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RESEARCH PAPER

Enabling Combinatorial siRNA Delivery against Apoptosis-Related Proteins with Linoleic Acid and α-Linoleic Acid Substituted Low Molecular Weight Polyethylenimines

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ABSTRACT

Purpose Short interfering RNA (siRNA) therapy promises a new era in treatment of breast cancers but effective delivery systems are needed for clinical use. Since silencing complementary targets may offer improved efficacy, this study was undertaken to identify non-viral carriers for combinatorial siRNA delivery for more effective therapy.

Methods A library of lipid-substituted polymers from low molecular weight polyethyleneimine (PEI), linoleic acid (LA) and α-linoleic acid (αLA) with amide or thioester linkages was prepared and investigated for delivering Mcl-1, survivin and STAT5A siRNAs in breast cancer cells.

Results The effective polymers formed 80–190 nm particles with similar zeta-potentials, but the serum stability was greater for complexes formed with amide-linked lipid conjugates. The LA and αLA substitutions, with the low molecular weight PEI (1.2 kDa and 2.0 kDa) were able to deliver siRNA effectively to cells and retarded the growth of breast cancer cells. The amide-linked lipid substituents showed higher cellular delivery of siRNA as compared to thioester linkages. Upon combinational delivery of siRNAs, growth of MCF-7 cells was inhibited to a greater extent with 2.0PEI-LA9 mediated delivery of Mcl-1 combined survivin siRNAs as compared to individual siRNAs. The qRT-PCR analysis confirmed the decrease in mRNA levels of target genes with specific siRNAs and 2.0PEI-LA9 was the most effective polymer for delivering siRNAs (either single or in combination).

Conclusions This study yielded effective siRNA carriers for combinational delivery of siRNAs. Careful choice of siRNA combinations will be critical since targeting individual genes might alter the expression of other critical mediators.

KEY WORDS anti-apoptotic proteins · breast cancer · linoleic acid · lipopolymers · non-viral delivery · siRNA delivery

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INTRODUCTION

RNA interference (RNAi) is a promising strategy for treatment of wide ranging diseases by undertaking silencing of specific gene expression post-transcriptionally (1). RNAi could an alternative strategy for breast cancer treatment since inherent or acquired resistance against conventional chemotherapy is a critical problem resulting in the failure of clinical therapy (2,3). Endogenous RNAi pathway is triggered by a long double strand RNA (dsRNA), which is cleaved to smaller length RNA molecules of about 21-23 nucleotides by the Dicer enzyme. Then, the short interfering RNA (siRNA) is incorporated into the protein complex, named RNA induced silencing complex (RISC), which is able to recognize specific complementary messenger RNAs (mRNAs). Although siRNA is intrinsically produced from long dsRNA in eukaryotic cell, exogenous introduction of synthetic siRNA can also effectively trigger silencing of targeted mRNA in mammalian cells (4–6). For siRNA therapy, the genes involved in cellular apoptosis pathways are promising targets because alteration of apoptosis

regulation in cancerous cells is one of the hallmarks of neoplastic transformation, progression and metastasis (7,8). Among the proteins involved in apoptosis regulation, three important mediators were implicated in aberrant regulation of apoptosis in breast cancer cells, namely Myeloid Cell Leukemia-1 (Mcl-1), survivin and Signal Transducer and Activator of Transcription-5 (STAT5) (9–12). Mcl-1 is an anti-apoptotic member in the Bcl-2 family that was first identified during the differentiation of myeloid leukemia ML-1 cells in monocyte/macrophage pathway (13). Mcl-1 is frequently overexpressed in several cancers and leads to extended survival and apoptosis resistance in breast cancer cells (14–16). Mcl-1 may function as an anti-apoptotic factor by sequestering Bak on the outer mitochondrial membrane and preventing cytochrome c release from the mitochondria (17). Survivin is a member of the inhibitor-of-apoptosis protein (IAP) family and controls cell division and inhibition of apoptosis (18–20). Survivin plays a critical rule in the chemo- and radio-resistance of tumor cells (21,22). It has been reported that survivin is expressed in the majority of human tumor types including lung, colon, gastric, oesophageal, pancreatic, liver, bladder, uterine and ovarian cancers, brain tumors and skin cancers (19), as well as breast cancers (23,24). Finally, STAT5 is a component of diverse signal transduction pathways that control cellular proliferation, differentiation, development and survival (25,26). The abnormal activity of STAT family members is associated with several human malignancies, including hematologic, head and neck, prostate and breast cancers (27). The STAT5A has been particularly reported to be over-expressed in breast cancer (28,29)

The major challenge for successful siRNA therapy is the need for effective and safe siRNA delivery methods. Different delivery systems have been developed for this end using different types of biomaterials. Among the biomaterials, cationic polymers, especially polyethylenimines (PEIs), have been promising since they is able to complex with siRNA avidly, package it into suitable nanoparticulate size, neutralizing the anionic charge of siRNA and making it amenable for cellular uptake (30–33). The low molecular weight (LMW) PEIs with <2 kDa display low cytotoxicity, and could be readily eliminated from circulation, but they show poor transfection efficiency (30,34,35). To overcome this limitation, LMW PEIs have been chemically modified to improve the transfection efficiency while preserving the low toxicity. The lipid moieties on PEI have been particularly beneficial to improve the interactions with the plasma membrane, promote stable complex formation and facilitate endosomal release during the delivery process (36,37). Teo *et al.* reported that LMW-PEI (1.8 kDa) modified polymers were able to condense plasmid DNA into cationic nanoparticles and increases the zeta potential of polymer/DNA complexes. *In vitro* transfection efficiency of modified PEI-1.8 showed a higher transfection efficiency than the unmodified counterpart in SKOV-3 and HepG2 cell lines (38). Octylacrylamide grafted on LMW PEI (600 Da) was able to self-assemble siRNA into nanoparticles and gave higher cellular uptake with 80–90% knockdown of reporter (luciferase) expression in A549-luc cells (39). The hydrophobic modification of LMW PEI (800 Da) with ethyl, butyl, and hexyl acrylate showed good efficiency for siRNA delivery including colloidal stability of the nanoparticles and lytic properties. Among these substituents, PEI modified by ten hexyl acrylates was the most favorable for siRNA delivery due to fast degradation even at neutral pH (40). The modification of branched PEI with longer lipids oleic and stearic acid showed better complex formation and stability in the presence of serum. The siRNA delivery to B16 cells showed effective reduction of integrin α(v) levels by using these modified polymers (41).

In this study, LMW PEIs (0.6–2.0 kDa) substituted with longer aliphatic lipids, linoleic acid (LA), α-linoleic acid (αLA), were investigated for siRNA delivery in breast cancer cells. These lipids differed in the degree of unsaturation (2 for LA and 3 for αLA) in the aliphatic chain; since unsaturated bonds can induce lipid membrane fluidity (42), we wanted to investigate if the increased unsaturation on the lipid substituent would have altered siRNA delivery into cells. The chosen lipids were attached to the polymers with 2 different linkages, namely amide *vs.* thioester linkages. While the amide linkage is relatively stable, thioester can undergo hydrolytic degradation, releasing the lipids from siRNA complexes. We wanted to further investigate the influence of degradable linkages on anchored lipids on siRNA delivery and silencing efficiency. The specific siRNA targets explored were Mcl-1, survivin and STAT5A, delivered either as individual siRNAs or in combination for a more potent effect. The complex properties, cellular uptake and gene silencing ability were investigated to reveal the underlying features of the most promising polymeric siRNA carriers.

MATERIALS AND METHODS

Materials

The 25 kDa branched PEI (PEI25; Mn: 10 kDa, Mw: 25 kDa), 2 kDa PEI (PEI2; Mn 1.8 kDa, Mw 2.0 kDa), 1.2 kDa PEI (PEI1.2; Mn 1.1 kDa, Mw 1.2 kDa), 0.6 kDa PEI (PEI0.6), linoleyl chloride (C18:2 9Z, 12Z; 99%), α-linoleyl chloride, methylthiazolyldiphenyl-tetrazolium bromide (MTT) were purchased form Sigma-Aldrich (St. Louis, MO). Hank's Balanced Salt Solution (HBSS with phenol red) was purchased from Lonza (Walkersville, MD). Dulbecco's Modified Eagle's Medium (DMEM), glutaMAX- 1, penicillin/streptomycin (10,000 U/ml and 10,000 μg/ml) solution and UltraPure™ agarose were purchased from Invitrogen (Carlsbad, CA). Fetal Bovine Serum (FBS) was from VWR (Mississauga, Ontario, Canada). SYBR® Green

II was purchased from Cambrex Bio Science (Rockland, MD). The scrambled siRNA and 6-carboxyfluorescein (FAM)-labelled scramble siRNA (Cat. No. AM4620) was obtained from Ambion (Austin, TX). The siRNA targeting myeloid leukemia cell differentiation protein (Mcl-1; catalogue no: SI02781205; Flexitube® siRNA) was purchased from Qiagen 1 (Mississauga, ON). The siRNA against STAT5A (HSC.RNAI.N003152.12.3) and siRNA against survivin (HSC.RNAI.N001012271.12.1) were purchased from IDT Technologies (Coralville, Iowa).

