. The rapid degradation of siRNAs in the extracellular environment with RNase A type nucleases combined with the poor cellular uptake of anionic siRNA has made it a practically incompetent silencing agent on its own.

Advanced materials are needed for therapeutic delivery of siRNA molecules and cationic polymers are attractive for this purpose since they can be tailored to neutralize the anionic charge of nucleic acids and are not hampered by the safety concerns associated with viral carriers. The electrostatic interactions between the anionic phosphates in siRNA and cationic moieties in polymers can assemble the siRNA molecules into nanoparticles suitable for cellular uptake. High-molecular-weight polyethylenimines (PEIs) are one class of polymers that have been shown to be effective siRNA delivery agents.[5–7] While the unprotonated amines of PEI create an opportunity for endosomal escape due to the "proton sponge effect",[8] the high density of positive charges facilitates strong binding to siRNA,

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which in turn creates a strong protection effect against enzymatic degradation. However, even though high molecular weight PEI has been used extensively in vivo,[9] and even commercialized,[10] the toxicity of high molecular weight PEIs has been a hurdle for clinical use.[11–16] Lower molecular weight PEIs present acceptable toxicity profiles but, unfortunately, the small polymers do not display efficacious siRNA delivery into cells. A promising approach to improve nucleic acid delivery into cells is to incorporate hydrophobic moieties onto the polymer amines, since hydrophobic substituents are expected to increase polymer interactions with lipophilic cell membranes and facilitate the uptake of the cargo. Such a beneficial effect of lipid substitution has been established in the context of plasmid DNA delivery for several cationic carriers, where enhanced gene expressions were typically obtained when plasmid DNA was delivered with lipid-substituted polymers.[17] However, whether lipid substitution on polymers are also beneficial for siRNA delivery remains to be investigated. A cholesterol-substituted 1.8 kDa PEI was recently shown to be suitable for siRNA delivery,[18] but the role of the lipid substituent on siRNA delivery could not be assessed, owing to lack of comparative studies with native (i.e., unmodified) polymers. No other lipids apart from the cholesterol were investigated and it is not known if other lipids are functional for siRNA delivery. The present study systematically investigated siRNA delivery systems based on lipid substitution on cationic polymers, with the purpose of (i) identifying advanced materials for siRNA delivery and (ii) better understanding of substituent effects on siRNA complex properties, cellular delivery and targeted gene silencing.

Results and Discussion

A library of lipid-substituted PEIs from a low-molecular-weight PEI (2 kDa; PEI2) was designed in the present study for siRNA delivery. Several lipids from eight to eighteen carbon chains were employed for polymer substitution. We investigated the assembly of siRNA with these lipid-substituted polymers with the purpose of revealing the underpinnings of these advanced materials for effective siRNA delivery. The functional performance of the materials was investigated by assessing the cellular uptake of siRNA and down-regulating a housekeeping enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and a membrane drug transporter (P-glycoprotein), believed to be a key component in multidrug resistance (MDR) against chemotherapy in cancer.

Three series of lipid substitutions (with lipid:PEI2 amine mole ratios of 0.066, 0.1 and 0.2) were performed on PEI2 with caprylic acid (CA), myristic acid (MA), palmitic acid (PA), stearic acid (SA), oleic acid (OA), and linoleic acid (LA) based on a method described elsewhere (Table 1).[19] There was a general increase in lipid substitution as the lipid:PEI ratio was increased during the synthesis (determined by ^{1}H NMR spectroscopy) and the highest number of lipids substituted was achieved with CA at lipid:PEI amine ratio of 0.2 (6.9 CAs/PEI). All polymers remained water soluble, except PEI-SA20 that had the highest number of lipid methylene groups substituted per PEI2 chain (89.0) and, hence, was excluded from the study.

The ability of each polymer to complex with siRNA was determined by binding affinity of the SYBR Green to free siRNA in polymer/siRNA complexes prepared with different weight/weight polymer:siRNA ratios (Figure 1a; 'ratio' denotes a weight ratio throughout the manuscript). Among the polymers derived from lipid:PEI ratio of 0.066, PEI-CA1 showed a lower binding affinity, while all other polymers performed similarly. Among the polymers derived from lipid:PEI ratio of 0.1, there was no difference among the polymers in the siRNA binding. For the polymers derived from lipid:PEI ratio of 0.2, the polymers PEI-CA20, PEI-LA20, and PEI-OA20 showed lower affinity than the others. Complete siRNA binding was typically achieved at poly-

Table 1. Lipid-substituted PEI 2K library.

Polymer	Substituted lipid	Lipid:PEI ratio[a)]	Lipid/PEI[b)]	Methylene/PEI[c)]
PEI-CA1	caprylic acid	0.066	1.1	8.8
PEI-CA10		0.1	2.4	19.0
PEI-CA20		0.2	6.9	56.8
PEI-MA1	myristic acid	0.066	0.6	8.3
PEI-MA10		0.1	1.7	24.1
PEI-MA20		0.2	1.5	20.8
PEI-PA1	palmitic acid	0.066	0.6	9.5
PEI-PA10		0.1	0.8	12.6
PEI-PA20		0.2	1.1	18.0
PEI-SA1	stearic acid	0.066	0.5	8.4
PEI-SA10		0.1	3.6	66.6
PEI-SA20		0.2	4.9	89.0
PEI-OA1	oleic acid	0.066	1.0	18.1
PEI-OA10		0.1	1.7	30.0
PEI-OA20		0.2	2.5	44.1
PEI-LA1	linoleic acid	0.066	1.0	17.3
PEI-LA10		0.1	1.8	33.2
PEI-LA20		0.2	3.2	57.7

[a)]Molar ratios used for synthesis; [b)]Extent of lipid substitution per PEI calculated from ^{1}H NMR analysis; [c)]Extent of methylene substitution per PEI, calculated based on the extent of substitution (from ^{1}H NMR) and number of methylene groups in each lipid.

