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## Co-delivery of DNA and siRNA via Arginine-Rich PEI-based Polyplexes

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### Abstract

In this study, we formulated polyplexes with different compositions for co-delivery of DNA and small-interfering RNA (siRNA). Since DNA and siRNA have distinctive and complementary morphological characteristics (DNA is long and winding and siRNA is short and rigid), we hypothesized that their co-delivery using polyplex would enhance each other's transfection. To test this hypothesis, cationic polymer branched polyethylenimine (bPEI) as a standard transfecting agent and its derivative arginine-rich oligopeptide-grafted bPEI modified with polyethylene glycol (P(SiDAAr)<sub>5</sub>P<sub>3</sub>), synthesized in our laboratory, were used as carriers for transfection. Polyplexes at different nucleic acid to polymer weight ratios were characterized for transfection in breast cancer sensitive (MCF-7) and resistant (MCF-7/Adr) cell lines. Gene silencing effect of polyplexes was determined in MDA-MB-231-luc-D3H2LN cell line. The results demonstrated that the polyplexes formed with derivative P(SiDAAr)<sub>5</sub>P<sub>3</sub> show significantly lower toxicity compared to polyplexes formed using bPEI. Further, co-delivery resulted in 20-fold higher DNA transfection and 2-fold higher siRNA transfection as compared to the respective single nucleotide delivery. DNA transfection was ~100 fold lower in resistant MCF-7/Adr cells than in sensitive MCF-7 cells. Confocal imaging and flow cytometry data demonstrated that enhanced transfection does not solely depend on DNA's cellular uptake, suggesting that other mechanisms contribute to increased transfection. DNA-co-siRNA delivery could be a promising therapeutic approach to achieve synergistic effects, because it can simultaneously target and interfere with multiple regulatory levels in a cell to halt and reverse disease progression.

### Keywords

combination therapy; non-viral gene delivery; cancer therapy; cationic polymers; cellular uptake

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## 1. Introduction

The discovery of siRNA has drawn much attention to gene therapy in the last few years, particularly for its usefulness in targeting a number of genetic diseases.<sup>1-4</sup> However, challenges remain in gene therapies, including low transfection efficiencies, particularly with non-viral vectors, as well as in achieving therapeutic effects at a tolerable dose. Many diseases are the result of single gene mutations. Yet, single gene disorders are not common in the population. Therefore, simultaneously targeting multiple genetic components and regulatory pathways pose the possibility of reaching a greater therapeutic potential for a number of diseases, including cancer.<sup>5</sup> In addition, DNA and siRNA's different physical characteristics (size, morphology, rigidity and charge) could cause them to have different electrostatic interaction strength with cationic polymers like PEI, thus changing the binding affinity of the polyplexes.<sup>6</sup>

Polyethylenimine (PEI) is a commonly used nucleic acid delivery vehicle.<sup>7</sup> It condenses nucleotides through electrostatic interactions, protects them from nuclease degradation and facilitates endosomal escape.<sup>8,9</sup> The cationic charges on the polyplex surface also promote cellular uptake through adsorptive endocytosis.<sup>10,11</sup> Despite its promising features, a number of drawbacks hinder it from achieving its therapeutic potential. For example, interaction of the high cationic charge of PEI with the anionic cell surface can destabilize the plasma-membrane and induce toxicity.<sup>12,13</sup> PEI could also interact with negatively charged serum proteins (such as albumin) and red blood cells, causing protein precipitation or red blood cell aggregation and lysis.<sup>14</sup> Therefore, chemical modifications are usually made to mitigate the effect of high cationic charge of PEI.<sup>15</sup>

In this study, to attenuate toxicity, enhance biocompatibility and improve cellular uptake, we modified PEI with 5 molar equivalents of L-arginine modified oligo (alkylaminosiloxane) graft and 3 molar equivalents of PEG, forming P(SiDAAr)<sub>5</sub>P<sub>3</sub>. The previous study has shown better transfection with P(SiDAAr)<sub>5</sub> than with PEI *in vitro* due to L-arginine residues that assist in adsorptive endocytosis, enhancing membrane permeability, and facilitating nuclear localization.<sup>16</sup> P(SiDAAr)<sub>5</sub>'s siloxane derivative is biodegradable and is excreted by urine.<sup>17</sup> Further, P(SiDAAr)<sub>5</sub> following PEG and folic acid conjugation showed greater tumor accumulation than unconjugated polymer.<sup>18</sup>

DNA and siRNA delivery systems have many similar criteria, such as facilitating efficient cellular uptake and rapid endosomal escape. However, their distinctive structural and chemical characteristics determined their different complex formation properties. DNA is usually several kilobase pairs long while siRNA is only 21-23 base pairs. Topologically, siRNA forms A-form helices, while DNA forms B-form helices.<sup>19</sup> The A-form has a larger diameter and a smaller rise per base pair, and thus is stiffer than the B-form.<sup>19</sup> Therefore, the structural differences between DNA and siRNA may result in DNA-co-siRNA polyplexes to have different structural properties than DNA- or siRNA-only polyplexes.<sup>20</sup> This study shows that DNA-siRNA co-delivery significantly enhances gene transfection levels of both the nucleotides (DNA and siRNA) compare to that of a single-nucleotide (DNA or siRNA). Further, comparing polyplexes, P(SiDAAr)<sub>5</sub>P<sub>3</sub> polyplexes have significantly better cytocompatibility than PEI polyplexes with the same nucleotide composition.