Cell Culture

Human breast adenocarcinoma cell line, MCF-7 and MDA-231 cell were kindly provided by Dr. Afsaneh Lavasanifar (Faculty of Pharmacy & Pharmaceutical Sciences, U. of Alberta) and Dr. Michael Weinfeld (Department of Oncology, U. of Alberta), respectively. The cells were cultured in DMEM containing 10% FBS, 100 unit/mL Penicillin, 100 μg/mL Streptomycin under a humidified atmosphere, 95% air, 5% CO_2 at 37°C. Cells were weekly sub-cultured using 0.25% trypsin/EDTA and the subcultivation ratio was 1:10.

Synthesis of Lipid Substituted LMW PEIs

Chemical modification of 0.6, 1.2 and 2.0 kDa PEIs was performed via *N*-acylation using aliphatic lipids LA and αLA (Scheme 1**a**), as described before (43). The PEI bearing thioester-linked LA and αLA were obtained by using mercaptopropionic acid end-capped aliphatic lipids (Scheme 1**b**), as described earlier (44). Briefly, in a typical preparation to obtain N-acylated PEIs, αLA (2.0 mM) and 2.0 kDa PEI (1.0 mM) were separately dissolved in anhydrous chloroform and cooled in ice bath for 30 min. To the 2.0 kDa PEI solution, 100 μL of triethylamine (TEA) was added and homogenized. Then αLA solution was then added to 2.0 kDa PEI solution under stirring on ice bath and left stirring over night at room temperature. The crude product of αLA grafted PEIs (hereafter referred as PEI-LA, PEI-αLA) was precipitated (3X) in ice cold diethyl ether and dried under vacuum for 48 h.

Likewise, to obtain thioester-linked lipids, mercaptopropionic acid end-capped aliphatic lipids were grafted onto PEIs after EDC/NHS activation (hereafter referred as PEI-tLA and PEI-tαLA). In typical reaction, tLA (0.2 mmol in 20 mL $CHCl_3$) was mixed with EDC (0.4 mmol in 1 mL $CHCl_3$) and stirred for 1 h at room temperature. Then, NHS (0.4 mmol in 1 mL methanol) was added dropwise and stirred for another 1 h. The activated tLA solution was then added to 1.2 kDa PEI solution (0.1 mmol in 100 mL $CHCl_3$) and the mixture was stirred overnight at room temperature. Reaction solution was concentrated by removing $CHCl_3$ though rotary evaporator. The crude product was precipitated (×3) in ice cold diethyl ether and dried under vacuum for 48 h.

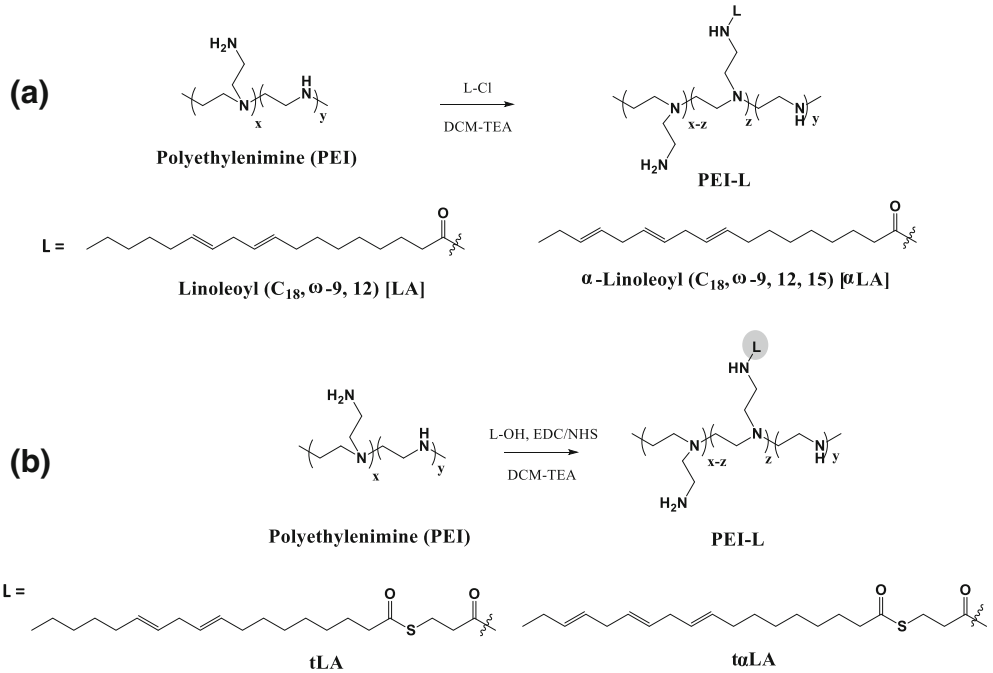
Structural compositions of the lipid-grafted polymers were elucidated through ^{1}H-NMR spectroscopy (Bruker 300 MHz, Billerica, MA) using TMS as an internal standard in D_2O and the corresponding grafting levels were quantified. The polymers were designated based on their MW, the substituent and feed ratio (mol ratio of lipid to PEI used in synthesis): e.g., 2.0PEI-LA9 corresponds to 2.0 kDa PEI with LA grafting with an amide linkage at feed ratio of 9, and 1.2PEI-tLA10 corresponds to 1.2 kDa PEI with LA grafting with a thioester linkage at feed ratio of 10.

Polymer Library Screening of siRNA Targeting Mcl-1, Survivin, and STAT5A

The polymer screening for siRNA delivery was performed with three specific siRNAs (Mcl-1, survivin and STAT5A) and a control (scrambled) siRNA (CsiRNA) in MCF-7 cells. The inhibition of cell proliferation as a result of siRNA treatment was determined by the MTT assay. Cells were seeded in 48 well plates at a density of 3×10^4 cells/well and incubated for 24 h. Polymer/siRNA complexes were prepared in serum-free DMEM at weight ratio of 5. The complexes were added to give a final siRNA concentration of 60 nM in each well and the treated cells were incubated for 72 h under normal maintenance condition. MTT solution (50 μL, 5 mg/mL in HBSS) was added to each well and further incubated for 1 h. The media was removed and 250 μL of DMSO was added to dissolve the formazan crystals. The optical density was measured at 550 nm using ELx800 universal microplate reader (BioTek Instruments; Winooski, VT, USA). The percentage of cell proliferation was calculated as compared to the non-treated group (taken as 100%).

SYBR® Green Assay for Binding Affinity of Polymers with siRNA

The binding affinity of effective polymers with siRNA was determined by SYBR® Green assay. Polymers/siRNA complexes were prepared in ddH_2O at different weight ratios from 0.025 to 5 containing 0.16 μg of CsiRNA in each sample. After incubated the complexes for 30 min, 100 μL of 1X SYBR Green II was added and fluorescence intensity was measured at $\lambda_{ex}/\lambda_{em} = 485/527$ to quantify the amount of free siRNA. The percentage of binding was calculated by taken the siRNA solution without polymers as 0% binding. The sigmoidal curves were generated by plotting %binding *vs.* polymers/siRNA weight ratios and the ratio for 50% binding affinity (BC50) was then calculated.



Scheme I Synthesis of amide-linked LA and αLA substituted LMW PEIs (a) and thioester-linked LA and αLA substituted LMW PEIs (b)

Serum Stability of Complexes by Gel Electrophoresis

To evaluate stability of complexes in serum, the effective polymers/siRNA complexes were prepared at same weight ratio of 5. Briefly, 2 μL of siRNA (10 μM) complexed with polymers in serum free media and incubated at room temperature for 30 min. FBS was then added to the complexes to give the final concentration of 10% FBS and incubated at 37°C for 3, 6, 24 and 48 h. The complexes were mixed with 50% glycerol before loaded to 1% agarose gel containing ethidium bromide. The electrophoresis was carried out in 1X TBE buffer at 120 V, 20 min. The siRNA release from complexes was visualized under UV illumination (Alpha Imager EC).