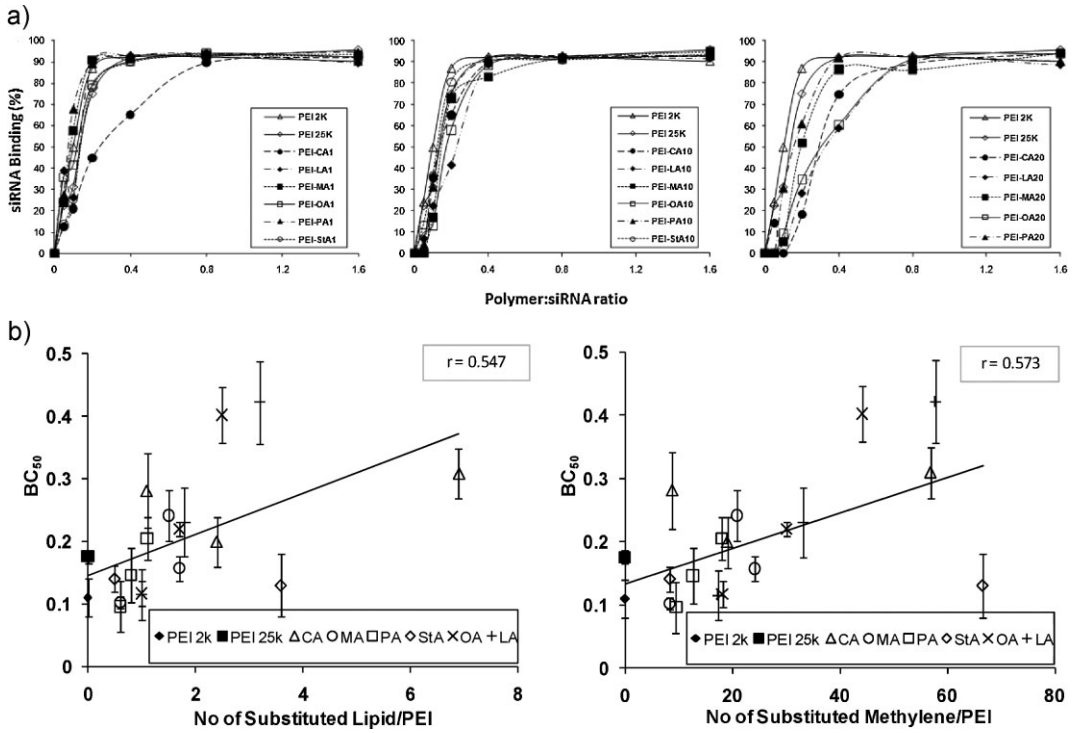

Figure 1. The binding affinity of lipid-substituted polymers to scrambled siRNA. (a) Percentage of siRNA bound to the polymer as a function of weight/weight polymer:siRNA ratio for lipid:PEI ratio of 0.066 (left panel), 0.1 (middle panel), and 0.2 (right panel). (b) Correlation between the polymer:siRNA ratio needed for 50% binding (BC_{50}) and the extent of lipid substitution, based on the number of lipids per PEI (left panel), and number of lipid methylene groups per PEI (right panel). There was a decrease in binding affinity of the polymers with increasing lipid substitution.

mer:siRNA ratio of <0.5. The polymer:siRNA ratio needed for 50% binding (BC_{50}) was calculated for each polymer from the binding curves and correlations between the extent of lipid substitution and the BC_{50} (for number of lipids per PEI and number of lipid methylene groups per PEI), are summarized in Figure 1b. The BC_{50} was generally increased with the extent of lipid substitution (either the number of lipids or lipid methylene groups per PEI), indicating an adverse effect of lipid substitution on siRNA complexation. The higher BC_{50} values are likely to reflect the lower amine content on polymers due to higher lipid substitution, which reduced the polymer affinity to siRNA. Since the results of siRNA binding did not reveal obvious differences among the lipids, the MA, and SA substituted polymers were eliminated from the rest of the study to minimize the scope of the conducted experiments.

Particle size analysis showed a range of 300 to 600 nm for the polymer/siRNA complexes (Figure 2a), with the exception of OA10 at the polymer:siRNA ratio of 10:1 that resulted in larger complexes. Particles formed with the PEI25 were relatively small (220 to 290 nm). No specific trends were observed in the complex sizes; i.e., lipid substitution did not alter the size of the complexes in an obvious way. The siRNA complexes formed with the native PEI2 showed negative zeta (ζ)-potential, even at the highest polymer:siRNA ratio (10:1), indicating weak assembly of the polymer with siRNA in solution (Figure 2b). The PEI25 complexes, on the other hand, showed positive ζ-potential for all ratios studied, indicating stronger affinity of the higher MW polymer to siRNA (as compared to 2 kDa PEI). For all lipid-substituted polymers, a continuous increase in the ζ-potential was observed with increasing polymer:siRNA weight ratio, and all polymers showed positive ζ-potential at the ratio of 10:1 (except PEI-CA1, which is consistent with the lower binding affinity of CA substituted polymers). Figure 2c summarizes the ζ-potentials of complexes with increasing polymer:siRNA ratio; while PEI2 and PEI25 were at the two ends of the spectrum, substituting the PEI2 with lipids brought the ζ-potentials of complexes closer to the ζ-potential of the PEI25 complexes. The PA, OA, LA and CA, in that order, were more effective in enhancing the ζ-potentials of complexes.

Given the sensitivity of siRNA to serum nucleases, and the need to protect siRNA in serum, the siRNA integrity was determined after incubation of complexes in serum. The percentage of intact siRNA was determined after 24 h by using complexes prepared with polymer:siRNA ratios of 0.2, 0.5, and 1:1 (Figure 3). With the polymer:siRNA ratio of 1:1,