Hydrodynamic Size and Zeta-Potential Analysis

Hydrodynamic size and zeta potential of polymer/CsiRNA complexes were measured using Zetasizer Nano ZS (Malvern, UK). Polymers/siRNA complexes containing 0.72 μg CsiRNA were prepared in ddH_2O at weight ratio of 2.5, 5 and 10. The complexes were diluted with ddH_2O to the final volume of 1 mL before measured hydrodynamic size and zeta potential.

Cellular Uptake of siRNA Nanoparticles

Cellular uptake of siRNA complexes was evaluated in MCF-7 and MDA-231 cells with the effective polymers from the polymer library screening. Cells were seeded in 24 well plates at the density of 7×10^4 cells/well (in complete cell culture medium) for 24 h before transfection with siRNA complexes. Polymers/siRNA complexes were prepared in serum-free media at weight ratio of 2.5, 5 and 10 using FAM-labeled scrambled siRNA. The complexes were added to give the final concentration of siRNA at 60 nM in each well. After 24 h of transfection, cells were washed three times with HBSS, trypsinized and fixed in 3.7% formaldehyde solution (in HBSS). The cellular uptake was quantified by flow cytometry (Cell Lab Quanta^{TM} SC; Beckman Couter) using FL-1 channel. The mean fluorescence in the total cell population and percentage of fluorescence-positive cells were determined. The autofluorescent cell population was calibrated by gating no-treated group to 1–2% of the total cell population.

For confocal microscopy study, MCF-7 cells were seeded on cover slips in 24 well plates for 24 h before transfection. Polymers/FAM-labeled siRNA complexes were prepared at weight ratio 5 and added to wells at final concentration of 60 nM. At designated time points, cells were washed with HBSS (×3), fixed in 3.7% formaldehyde solution for 15 min and washed with HBSS. The cytomembrane was stained with rhodamine-labeled wheat germ agglutinin (1 μg/mL in HBSS) for 30 min and then washed with HBSS (2X) and ddH_2O (1X). The cover slips were mounted on glass slides with mounting media containing DAPI. The internalization of complexes was observed under confocal laser scanning microscope (LSM710, Carl Zeiss AG, Oberkochen, Germany) and the amount of polymers/siRNA complexes per cell was determined by Imaris software (Bitplane, Belfast, UK).

Combinatorial siRNA Targeting against Mcl-1, Survivin and STAT5A

The effect of single and combinatorial siRNAs on cell proliferation was determined by the MTT assay. Cells

were transfected with individual siRNAs, and combinations of Mcl-1/survivin, Mcl1/STAT5A and survivin/STAT5A siRNAs using 2.0PEI-LA9, 1.2PEI-αLA4, 1.2PEI-tLA10 and 1.2PEI-tαLA6 polymers (see Results for the reason for the choice of this polymer). Lipofectamine™ 2000 and PEI25 were used as positive controls. MCF-7 and MDA-231 cells were seeded in 48 well plates 24 h prior to transfection. Polymers/siRNAs complexes were prepared at weight ratio of 5 and then added to give the final concentration of 60 nM in each well. After incubating the transfected cells for 72 h, MTT solution was added to the wells and processed as above. Percent inhibition of cell proliferation was calculated by taking no-treated cell as a control (taken as 100%).

Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

The reduction of mRNA levels after treatment with specific siRNAs was investigated by qRT-PCR. MCF-7 cells were seeded in 6 well plates and incubated for 24 h. The single target siRNAs and combinatorial siRNAs were complexed with 2.0PEI-LA9 or 1.2PEI-αLA10 at weight ratio 5 and added to give the final concentration of 60 nM in each well. After incubation under normal culture conditions for 24 and 48 h, the medium was removed and cells were washed with HBSS and 1 mL of Trizol® was added to lyse the cells. Chloroform was added to lysed cell solution, vigorously mixed and incubated at room temperature for 3 min. The solution was centrifuged to collect the aqueous phase. RNA was precipitated by adding isopropanol and then RNA pellets were washed with 75% ethanol. RNA pellets were dissolved in RNase free water and the concentration was determined using Nanodrop (Thermo Fisher Scientific, Wilmington, DE). RNA was then reversed transcription to cDNA using random hexamer primer, oligo (dt) primer and dNTP mix at 65°C for 5 min. Synthesis buffer (5×), DTT (0.1 M), RNAout RNase inhibitor (1.8 U/μL) and MMLV RT enzyme were then added and samples were incubated at 25°C for 10 min, 37°C for 50 min, and 70°C for 15 min. qRT-PCR was performed on StepOnePlus™ RT-PCR system (Applied Biosystems, Foster City, CA, USA) and quantified by using SYBR green master mix with ROX (MAF Center, University of Alberta, Edmonton, AB, Canada). Beta actin (forward primer: 5' CCA CCC CAC TTC TCT CTA AGG A 3′; reverse primer: 5′ AAT TTA CAC GAA AGC AAT GCT 3′) was used as housekeeping gene to evaluate transcript of Mcl-1 (forward primer: 5′ GTG CCT TTG TGG CTA AAC ACT TG 3′; reverse primer: 5' ACG AGA ACG TCT GTG ATA CTT TCT G 3′), Survivin (forward primer: 5′ TGA GAA CGA GCC AGA CTT GG 3′; reverse primer: 5′ ATG TTC CTC TAT GGG GTC GT 3′) and STAT5A (forward primer: 5' CCG ACG GGA CCT TCT TGT TG 3′; reverse primer: 5' TGC GTT CCG GGG AGT CAA AC 3′). The amplification cycle was performed by heating samples at 95°C for 5 min and followed with 40 cycles of denaturation at 95°C for 10 s and annealing/elongation at 60°C for 30 s. The mRNA expression level was determined by comparing threshold cycle value (CT) of target gene with beta actin and relative quantity (RQ) were reported as fold change compared to no-treated group.

Statistical Analysis

All results were presented as mean ± standard deviation (SD) of triplicate sample. The statistical significant of data were analyzed using unpaired student's t test at the significance level of 0.05. The significance was determined by comparing target siRNA treated group with no-treated group or CsiRNA treated group as control.

RESULTS AND DISCUSSION

The delivery by an effective and safe carrier is a critical requirement for clinical translation of the siRNA therapy. The carrier should able to deliver siRNAs targeting different mRNAs and preferably deliver multiple siRNAs in the case of combinatorial siRNA therapy (45) in order to overcome the limitations of single agent therapies. From previous reports, lipid substituted LMW PEI efficiently delivered specific siRNAs to different cell lines (46–48). We prepared a new lipid-substituted LMW PEI library (Fig. 1) and investigated their delivery efficacy with three siRNAs targeting genes involved in the apoptosis pathway, namely Mcl-1, survivin and STAT5A in MCF-7 cells. The lipid substituents were LA, which was previously found to be effective in other breast cancer cells (49), and αLA, which contains an additional degree of unsaturation to induce more disorder in lipid membranes. The lipid-substituted polymers were constructed with the conventional amide linkage, but also with a thioester linkage to explore the influence of this labile linkage on siRNA delivery features. The level of substitution on the polymers were controlled by the lipid/polymer feed ratio during synthesis; the feed ratios as well as the final composition of the polymers, are shown in Fig. 1. It was evident that the feed ratio effectively controlled the extent of lipid substitutions in the designed polymer library (Fig. 1b). With both lipids LA or αLA, a similar lipid substitution pattern was obtained with the *N*-acylation reaction irrespective of the MW of the PEI employed. Thioester substitution of both lipids also followed a similar pattern, where the extent of substitutions varied similarly for both chemistries (Fig. 1b).