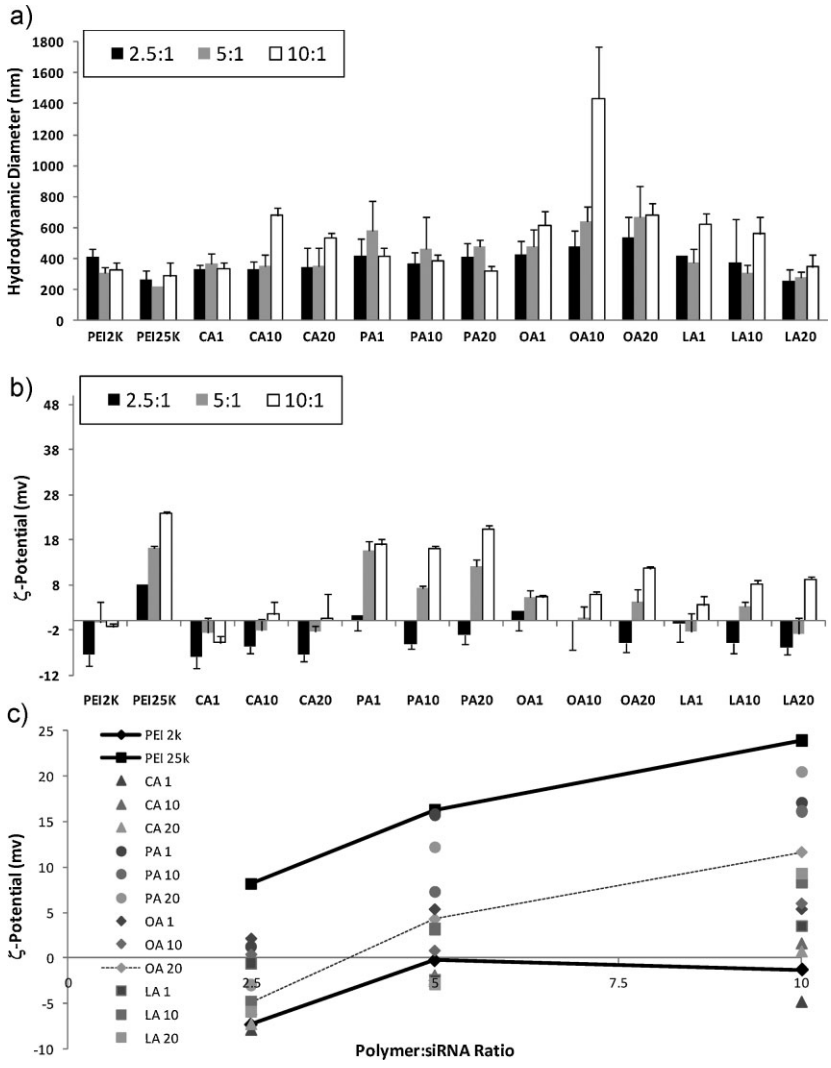

Figure 2. (a) Hydrodynamic diameter, (b) ζ-potential, and (c) ζ-potentials of complexes formed with lipid-substituted polymers in comparison to PEI2 and PEI25 complexes. In (a) and (b), the complexes were formed at weight/weight polymer:siRNA ratios of 2.5:1, 5:1 and 10:1, while the complexes for the serum stability was formed at the ratios of 0.2:1, 0.5:1 and 1:1. While there was no obvious trend in the hydrodynamic diameter of complexes (a), substituting the PEI2 with lipids increased the ζ-potentials of complexes and brought it closer to the ζ-potential of the PEI25 complexes (c).

all lipid-substituted polymers showed complete protection against degradation, while naked siRNA was readily degraded (<5% intact siRNA remaining) and only ≈68% of siRNA bound with PEI2 remained intact under the experimental conditions. At lower ratios, the protective effect decreased for all the polymers studied, with CA-substituted polymers showing minimal protection (consistent with the lower binding affinity and negative ζ-potential for these polymers) and PA- and OA-substituted polymers generally showing better protection. The minimum lipid substitution obtained with polymers (i.e., ≈1 lipid/PEI) was sufficient to provide better protection, whereas higher substitutions generally reduced the integrity of siRNA in complexes.

Cytotoxicity has been a major concern for polymeric systems for siRNA delivery. The low-molecular-weight PEI2 is known to be relatively biocompatible, but lipid substitution may impact its cellular interactions and alter its toxicity. In vitro toxicity of the lipid-substituted polymers was accordingly assessed with the MTT assay after forming polymer complexes with a scrambled siRNA. Human melanoma MDA-MB-435 cells, stably transfected with P-glycoprotein (P-gp) and serving as a model for MDR, were used for this purpose. Figure 4 summarizes the cell viability after 24 h exposure to polymer/siRNA complexes. While PEI2 complexes showed almost no toxic effect (even at 10 $\mu g \cdot mL^{-1}$), the PEI25 complexes were significantly toxic at 5 $\mu g \cdot mL^{-1}$ and higher concentrations. Lipid substitution on PEI2 increased the toxicity of the complexes, especially for CA- and OA-substituted PEI2. However, the observed toxicity of the complexes with lipid-substituted polymers was significantly lower than the PEI25 complexes.

The uptake of polymer/siRNA complexes was evaluated in MDA-MB-435 MDR cells at 2 different polymer:siRNA ratios (2:1 and 8:1, Figure 5). As expected, while PEI2 yielded minimal siRNA delivery into the cells, PEI25 was among the most effective polymers at both ratios (Figure 5a). The complex uptake at 8:1 ratio was higher than the 2:1 ratio and, for most lipid-substituted polymers, >90% of the cells were positive for the siRNA after 24 h of incubation (Figure 5b). When the siRNA delivery was correlated to the extent of lipid substitution, a different pattern was observed for individual polymers at the two ratios employed. At the 2:1 ratio, all polymers showed a higher efficacy in siRNA delivery with higher lipid substitution (Figure 5c). At the 8:1 ratio, on the other hand, this trend was observed only for CA- and OA-substituted PEI2 (Figure 5c). For PA, an inverse correlation was seen between the substitution level and siRNA uptake and no obvious trend was observed for LA-substituted PEIs, even though LA was among the most effective substituent for siRNA delivery. Therefore, while

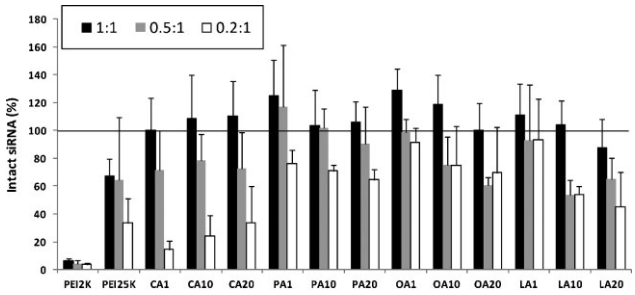

Figure 3. Serum stability of polymer/siRNA complexes; the percentage of intact siRNA was determined after 24 h exposure of complexes to 25% FBS. Lipid-substitution on PEI2 was able to better protect siRNA in serum at the low weight/weight polymer:siRNA ratios (0.2:1), while full protection was afforded at 1:1 ratio.

the lipid substituent clearly helped the cellular uptake of siRNA complexes, a direct relationship between the extent of lipid substitution and the siRNA delivery was dependent on the polymer:siRNA ratio used to form the complexes.

The functional performance of the lipid-substituted PEIs was evaluated based on down-regulation of GAPDH and P-gp in MDA-MB-435 MDR cells. All the down-regulation experiments were performed in presence of 10% fetal bovine serum (FBS). While GAPDH is a housekeeping enzyme commonly used as a prototypical target for silencing,[20,21] P-gp is a drug transporter whose up-regulation has been linked to resistance to chemotherapy in several types of cancers.[22–26] The complexes were prepared at the polymer:siRNA ratios of 2:1, 4:1, and 8:1 for GAPDH silencing (Figure 6a). At the ratio of 2:1, the down-regulation was minimal, and was only observed for PEI25 and LA20 (12–16%). The silencing effect was more significant at the 8:1 ratio, especially for all LA-substituted polymers that gave 23–32% silencing. The toxic effect of PEI25 was significant at this ratio (note the low level of GAPDH recovered), whereas PEI2-based polymers did not result in significant toxicities under equivalent conditions (i.e., polymer concentration of 8 $\mu g \cdot mL^{-1}$). Among other lipid-substituted polymers, only PEI-CA20 showed significant GAPDH down-regulation (≈17%) at this ratio. The down-regulation of GAPDH was higher at the 4:1 ratio as compared to the other two ratios, with almost all polymers showing some effect (Figure 6a). The PEI2-LA20 showed the maximum effect at ≈66%, higher

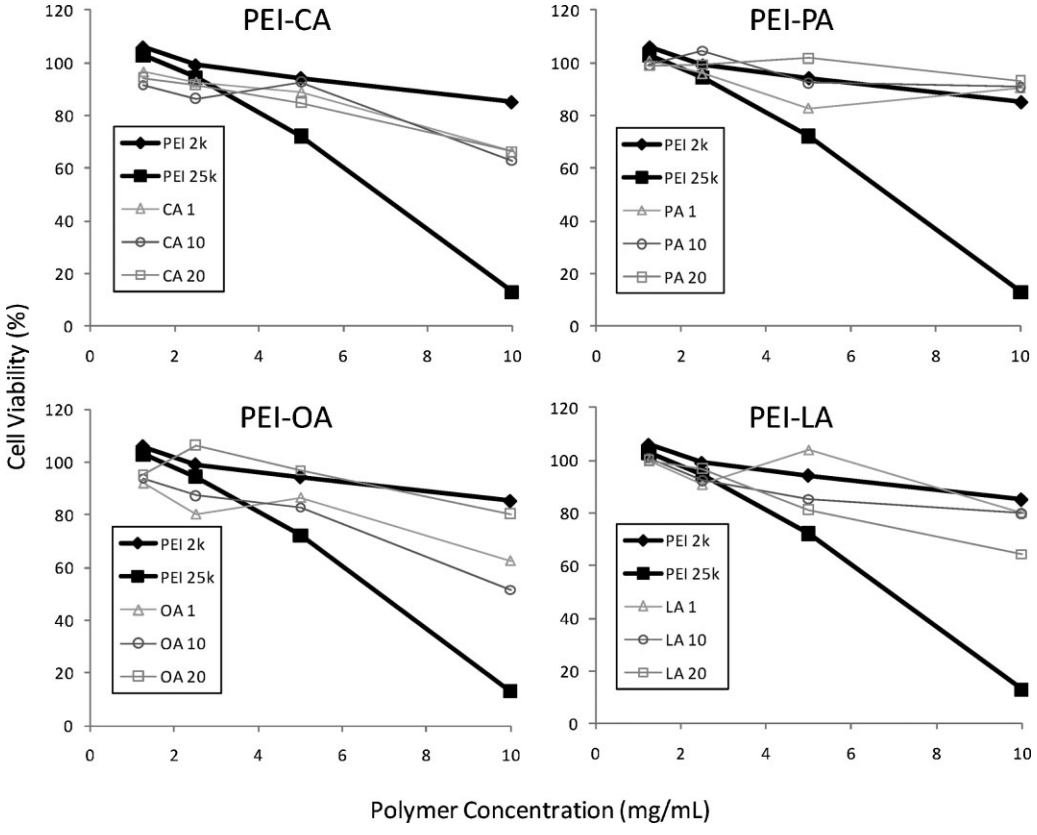

Figure 4. The viability of the P-gp transfected human MDA-MB-435 cells after 24 h exposure to polymer/siRNA complexes prepared using increasing polymer concentrations. While PEI25 was obviously toxic to the cells at concentrations above 2.5 $\mu g \cdot mL^{-1}$, the toxicity profiles of the lipid substituted polymers were similar to the relatively non-toxic PEI2, with OA- and CA-substituted polymers showing most toxicity among the lipid-substituted polymers.