(a)

Polymer ID	Feed Ratio (mol/mol)	Calculated, NMR (mol/mol)	Polymer ID	Feed Ratio (mol/mol)	Calculated, NMR (mol/mol)
0.6PEI-LA1	1.0	0.3	0.6PEI-αLA1	1.0	0.1
0.6PEI-LA2	2.0	0.7	0.6PEI- αLA2	2.0	0.8
0.6PEI-LA4	4.0	1.1	0.6PEI- αLA4	4.0	2.3
1.2PEI-LA2	2.0	0.4	1.2PEI- αLA1	1.0	0.7
1.2PEI-LA4	4.0	1.6	1.2PEI- αLA2	2.0	0.9
1.2PEI-LA6	6.0	2.5	1.2PEI- αLA4	4.0	2.4
1.2PEI-LA8	8.0	3.5	1.2PEI- αLA6	6.0	3.2
2.0PEI-LA1.5	1.5	0.6	2.0PEI- αLA2	2.0	1.3
2.0PEI-LA3	3.0	1.9	2.0PEI- αLA4	4.0	2.7
2.0PEI-LA6	6.0	2.3	2.0PEI- αLA8	8.0	3.6
2.0PEI-LA9	9.0	3.2			
1.2PEI-tLA4	4.0	2.7	1.2PEI- tαLA2	2.0	0.8
1.2PEI-tLA6	6.0	3.1	1.2PEI- tαLA4	4.0	1.9
1.2PEI-tLA10	10.0	6.5	1.2PEI- tαLA6	6.0	2.7

(b)

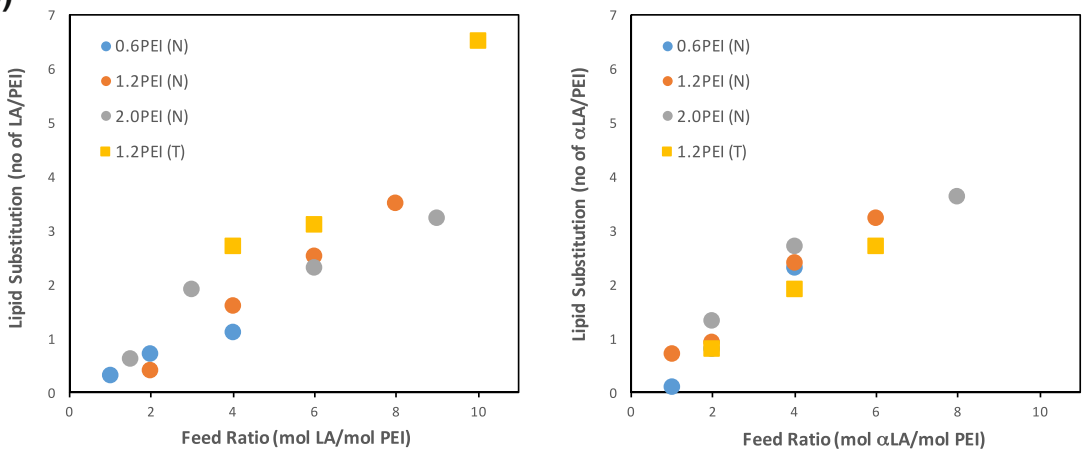


Fig. 1 (**a**) The polymer library prepared with the LA and αLA substituents, summarizing the feed ratio (i.e., lipid to PEI mol ratio) used during the synthesis and the resultant level of substitution on the polymers prepared for this study. (**b**) Correlation between the feed ratios and the final lipid substitutions for LA- substituted (**left**) and αLA-substituted polymers (**right**).

Screening for Effective Polymers to Deliver Mcl-1, Survivin and STAT5A siRNAs

To identify effective polymers among the prepared library, the polymers were used for siRNA delivery with the intention of inhibiting growth of breast cancer MCF-7 cells. This endpoint provides a direct measure of the intended use the siRNA therapy, where the treatment with a specific siRNA will Polymer ID Feed Ratio ideally inhibit growth of malignant cells. A CsiRNA treatment was employed with each polymer as negative control treatment, to rule out non-specific toxicity associated with the siRNA complexes. All cell growth data was normalized against non-treated cells (taken as 100%) to reveal the differences between CsiRNA treatment and specific siRNA treatments (Fig. 1S). The heat plot generated from all siRNA treatments (in term of percent inhibition of growth over the CsiRNA treatments) is summarized in Fig. 2. For LA6 and αLA-substituted polymers, the transfection efficiency depended on the lipid substitution level, where the transfection efficiency was improved at higher lipid substitutions for both lipids. This result was in line with previous reports that showed higher lipid substitutions (with caprylic acid and LA) on 2.0 kDa PEI improved transfection efficiency of siRNA (45,50–52). It is noteworthy that the lower MW PEI substituents were not effective for siRNA delivery, and that amide and thioester linkages functioned equally well in this functional assay. The PEI with intermediate MW (1.2 kDa) appeared to act as a better delivery system after lipid

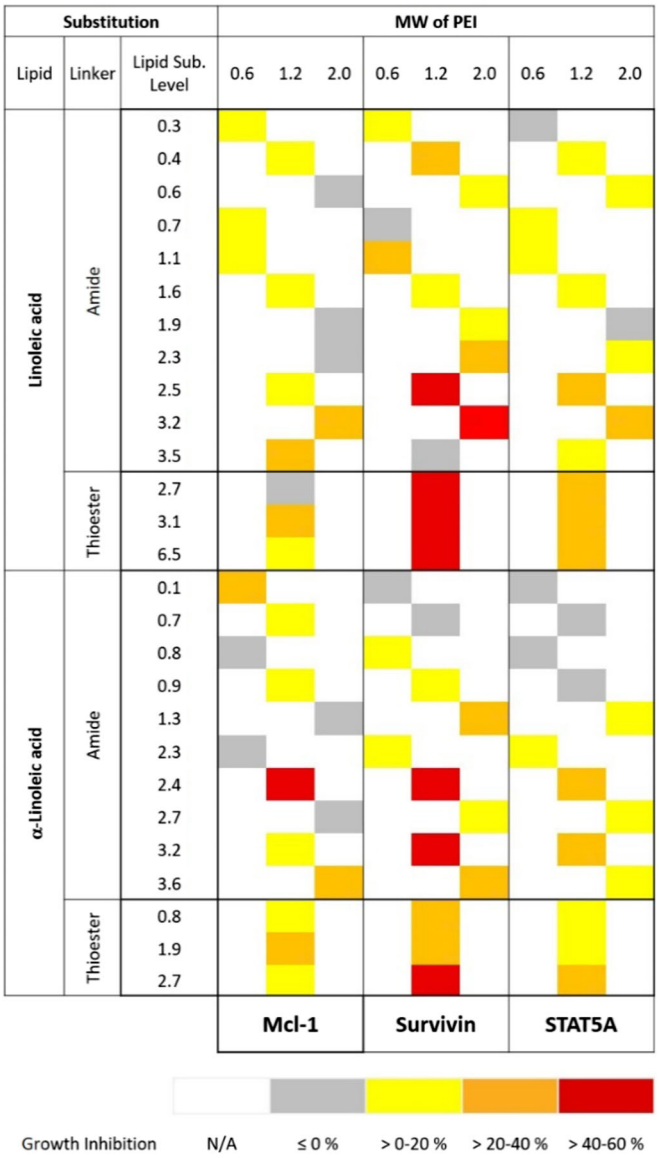


Fig. 2 Heat plot representing the percentage of growth inhibition in MCF-7 cell after treatment with specific siRNAs. The polymers/siRNAs weight ratio was 5/1, and cell growth was determined 3 days after treatment by the MTT assay. N/A: not available.

substitution. Among the screened polymers, most effective four polymers were chosen for further analysis and combinatorial siRNA delivery. These polymers were (i) LA-substituted 2.0 kDa PEI with an amide linkage (2.0PEI-LA9), (ii) LA-substituted 1.2 kDa PEI with a thioester linkage (1.2PEI-tLA10), (iii) αLA-substituted 1.2 kDa PEI with an amide linkage (1.2PEI-αLA4) and (iv) αLA-substituted 1.2 kDa PEI with a thioester linkage (1.2PEI-tαLA6).

siRNA Binding Affinity and Complex Stability of Selected Polymers

The siRNA binding affinity of four selected polymers was determined by the SYBR Green assay in triplicate. As summarized in Fig. 3, all polymers were able to bind to siRNA completely at polymer/siRNA ratios of < 2. Among these polymers, 1.2PEI-αLA4 showed the lowest BC50 at polymer/siRNA weight ratio of 0.09 ± 0.01 ($n = 3$), while 1.2PEI-tLA10 showed the highest BC50 at weight ratio of 0.84 ± 0.20 (n = 3). In addition, a general trend of increasing BC50 with increasing degree of substitution was observed and thioester linkages showed higher BC50 values than amide linkage with the same lipid substituent. These were in line with our expectations that complete siRNA binding would require more polymers in case of the higher degree of lipid substitution and longer linker length, both contributing to increased steric hindrance to binding.