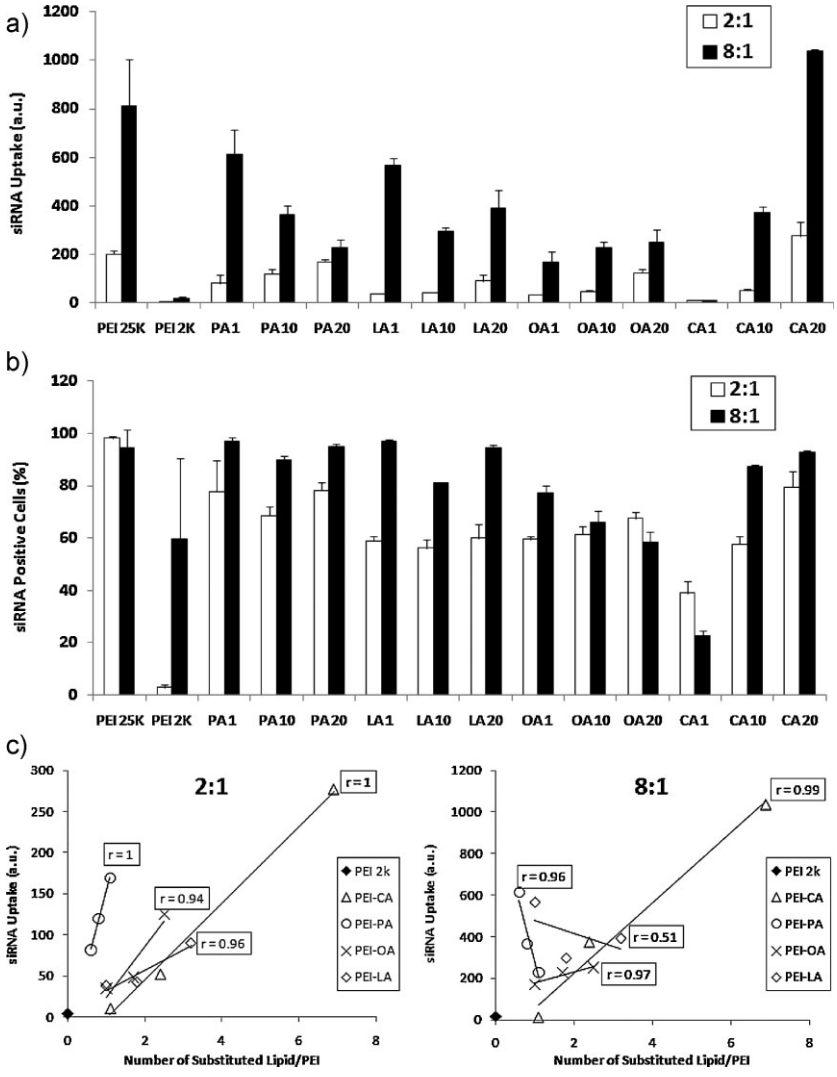

Figure 5. Cellular uptake of polymer/siRNA complexes. (a) The mean fluorescence of the MDA-MB-435 MDR cells after 24 h exposure to complexes formed with FAM-labeled siRNA at weight/weight polymer:siRNA ratios of 2:1 and 8:1. (b) The percentage of cells positive for FAM-siRNA after 24 h exposure to siRNA complexes. (c) The correlation between the extent of cellular uptake [i.e., mean fluorescence from (a)] and the extent of lipid substitution based on the number of lipids per PEI for weight/weight polymer:siRNA ratios of 2:1 (left panel), and 8:1 (right panel). Hydrophobic modification enhanced the siRNA cellular uptake significantly, even more than the uptake with PEI25 (in case of CA20). There was a positive correlation between the substitution level and uptake at ratio of 2:1 for all hydrophobic moieties; such a correlation was only observed for CA- and OA-substituted polymers at 8:1.

than the down-regulation achieved with PEI25 (≈55%). Figure 6b shows the correlation between the substitution level and GAPDH down-regulation for individual polymers. An increase in GAPDH down-regulation was observed with increasing substitution levels for both LA- and CA-substituted polymers, consistent with the trends observed for the siRNA delivery results. In PA-substituted polymers, however, a reverse trend was observed: the highest down-regulation was observed for PA1, similar to the siRNA uptake pattern seen in PA-substituted polymers.

The down-regulation of P-gp by the synthesized polymers is summarized in Figure 7, where the complexes were prepared at the polymer:siRNA ratios of 2:1 and 8:1. The complexes at the ratio of 8:1 generally showed a more effective down-regulation as compared to the 2:1 ratio (consistent with GAPDH results for these ratios; note that 4:1 ratio was not attempted for P-gp). At the polymer:-siRNA ratio of 2:1, the highest down-regulation was achieved with PEI-CA20 (≈43%), followed by PEI-LA20 (35%), both of which were more effective than PEI25 (≈28%) and Lipofectamine 2000 (≈3.6%). At this ratio, the PEI-PA20 was ineffective, and a trend of increasing P-gp down-regulation was observed with an increase in the substitution level in other polymers (Figure 7d). At the polymer:-siRNA ratio of 8:1, the PEI-LA1 was the most effective (≈67%), which was higher than the P-gp down-regulation achieved with the PEI25 (≈61.2%). At this ratio, only PEI-PA20 showed some effect (≈15.4%) among the PA-substituted polymers, while OA-substituted polymers and Lipofectamine 2000 (≈3.9%) were ineffective. Figure 7b and 6c show the correlation between uptake and down-regulation of P-gp for the two polymer:-siRNA ratios used. At the 2:1 ratio, the PEI-CA20 had both the highest uptake and highest down-regulation and this trend was more apparent in OA- and CA-substituted polymers. However, such a positive correlation was not observed for the LA- and PA-substituted polymers. A fairly strong correlation was also observed between the number of substituted methylene groups and P-gp down-regulation. Looking at individual polymers, however, such a strong correlation was only observed for CA-substituted polymers. No specific correlation was observed between ζ-potential and P-gp down-regulation at the ratio of 2:1 (not shown). The correlation between the substitution level and P-gp down-regulation (Figure 7d,e) was different for each polymer at the 8:1 ratio; while LA- and OA-substituted polymers showed lower down-regulation with higher substitution levels, the CA- and PA-substituted polymers showed the reverse trend, but not with a very strong correlation.

Our results showed that increases in number of hydrophobic substitutions on PEI2 were correlated with a decrease in the binding affinity of the polymer to the siRNA. This could be due to interference of the substituted hydrophobic entities with the electrostatic interaction between the polymer and siRNA. However, these hydrophobic groups did increase the ζ-potential of the complexes formed with substituted PEI2s significantly and, in some cases such as the PA-substituted polymers, to the same level as PEI25 complexes. This increase was indicative of better interaction between polymer molecules involved in each particle. Complexes formed with the PEI2 did not show a positive surface charge, even with a polymer:siRNA ratio of 10:1. This was unlike the ability of PEI25 to assemble high ζ-potential complexes at low polymer:siRNA ratios. Adding the hydrophobic entities to PEI2 enhanced the interaction of the polymer molecules to facilitate the formation of highly cationic (i.e., ζ-potential) siRNA complexes. This effect might be the underlying basis of the increased transient toxicity manifested by the complexes, but the observed toxicities were still significantly lower than the toxicity of PEI25 (Figure 4). However, the ζ-potential did not seem to be the driving force for the cellular uptake, as we did not observe a strong correlation between the ζ-potential of the complexes and the corresponding cellular uptake (not shown). On the other hand, increasing the substitution level does seem to have a positive effect on the cellular uptake of the complex. This correlation is especially obvious at the ratio of 2:1 for each lipid (Figure 5c). We can conclude that the hydrophobic substitution on low-molecular-weight PEI significantly increases the cellular uptake of the siRNA complexes, which correlates with the substitution level, without increasing the toxicity of the complexes significantly.

Functional down-regulation of GAPDH expression was best achieved with a polymer:siRNA ratio of 4:1, especially for LA- and CA-substituted polymers (Figure 6). The higher ζ-potential at the ratio of 8:1, which seems to be an advantage in increasing the cellular uptake (Figure 5a) could have became an obstacle since the stronger binding may prevent siRNA availability in free form to reach their site of action.