The complex stability in the presence of serum was investigated by gel electrophoresis after incubating the siRNA complexes (polymer/siRNA weight ratio of 5) with serum for 3, 6, 24 and 48 h (Fig. 4). All selected polymers were able to completely bind with siRNA and no siRNA was released from the

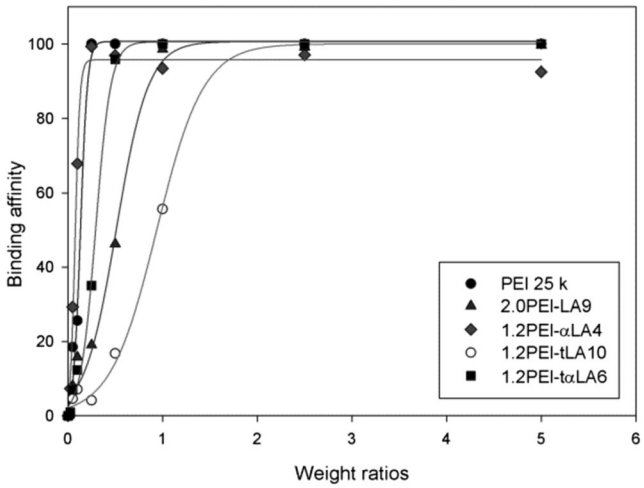


Fig. 3 Binding affinity of 2.0PEI-LA9, 1.2PEI-αLA4, 1.2PEI-tLA10 and 1.2PEI-tαLA6 with siRNA at various polymer/siRNA weight ratios (as determined by SYBR Green assay). The calculated BC50 values were 0.5, 0.1, 0.9, and 0.3 for these polymers, respectively. Three independent binding curves were generated for each polymer and the mean ±SD of BC50 values were calculated for each polymer (indicated in the text).

complexes after 3 and 6 h of serum incubation. Upon longer incubation, siRNA release from the complexes of 1.2PEI-tLA10 was observed at 24 h, while siRNA from the complex of 1.2PEI-αLA4 was observed after 48 h of serum incubation. It is not surprising that thioester linked LA will release the siRNA since it is expected to be a fairly labile linkage in aqueous medium. The amide linkage in 1.2PEI-αLA4 was expected to be stable, and it is likely that the enhanced unsaturation in this lipid conjugate might have created a relatively dynamic complex with possibility of siRNA displacement with serum components. The siRNA complexes of 1.2PEI-tαLA6 were relatively more stable (despite the presence of thioester) and this might be due to increased lipid substitution on these polymers contributing to improved stability of the complexes.

Hydrodynamic Size and Zeta-Potential

Hydrodynamic sizes and zeta-potentials of the polymers/siRNA complexes at weight ratios of 2.5, 5 and 10 are shown in Fig. 5. The hydrodynamic size of the complexes was in the range of 80–190 nm, which varied as a function of weight ratio and type of polymer used for complexation. At the intermediate polymer/siRNA weight ratio of 5 (which was used in initial polymer screen and subsequent studies), the complexes of 1.2PEI-tLA10 had the smallest size while the complexes of 1.2PEI-αLA4 had the largest size. Although the 1.2PEI-tLA10 complex had the lowest binding affinity to siRNA and the complex was less stable than the other complexes, its size was still relatively compact, which should enhance the cellular siRNA delivery. The sizes of the polymeric complexes were generally less than 150 nm and comparable with the complexes formed by the reference reagent PEI25, which is known to give the suitable complexes for cellular uptake.

The zeta-potential of the polymers/siRNA complexes was positive at all investigated weight ratios. For 2.0PEI-LA9/siRNA complexes, the zeta-potential appeared to increase with increasing weight ratio, similar to the PEI25 complexes of siRNA. On the other hand, the zeta-potential of other polymers/siRNA complexes decreased at the high weight ratio of 10. It might be possible that longer thioester linkage and more unsaturated double bond in αLA affected the electrostatic interactions of polymers with siRNA and shielded the cationic charge of complexes at the high weight ratio. At the

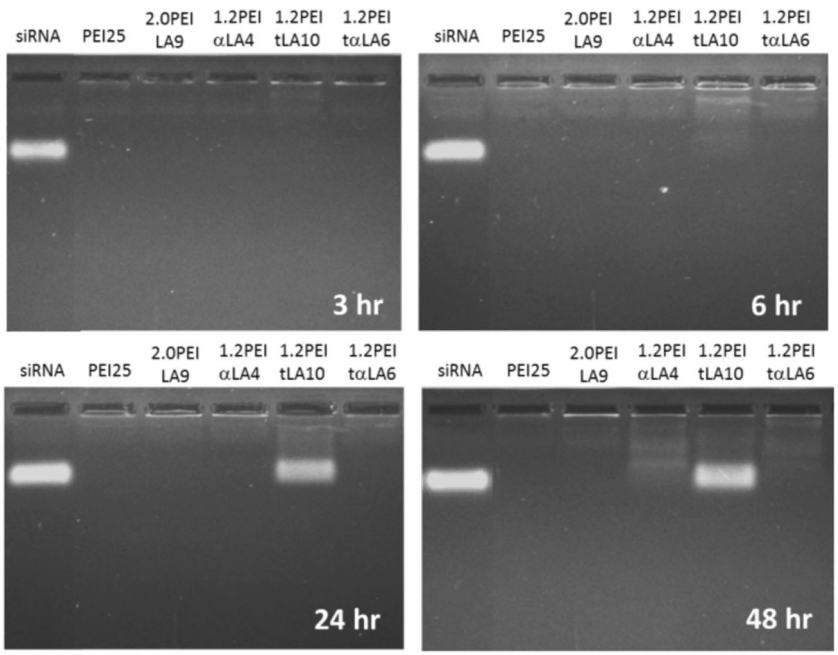


Fig. 4 Gel electrophoresis assessment of the stability of polymers/siRNA complexes after incubating the complexes in the present of serum for 3, 6, 24 and 48 h.

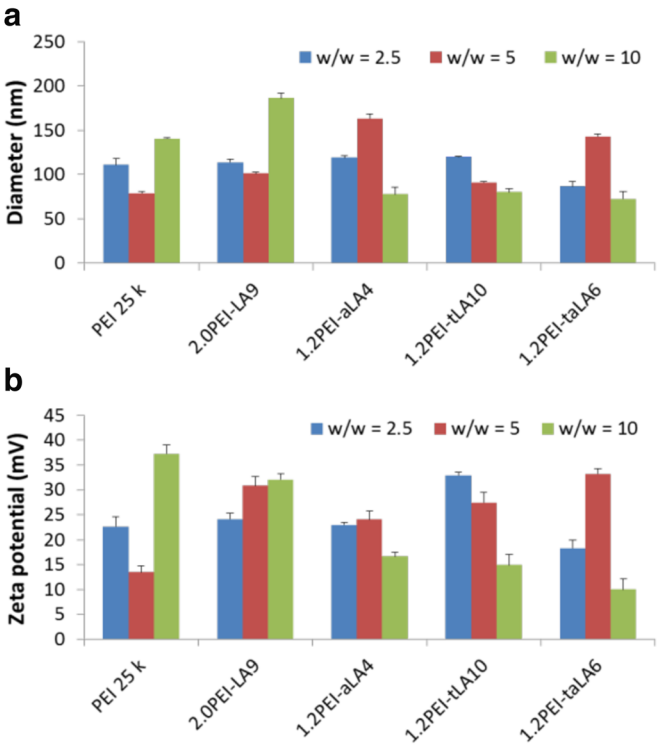


Fig. 5 The hydrodynamic size (**a**) and zeta-potential (**b**) of polymers/siRNA complexes prepared at the weight ratios of 2.5, 5 and 10. The selected polymers were able to formulate the siRNA into 80–190 nm particles with positive zeta-potential for all complexes. The weight ratios affected the size and zeta-potential but with different trends for each polymer.

weight ratio of 5, 1.2PEI-tαLA6/siRNA complexes showed the highest zeta-potential while 1.2PEI-αLA4/siRNA complexes showed the lowest zeta-potential, which was still higher than PEI25/siRNA complexes.