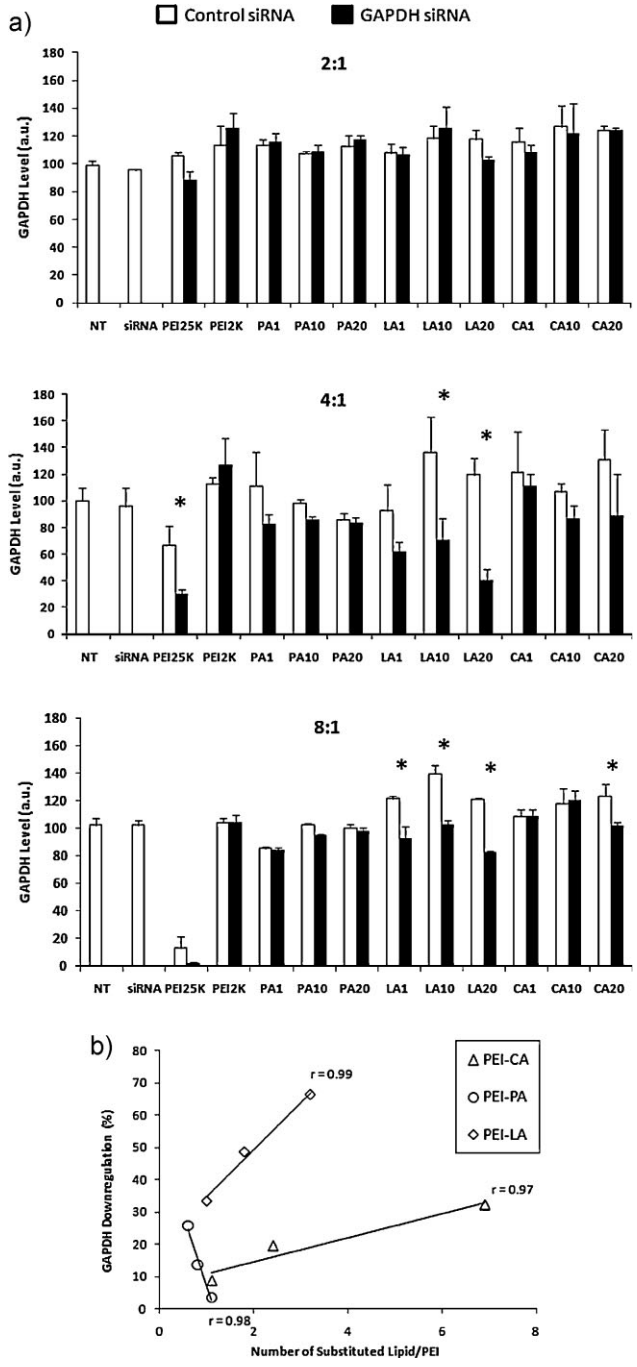

Figure 6. Down-regulation of GAPDH expression by polymer/siRNA complexes. (a) The GAPDH levels in MDA-MB-435 MDR cells after 72 h exposure to polymer/siRNA complexes at weight/weight polymer:siRNA ratios of 2:1 (top panel), 4:1 (middle panel), and 8:1 (bottom panel). The white bars represent GAPDH levels for scrambled siRNA treated cells, whereas the black bars represents cells treated with GAPDH specific Silencer siRNA. NT (No Treatment) refers to cells treated with buffer alone. Asterisks represent significant down-regulation compared to scrambled siRNA treated cells (t-student's test; p 0.05). (b) Correlation between the level of GAPDH down-regulation (represented by the level of GAPDH expression as a percentage of scrambled siRNA treated cells) for weight/weight polymer:siRNA ratio of 4:1 and the extent of lipid substitution based on the number of lipids per PEI. Correlations with 2:1 and 8:1 ratios were not shown due to relatively weaker GAPDH down-regulation. While none of the complexes were effective in down-regulating GAPDH expression at 2:1 ratio, significant down-regulation was observed with some of the lipid-substituted polymers at higher ratios, most notably with LA20 at ratio of 4:1. Naive PEI2 was ineffective in all polymer:siRNA ratios and a positive correlation was observed between the GAPDH down-regulation and the substitution level for more effective CA- and LA-substituted polymers.