Cellular Uptake of Polymers/siRNA Complexes

The efficiency of selected polymers for cellular delivery of FAM-labeled siRNA was determined by flow cytometry. Cells were transfected with the polymers/siRNA complexes at weight ratios of 2.5, 5 and 10 and cell uptake was determined after 24 h of transfection. This time period was considered optimal for uptake, striking a balance between suboptimal uptake for reduced times and excess complex processing (siRNA dissociation and RNAi engagement) at excessive times. The polymers with amide linkage showed higher cellular uptake than those with thioester linkage. The ability to deliver siRNA increased with increasing of weight ratio for 2.0PEI-LA9, 1.2PEI-tLA10 and 1.2PEI-tαLA6, whereas it was a reverse trend for the 1.2PEI-αLA4, showing low siRNA delivery at the higher weight ratio (Fig. 6a). This result in delivery efficiency was also similar when the studies were performed with MDA-MB-231 cells (**Fig. S2A**). Comparing the average uptake (mean fluorescence intensity) at the weight ratio of 5, 2.0PEI-LA9 and 1.2PEI-αLA4 showed the highest siRNA delivery to MCF-7 cells, which was comparable with the PEI25. Only 1.2PEI-αLA4 showed the highest siRNA delivery in MDA-231 cell. These results revealed that the uptake of polymers/siRNA complexes was varied in difference cell types and depended on the weight ratio used to make complexes. The percentage of cells displaying uptake (percent FAM-positive cells) was 60–80% with MCF-7 cells (Fig. 6b) and 80–100% with MDA-MB-231 cells (**Fig. S2B**) with no large difference between type of polymers and weight ratios. Lipofectamine™ 2000 was additionally used to form and deliver siRNA complexes; it gave a similar percentage of siRNA-positive cells, but the mean uptake was higher than the other polymers. However, toxicity was also evident with this reagent so much that no cells were recovered at the weight ratio of 10 in this case.

The cellular uptake of polymers/siRNA complexes at different time points (3, 6, 24 and 48 h; weight ratio of 5) was also studied in MCF-7 cell (**Fig. S3**). The results indicated that the polymers/siRNA complexes were taken up as early as 3 h and the mean fluorescence intensity was equal or decreased with an extended time for 2.0PEI-LA9, 1.2PEI-tLA10 and 1.2PEI-tαLA6 polymers, whereas the cellular uptake of 1.2PEI-αLA4/siRNA complexes was highest at 24 h. The mean fluorescence intensity of cells treated with thioester linked polymers/siRNA complexes were still lower than amide linked polymers/siRNA complexes at all time. Although 1.2PEI-tLA10 and 1.2PEI-tαLA6 polymers gave relatively low siRNA uptake (based on mean fluorescence intensity), the cells treated with these polymers/siRNA complexes underwent significant retardation of growth in the initial polymer screening study. Therefore, the fluorescence emission from polymers/FAM-labeled siRNA complexes was determined in order to examine whether the polymers quenched

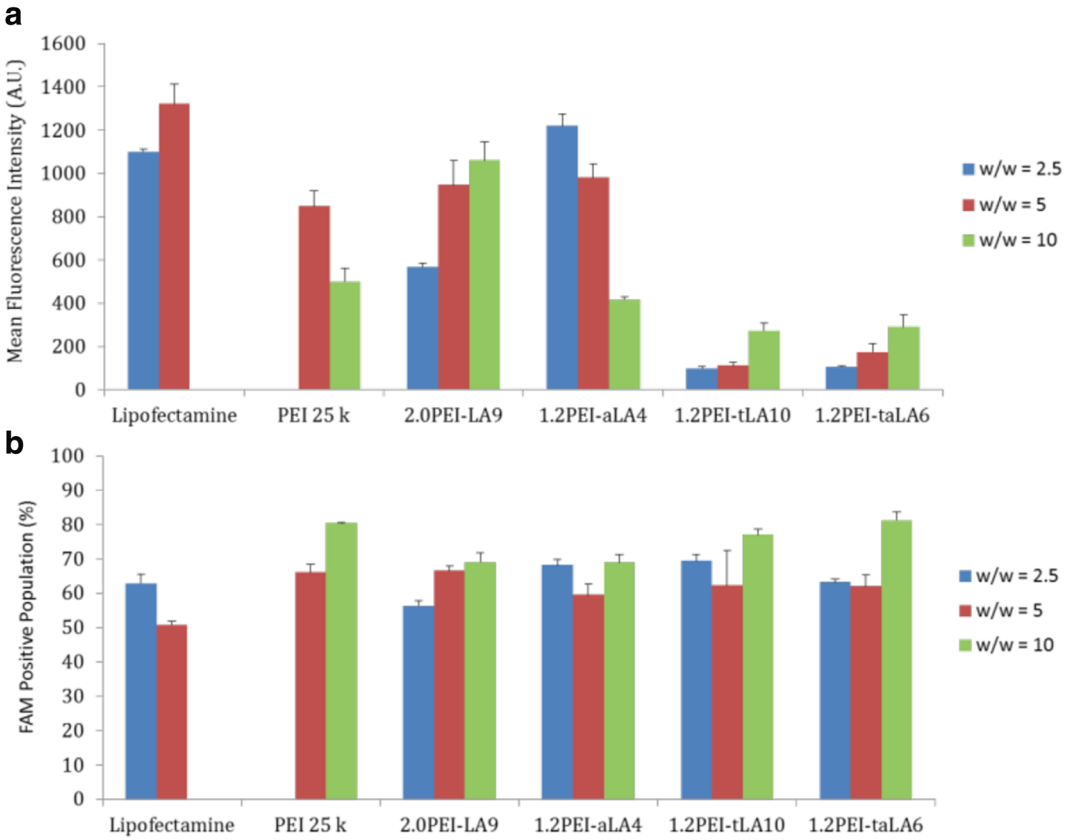


Fig. 6 Cellular uptake of selected polymers/FAM-siRNA complex at weight ratio of 2.5, 5 and 10 using FAM-siRNA at 60 nM after 24 h of transfection in MCF-7 cell. The data showed in term of the mean fluorescence intensity (**a**) and GFP positive population (**b**) analyzed by flow cytometer.

the fluorescence intensity differentially. This might have provided a better understanding as to why uptake with thioester polymers appeared low despite significant effects of target (Mcl-1, survivin and STAT5) silencing on the cells. The result indicated that the fluorescence intensity of FAM-siRNA was not quenched after complexing with these polymers (**Fig. S4**), and especially there was no reduction of fluorescence intensity with thioester polymers as compared to amide polymers. This rules out the possibility of more effective quenching of fluorescence as the reason for lower uptake in cells. Consequently, MCF-7 cells appeared to take up more 2.0PEI-LA9/siRNA and 1.2PEI-αLA4 complexes than 1.2PEI-tLA10/siRNA and 1.2PEI-tαLA6/siRNA complexes. We also noted that the size and the zeta-potential of the complexes (at weight ratio of 5 where the uptake was assessed) were not overly variable, unlike the uptake results. Hence the uptake of nanoparticles did not seem to rely on these nanoparticle features and other factors might have contributed to lower delivery with thioester polymers.

A confocal microscopy analysis of the MCF-7 cells transfected with polymers/siRNA complexes (weight ratio of 5) were also conducted using FAM-siRNA after 24 h of uptake (Fig. 7). The microscopy analysis showed that the polymers/siRNA complexes were taken up into the cells and most of complexes located in the cytoplasm, where the siRNA are expected to function for gene silencing. In addition, the number of complexes and fluorescence intensity of FAM-siRNA complexes in cells transfected with 2.0PEI-LA9/siRNA and 1.2PEI-αLA4/siRNA complexes were more than those in cells transfected with 1.2PEI-tLA10/siRNA and 1.2PEI-tαLA6/siRNA complexes. This was in line with the flow cytometry analysis of the cellular uptake.

Combinatorial siRNA Delivery

To investigate the ability of the polymers to deliver multiple siRNAs and affect the growth of breast cancer cells, MCF-7 cells were treated with polymers/siRNA complex bearing specific siRNAs against Mcl-1, survivin and STAT5A, and cell growth was assessed by the MTT Assay. The treatment was performed in three groups: 1) cells treated with polymers/siRNAs complexes with a single target-specific siRNA at 60 nM (final concentration in medium), 2) cells treated with polymers/siRNAs complexes containing a CsiRNA and a target-specific siRNA at 30 nM each, and 3) cells treated with polymers/siRNAs complexes containing two target-specific siRNAs at 30 nM each. The results, summarized in Fig. 8**,** revealed

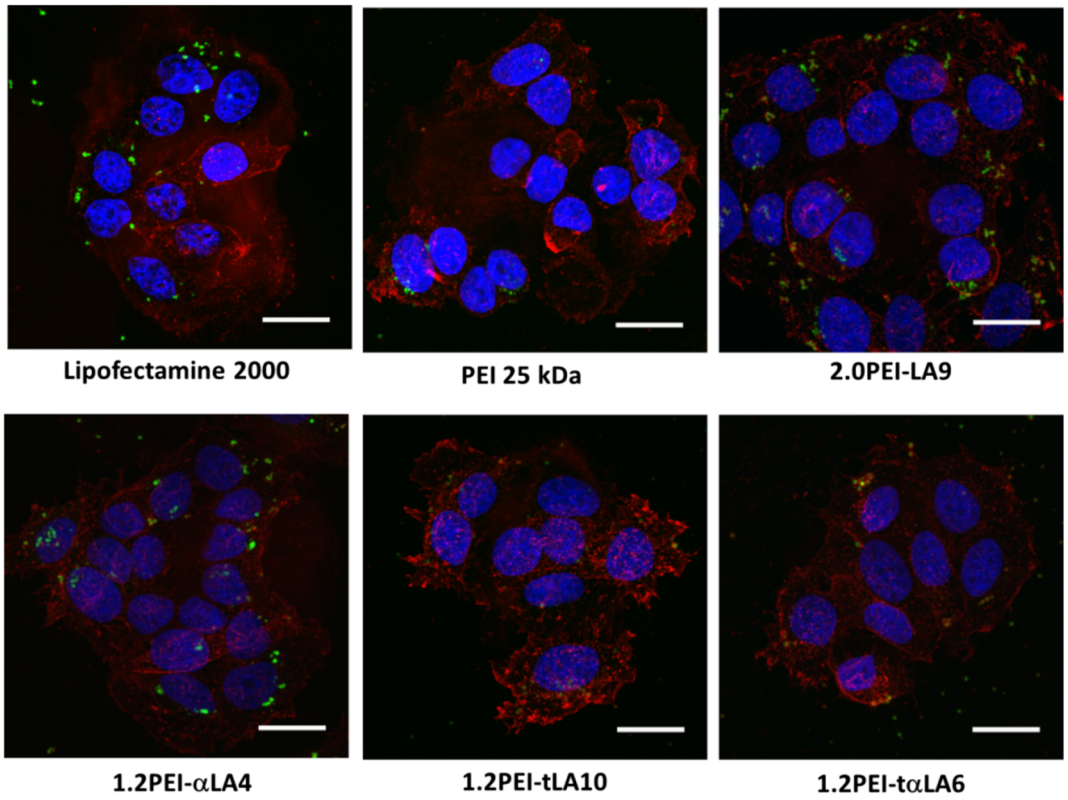


Fig. 7 Confocal images of MCF-7 cells after transfected with selected polymers/FAM-siRNA complexes (weight ratio of 5) for 24 h. Cytomembrane was stained with rhodamine-labeled wheat germ agglutinin (red) and nucleus was stained with DAPI (blue). The scale bars represent 20 μm.

that all four polymers were able to deliver the siRNAs effectively and affected the growth of treated cells as compared to treatment with polymers/CsiRNA complexes as a control. Among the targets, Mcl-1 was the most effective target in MCF-7 cells; the specific siRNA at 60 nM decreased the cell growth by 20–45% depending on the polymer used (2.0PEI-LA9/siRNA giving the most inhibition over control siRNA treatment). The effectiveness of the 1.2PEI-tαLA6 was relatively less compared to other polymers. These results were in line with other reports that indicated Mcl-1 to be an effective target in MCF-7 cells (12,53). The different concentration of the siRNA (60 *vs.* 30 nM) against the same protein did not show significant difference in inhibiting cell growth. For combinatorial siRNA treatment, the cell growth as a result of 2.0PEI-LA9 delivery of Mcl-1 + survivin siRNAs was significantly lower than the delivery of Mcl-1 siRNA alone at both 30 and 60 nM (Fig. 8). The cell growth as a result of 2.0PEI-LA9 delivery of Mcl-1 + STAT5A siRNAs was also significantly lower than the Mcl-1 siRNAs alone at 30 nM. These results indicated that 2.0PEI-LA9 was the most effective polymer for both single siRNA and combinatorial siRNA delivery in MCF-7 cells. No such advantage was evident with the three other polymers. Furthermore, the cytotoxicity of these polymers was low with cell viability more than 80% in polymers/scramble siRNA treated groups.

These results were consistent with the uptake results in some respects, where the 2.0PEI-LA9 polymer provided higher uptake as compared to thioester polymers (1.2PEI-tLA10 and PEI1.2-tαLA6), in line with better inhibition of cell growth. But the inhibition of MCF-7 proliferation by the 1.2PEI-tLA10 complexes was also relatively strong, despite little uptake of these complexes (from Fig. 6). It appears that this polymer might give more efficient silencing once the complexes are internalized, perhaps releasing the siRNA more effectively to exert its silencing activity. The labile thioester linkage might facilitate the siRNA release, but this needs to be confirmed with further studies looking at *in situ* release of internalized siRNA complexes. Even with the most effective polymer, 2.0PEI-LA9, the combinational delivery resulted in an additive (not synergistic) effect and it is likely that other target (and siRNA) pairings will be needed to achieve a synergistic effect.

qRT-PCR Analysis of Targeted mRNAs

While cell growth inhibition is the ultimate functional outcome desired when inspecting combinational delivery, a more direct assessment of siRNA delivery efficiency is the changes in the

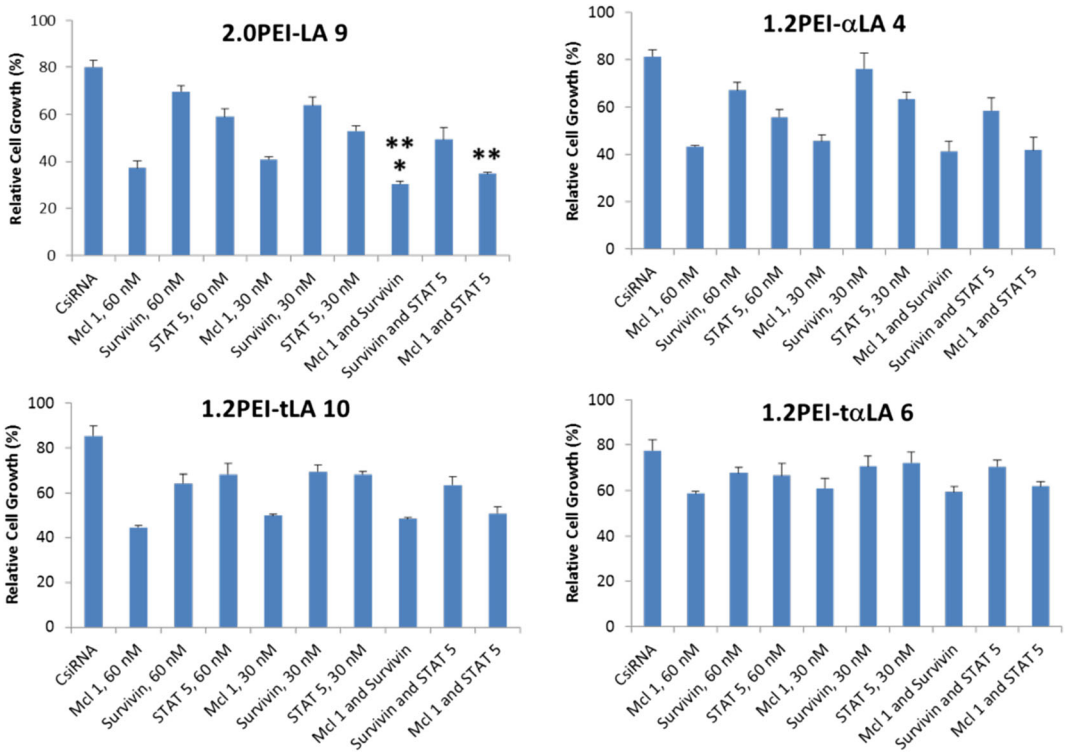


Fig. 8 Combinatorial siRNA target at Mcl-1, survivin and STAT5A in MCF-7 cell. The cells were transfected with polymers/siRNAs complexes at weight ratio of 5 using total siRNA at 60 nM in each group. Cell viability was performed by MTT assay after 72 h of transfection taken no-treated group as a control (100% cell viability). Single asterisk and double asterisks represent the significant decreasing in cell viability of combinational siRNA therapy compared to the most effective single siRNA at 60 nM and at 30 nM, respectively ($p < 0.05$).