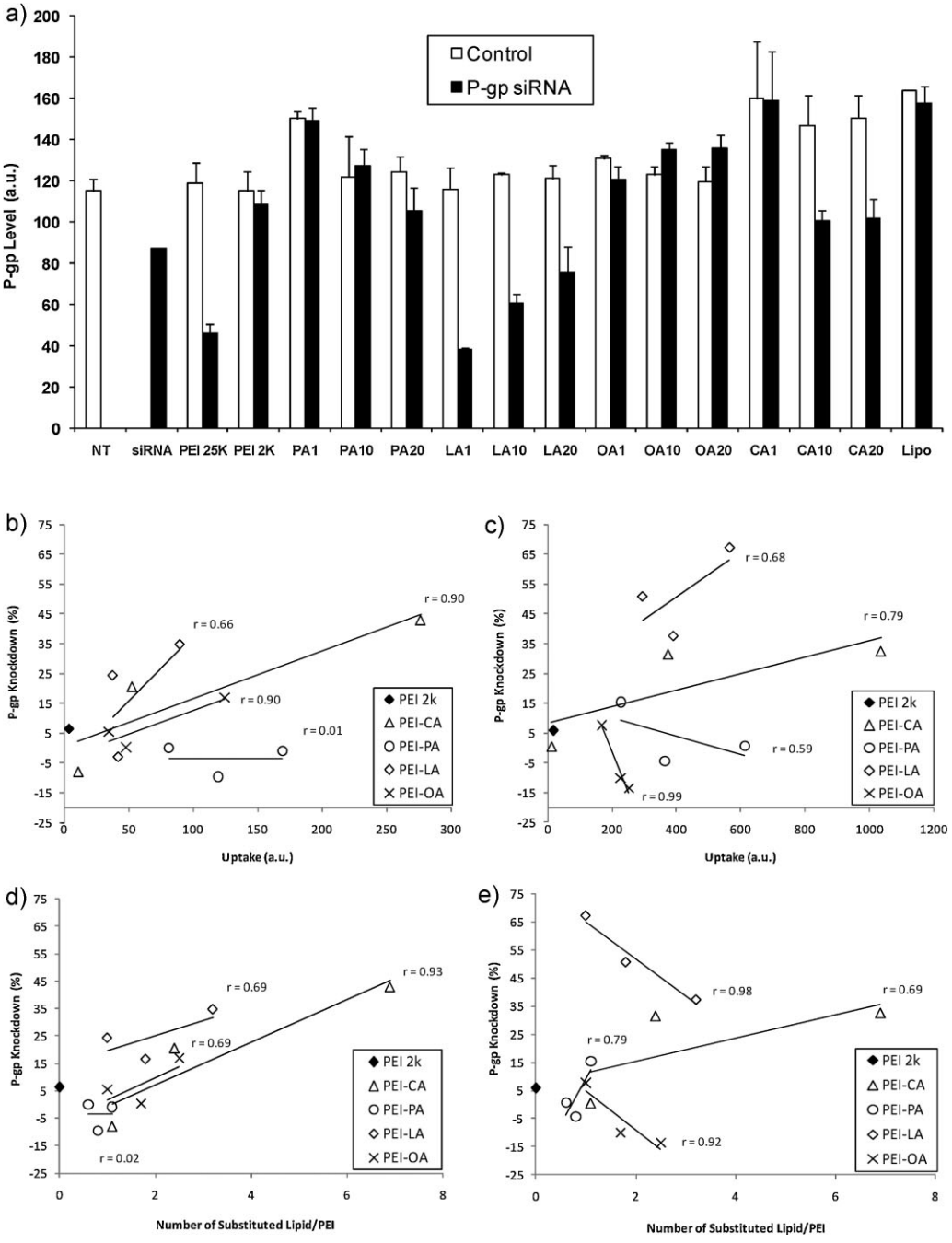

Figure 7. Down-regulation of P-gp expression by polymer/siRNA complexes. (a) The level of P-gp expression in MDA-MD-435 MDR cells after 48 h exposure to complexes. Prepared with ratio of 8:1. The white bars represent P-gp levels for scrambled siRNA treated cells, whereas the black bars represents cells treated with P-gp specific siRNA. NT (No Treatment) refers to cells treated with buffer alone. (b) The correlation between the extent of P-gp down-regulation and the cellular uptake of the polymer/siRNA complexes (data from Figure 5a) for a polymer:siRNA weight ratio of 2:1. (c) The correlation between the extent of P-gp down-regulation and the cellular uptake of the polymer/siRNA complexes (data from Figure 5a) for a polymer:siRNA weight ratio of 8:1. (d,e) The correlation between the extent of P-gp down-regulation and the extent of lipid substitution based on the number of lipids per PEI for the weight/weight polymer:siRNA ratio of 2:1 (d) and 8:1 (e). Highest level of P-gp down-regulation was achieved with CA-substituted polymers at the polymer:siRNA ratio of 2:1, and with LA-substituted polymers at 8:1. At ratio of 2:1, an increase in down-regulation was observed with an increase in uptake, and the extent of lipid substitution. At ratio of 8:1 such a correlation was not observed for all of lipid-substituted polymers.

This could explain the higher down-regulation efficiency achieved at the ratio of 8:1 compared to ratio of 2:1 (because of a higher uptake), and the higher efficacy at the ratio of 4:1 compared to 8:1 and 2:1 ratios (because of more free siRNA available after the uptake), which made the 4:1 the optimal ratio for siRNA silencing. The extent of down-regulation seems to correlate with the level of substitution at the ratio of 4:1; however, this trend was only confirmed for LA- and CA-substituted polymers. In addition to the GAPDH silencing, we evaluated the efficiency of the substituted polymers for down-regulating the therapeutically relevant P-gp expression (Figure 7). Similar to siRNA uptake results, P-gp down-regulation efficiency was higher with ratio of 8:1, except for OA20. There was a strong correlation between the level of substitution and P-gp down-regulation at the polymer:siRNA ratio of 2:1, but not at the 8:1 ratio (Figure 7d,e). This might be due to excessive polymer exposure to the cells, which could saturate the cellular uptake mechanism. At the latter ratio, only CA- and LA-substituted polymers were effective in down-regulating P-gp, which also had the lowest binding affinity to siRNA, which implies easier dissociation of siRNA from the complex to be beneficial for P-gp down-regulation.

Conclusion

In conclusion, lipid substitution on the low-molecular-weight PEIs was shown to lead to functional materials for siRNA delivery and effective gene knockdown with minimal cytotoxicities. The lipid substitution leads to better assembly of siRNA complexes, and higher intracellular delivery of therapeutic siRNA molecules. The gene knockdown efficiency was ultimately dependent on the nature of the substituted lipid, the level of substitutions, and the relative ratio of polymer to siRNA, which had to be tailored and optimized for therapeutic purposes.

Experimental Section

Cell Line

The P-gp transfected human MDA-MB-435 cells were kindly provided by Dr. Robert Clarke (Georgetown University, Washington, DC). Cells were cultured in RPMI 1640 medium (with 10% FBS, 100 $U \cdot mL^{-1}$ penicillin, and 100 $\mu g \cdot mL^{-1}$ streptomycin) in 37 °C and 5% CO_2. Cell culture was considered confluent when a monolayer of cells covered more than 80% of the flask surface. To propagate the cells, a monolayer was washed with Hank's balanced salt solution (HBSS), and subsequently incubated with 0.05% trypsin/ethylenediaminetetraacetate (EDTA) for 10 min at 37 °C. The suspended cells were centrifuged at 600 rpm for 4 min, and were re-suspended in the medium after removal of the supernatant. The suspended cells were either sub-cultured at 10% of the original count or seeded in multiwell plates for testing.

Synthesis of Lipid-Substituted Polymers

The process of lipid-substituted polymers synthesis has been described elsewhere.[19,27] Briefly, a 50% 2 kDa PEI solution (in water) was purified by freeze-drying, and substitution was performed by *N*-acylation of PEI with commercially available lipid chlorides. Acid chlorides were typically added to 100 mg of PEI in anhydrous dimethyl sulfoxide (DMSO). The lipid:PEI ratios were systemically varied between 0.012 to 0.2. The mixture was allowed to react for 24 h at room temperature under argon, after which excess ethyl ether was added to precipitate and wash the polymers. The substituted polymers were dried under vacuum at ambient temperature overnight. Polymers were analyzed by ^1H NMR (Bruker 300 MHz; Billerica, MA) in D_2O. The characteristic proton shift of lipids ($\delta \approx 0.8$; $-C\underline{H}_3$) and PEI ($\delta \approx 2.5$–2.8; $NH-C\underline{H}_2-C\underline{H}_2-NH-$) were integrated, normalized for the number of protons in each peak, and used to determine the extent of lipid substitutions on polymers (Table 1).

SYBR Green Dye Exclusion Assay

The affinity of the polymers to siRNA was assessed by the SYBR Green II binding assay. Briefly, scrambled siRNA solutions (1 $\mu g \cdot mL^{-1}$) were prepared in 0.15 M NaCl (in duplicate) and the polymer solutions (2.5 $\mu g \cdot mL^{-1}$) were added to the siRNA solution in different volumes to create different polymer:siRNA weight ratios from 0.05:1 to 1.6:1. After 30 minutes of incubation at room temperature, 200 μL of the SYBR Green II solution was added to the complexes and the fluorescence of the samples was measured in a 96-well plate (λ_{ex}: 485 nm, λ_{em}: 527 nm) to quantify the amount of free siRNA. The binding curves were generated by plotting the percentage of siRNA bound to the polymer vs polymer to plasmid ratio as described above, and the BC_{50} value was calculated based on the ratio that yielded 50% binding under the experimental conditions.