mRNA levels of targeted genes. To this end, qRT-PCR was used for evaluating the extent of changes in Mcl-1, survivin and STAT5A mRNAs in MCF-7 cells after 24 and 48 h of treatment. The 2.0PEI-LA9 and 1.2PEI-tαLA10 were chosen for this analysis as representative amide and thioester linked lipids that showed higher inhibition of cell growth. As seen in Fig. 9, the mRNA levels were decreased in cells transfected with 2.0PEI-LA9/single siRNAs complexes at both 24 and 48 h, compared with CsiRNA, which did not give any silencing at the mRNA level (as expected). The mRNA levels in cells transfected with combinatorial siRNA complexes also decreased at 24 h, except the mRNA level of Mcl-1 in cells transfected with 2.0PEI-LA9/Mcl-1 + STAT5A siRNA complexes. However, the mRNA level of STAT5A in the combinatorial STAT5A siRNA treatment groups showed lower than the single STAT5A siRNA treated group at 24 h, indicating sustained silencing. For longer incubation at 48 h, the mRNA level of survivin in the group treated with 2.0PEI-LA9/Mcl-1 + survivin siRNA complexes was lower than the single survivin siRNA treated group. Nevertheless, the STAT5A mRNA levels in all combinatorial siRNA and survivin siRNA treated groups was higher than the single STAT5A treated group even in combinatorial groups of STAT5A. This phenomenon was also same as Mcl-1 mRNA level in the groups treated with other siRNAs. These results indicated that STAT5A mRNA levels were decreased at early time point (24 h) but rebounded quite rapidly at 48 h indicating rapid adaptation. It is possible that the cells adopted to silencing of critical mediators by increasing the expression of other anti-apoptotic genes when we transfected with siRNA targeting of specific genes. However, this did not occur in the case of survivin mRNA. Although we transfected the cells with other target siRNA or combinatorial siRNA, the mRNA level of survivin did not increase. STAT5A were implicated in regulation of survivin expression (54, 55), so that th survivin mRNA reduction after the knock down of STAT5A mRNA might be a consequence of the cross-regulation between the two mediators. In this case, STAT5A siRNA might undertake its action via reduction in both STAT5 and survivin mRNA levels.

For the cells transfected with 1.2PEI-tLA10/siRNA complexes, the results showed a similar trend as the cells transfected with 2.0PEI-LA9/siRNA complexes, but the STAT5 mRNA levels did not decrease except the group treated with the single STAT5 siRNA at 24 h. By comparing these two polymers, 2.0PEI-LA9 was found to be more suitable for delivering siRNA into MCF-7 cells, which gave decreased mRNA levels for all targets. Combinational delivery of two specific siRNAs also gave more significant silencing of targets mRNAs (as much as ~90%), which was in line with more efficacious growth inhibition observed with combinational siRNA delivery. We noted that reduction in mRNA levels (Fig. 9) were more pronounced that the inhibition of cell

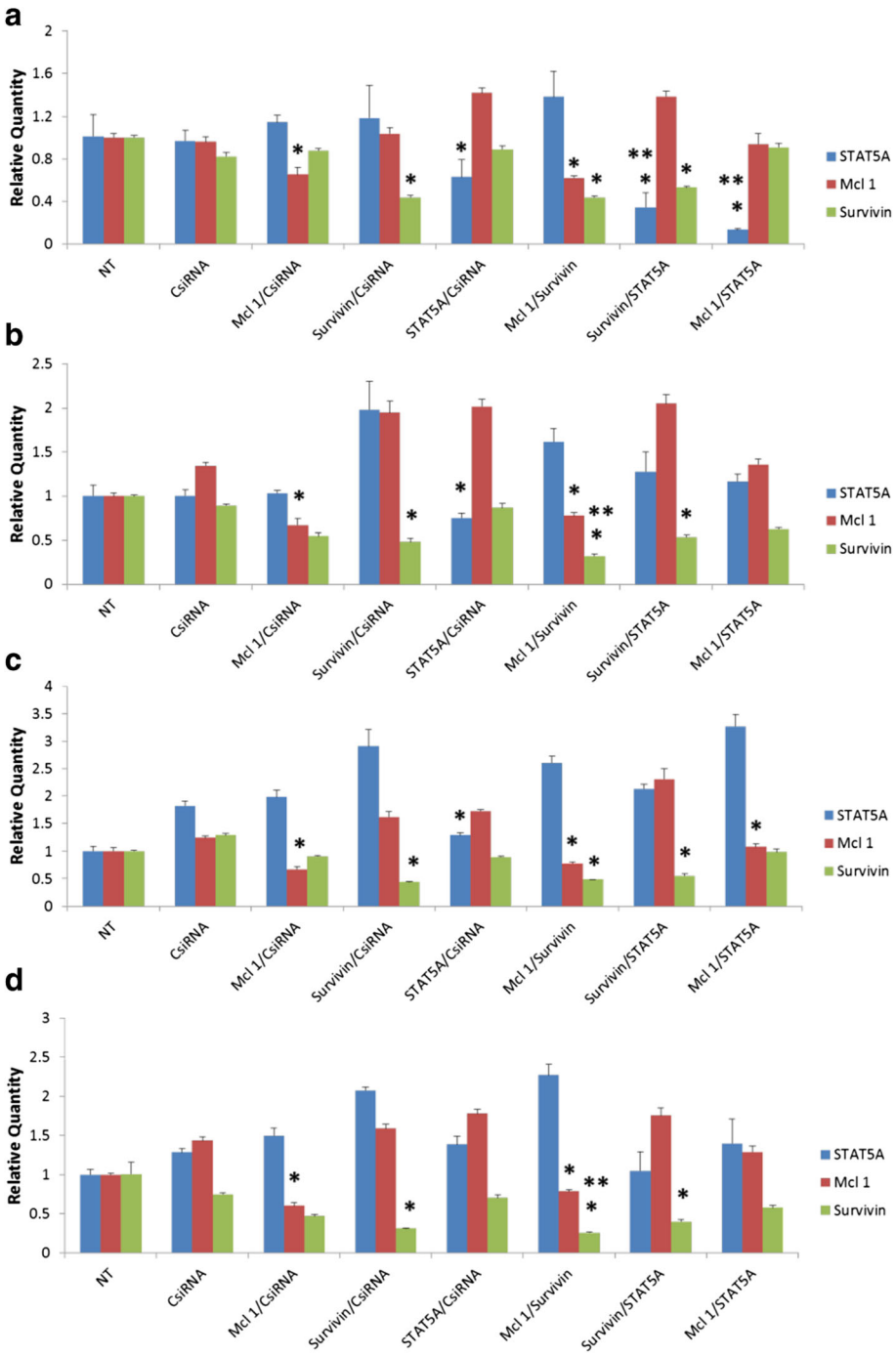


Fig. 9 Mcl-1, survivin and STAT5A gene silencing in MCF-7 cell evaluated by qRT-PCR. Cells were transfected with 2.0PEI-LA9/siRNAs complexes at 24 h (**a**) and 48 h (**b**) or 1.2PEI-tLA10/siRNAs complexes at 24 h (**c**) and 48 h (**d**) using total siRNAs concentration at 60 nM. Scrambled siRNA (CsiRNA) was used as a control. Single asterisk represents the significant reduction in the mRNA expression compared to CsiRNA ($p < 0.05$). Double asterisks represent the significant reduction in the mRNA expression compared to the most effective single siRNA treatment group ($p < 0.05$).

growth observed (Fig. 8), so that additional mediators are suspected to substituted to maintain the growth of MCF-7 cells. It will be important to identify such mediators for a better control of cell growth.

CONCLUSIONS

This study further contributed to development of lipid-substituted PEIs as siRNA carriers. The LA and αLA substitutions on low molecular weight PEI (1.2 kDa and 2.0 kDa), were able to provide carriers that delivered siRNAs effectively and caused a decrease in the growth of breast cancer cells *in vitro*. The additional unsaturation in αLA was not obviously beneficial in siRNA delivery, since the two substituents generally performed similarly for siRNA delivery. While lipid substitutions on smallest PEI (0.6 kDa) were not generally effective for siRNA delivery, intermediate MW PEI (1.2 kDa and 2.0 kDa) appeared to provide the optimal backbone for lipid substitution. The hydrodynamic

size (80–190 nm) of effective polymers/siRNA complexes were similar, as well as the zeta-potentials at tested weight ratios (2.5 to 10.0), so that the overall structures of the complexes did not depend on the nature of lipid or lipid linkage. The amide-linked lipid substituents showed higher cellular delivery of siRNA as compared to thioester linkages (2–6 fold difference depending on polymer:siRNA ratio in complexes), yet the latter carriers were also effective in silencing the chosen targets. The combinatorial siRNA delivery showed that growth of MCF-7 cells was inhibited to a greater extent with 2.0PEI-LA9 mediated delivery of Mcl-1 + survivin siRNAs as compared to cells treated with the individual siRNAs. The qRT-PCR result confirmed that the mRNA levels of target genes decreased by transfecting cells with specific siRNAs and 2.0PEI-LA9 was the most effective polymer for delivering siRNAs (either single or in combination) into MCF-7 cells. This study effective and safe siRNA delivery system for combinational delivery of siRNAs, but careful choice of siRNA combinations would be critical since knocking down individual genes might up-regulate other genes critical for survival of breast cancer cells.

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