Size and Zeta (ζ)-Potential Measurements

Polymer/siRNA complexes were formed in 0.15 M NaCl with the same method as above, but with polymer:siRNA weight ratios from 2.5:1 to 10:1. Size and ζ-potential of the complexes were measured at least three times each using a Zetasizer, (Nano ZS; Malvern Instruments, UK).

Serum Stability

Samples of unprotected ("naked") scrambled siRNA, and polymer/siRNA complexes prepared with polymer:siRNA weight ratios of 0.2, 0.5, and 1:1 were incubated in triplicates at 37 °C with 25% FBS for 24 h. At the end of incubation period, 100 μg heparin (2 μL of a 5% solution) and 3 μL of a $5 \cdot 10^{-4}$ M EDTA solution were added to the samples and incubated at 37 °C for 1 h to ensure complete

dissociation of remaining siRNA from the polymers. Samples were then analyzed for the remaining intact siRNA by agarose gel electrophoresis. Samples were loaded onto 2% agarose gel containing 0.2% $mg \cdot mL^{-1}$ ethidium bromide (EtBr). Electrophoresis was performed at 130 V for 30 min. The resulting gels were evaluated and photographed under UV illumination, and digitized pictures were analyzed with Scion image analysis software to determine the density of the siRNA band.

Cytotoxicity Evaluation by MTT Assay

The cytotoxicity of the polymers was evaluated in human MDA-MB-435 MDR cells using an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay in 48-well flat-bottomed plates. Confluent cell cultures were trypsinized, seeded in 48 well plates with 0.2 mL medium in each well, and allowed to reach ≈80% confluence (1–2 d). Polymer/siRNA complexes were prepared using the scrambled siRNA at the ratio of 8:1 and were added to the wells to give final polymer concentrations of 1.25, 2.5, 5, and $10\,\mu L \cdot mL^{-1}$ in triplicate. Cells were incubated for 24 h in their normal maintenance conditions and then 40 μL of MTT solution ($5\,mg \cdot mL^{-1}$ in HBSS) was added to each well. After 2 h of incubation in 37 °C, the medium was removed, and 500 μL of DMSO was added to each well to dissolve the crystals formed. The optical density of the wells was measured with an ELx800 Universal Microplate Reader (BioTek Instruments; Winooski, VT, USA) with cell-less medium as a blank. The absorbance of polymer-treated cells was compared to untreated cells (as 100% viability) and the % cell viability was calculated for each concentration of polymers.

Cellular Uptake of siRNA

Confluent cell cultures were trypsinized, re-suspended as described before and seeded in 48 well plates (0.35 mL in each well) at ≈50% confluence. After 24 h, 200 μL fresh medium was added to each well, followed by the addition of polymer/siRNA complexes. The complexes were prepared in sterile tubes using both 5-carboxy-fluorescein (FAM)-labeled scrambled siRNA and non-labeled scrambled siRNA (as a negative control) with polymer:siRNA ratios of 2:1 and 8:1 (corresponding $36 \cdot 10^{-9}$ M siRNA and 1 and $4\,\mu g \cdot mL^{-1}$ polymer in culture medium). The prepared complexes were added to wells in triplicates and were incubated in 37 °C for 24 h. After the incubation period, cells were washed with HBSS (x3) and trypsinized. A 3.7% formaldehyde solution was added to suspended cells and the siRNA uptake was quantified by a Beckman Coulter QUANTA SC flow cytometer using the FL1 channel to detect cell-associated fluorescence. The percentage of cells showing FAM fluorescence, the mean fluorescence in the positive cells, and the mean fluorescence in the total cell population were determined.

GAPDH Knockdown in MDA-MB-435 MDR Cells

Confluent cell cultures were trypsinized and seeded in 96 well plates (100 μL in each well) at ≈50% confluence in medium containing 10% FBS. The polymer/siRNA complexes were prepared at different ratios of polymer:siRNA in sterile tubes using the Silencer GAPDH siRNA and a manufacturer-supplied negative control siRNA (both from Ambion; Streetsville Ontario) with polymer:siRNA ratios of 2:1, 4:1, and 8:1 (corresponding to 71×10^{-9} M siRNA and 2, 4, and $8\,\mu g \cdot mL^{-1}$ polymer in cell culture medium), and were added to the wells in triplicates. The plates were incubated at 37 °C for 72 h, after which they were transferred to microcentrifuge tubes and were centrifuged at 1 000 rpm for 4 min. The pellets were washed with HBSS and the GAPDH enzyme expression was measured by the KDalert GAPDH Assay Kit (Ambion). Briefly, the cells were lyzed with 200 μL of lysis buffer and were incubated for 20 min at 4 °C. After the incubation time, 90 μL of the KDalert Master Mix reagent was added to 10 μL of the lyzed samples and the fluorescence of the samples were measured using a Fluoroskan Ascent plate reader (Thermo Fisher Scientific, Waltham, MA) with $\lambda_{ex} = 536$ and $\lambda_{em} = 604$ nm.

P-gp Knockdown in MDA-MB-435 MDR Cells

Confluent cell cultures were trypsinized and seeded in 24 well plates (200 μL in each well) at ≈50% confluence in medium containing 10% FBS. The polymer/siRNA complexes were prepared in sterile tubes using both scrambled siRNA (as a negative control) and P-gp specific siRNA (Qiagen; Huntsville, AL, USA) with polymer:siRNA ratios of 2:1 and 8:1 (corresponding to 36×10^{-9} M siRNA and 1 and $4\,\mu g \cdot mL^{-1}$ polymer in cell culture medium), and were added to the wells in triplicates. The plates were incubated in 37 °C for 48 h, after which the medium was removed from the wells, and 100 μL fresh medium was added to each well. Fluorescein isothiocyanate (FITC) labeled P-gp antibody (BD Pharmingen; Franklyn Lakes, NJ, USA) was added to each well (10 μL), and plates were incubated at room temperature for 45 min. The cells were then washed with HBSS (×3) and trypsinized. A 3.7% formaldehyde solution was added to suspended cells and the P-gp down-regulation was quantified by a Beckman Coulter QUANTA SC flow cytometer using the FL1 channel to detect the fluorescence. The percentage of cells showing FITC fluorescence, the mean fluorescence in the positive cells, and the mean fluorescence in the total cell population were determined.

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