## 2. Materials and Methods

### 2.1. Materials

3-(2-aminoethylamino) propyl-methyl-dimethoxysilane, 25-kDa branched PEI, L-arginine, O-(2-aminoethyl)-O'-(2-carboxyethyl), PEG-3kDa hydrochloride, 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), dicyclohexylcarbodiimide (DCC), N-hydroxysuccinimide (NHS) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO). Fetal bovine serum (FBS) and Trypsin/EDTA were obtained from Gibco (Grand Island, NY). The luciferase assay system and reporter lysis buffer were purchased from Promega (Madison, WI). Scrambled siRNA (s-siRNA) and YOYO-1 DNA intercalating dye were purchased from Life Technologies (Carlsbad, CA). The 1% Gold Agarose Reliant Gel System was purchased from Lonza (Allendale, NJ). Anti-luciferase siRNA (luc-siRNA) was purchased from Dharmacon, Inc. (Lafayette, CO). The BCA Protein Assay Kit was purchased from Thermo Scientific (Rockford, IL). Cell culture media Dulbecco's Modified Eagle's Medium (DMEM), DPBS, penicillin and streptomycin were purchased from our Cell Services' Media Preparation Core.

### 2.2. pDNA Amplification and Purification

The pGL3 (luciferase reporter vector) and control (noncoding) plasmid DNA from Promega (Madison, WI) were cloned via *E. coli* (JM109, Promega) according to the manufacturer's technical manual. pDNA was purified using Qiagen EndoFree Plasmid Mega Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions and re-suspended in DNase-free dH<sub>2</sub>O. pDNA concentration was measured with a Nanodrop 1000 (Thermo Scientific, Waltham, MA).

### 2.3. Polymer Synthesis

Arginine modified oligo-(alkylaminosiloxane)-graft-PEI (P(SiDAAr)<sub>5</sub>) (5:1 graft: PEI molar ratio) was synthesized as previously described.<sup>16</sup> Briefly, the free acid group of NH<sub>2</sub>-PEG-COOH was activated using EDC as a coupling agent and NHS as a catalyst in pH 6 MES buffer at 40 °C for 3.5 hr. Three molar equivalents of activated PEG were then reacted with 1 molar equivalent of P(SiDAAr)<sub>5</sub> overnight in PBS (pH=7.5) via carbodiimide chemistry. The synthesized polymer was dialyzed for 2 days using 12K MWCO dialysis membrane (Spectrum Labs, Rancho Dominguez, CA) to remove un-reacted elements.

### 2.4. Polymer/Nucleotide Complexation

Polymers were dissolved in MilliQ water (dH<sub>2</sub>O) and filtered through a 0.22 μm sterile polyethersulfone filter (Millipore, Darmstadt, Germany). SiRNA was dissolved at 1 mg/mL in RNase-free dH<sub>2</sub>O. For characterization studies, DNA and polymers were dissolved separately at 1 mg/mL in DNase- and RNase-free dH<sub>2</sub>O. For cell culture experiments, DNA and polymer were dissolved separately at 1 mg/mL in serum-free Dulbecco's Modified Eagle Medium (DMEM). Polyplexes were formed by combining DNA, siRNA and polymer solutions, pipetting to mix thoroughly, and self-assembling for 30 min. PEI was used in parallel with P(SiDAAr)<sub>5</sub>P<sub>3</sub> for comparison purposes.

## 2.5. Size/Zeta Potential Measurements

Polymers PEI and P(SiDAAr)<sub>5</sub>P<sub>3</sub>, and the corresponding polyplexes with different DNA/siRNA/polymer (D/S/P) ratios, were measured for mean hydrodynamic diameters and zeta potentials in dH<sub>2</sub>O via dynamic light scattering (DLS) using the NICOMP 380 ZLS Particle Sizing System (Santa Barbara, CA). All polymer and polyplex measurements were done in 3 replicates. Average and standard deviation were calculated for each polyplex composition.

## 2.6. Gel Retardation Assay

Agarose gel electrophoresis was used to determine if there was complete nucleotide complexation in each polyplex formulation. The polyplex solutions were prepared at varying polymer/nucleotide (P/N) weight/weight ratios. Free non-coding plasmid DNA (Ø-pDNA), luc-siRNA and Ø-pDNA-co-luc-siRNA P(SiDAAr)<sub>5</sub>P<sub>3</sub> polyplex solutions were loaded in a 1% Gold Agarose gel (Lonza, Allendale, NJ) in TrisAcetate-EDTA buffer at pH 8.0. For free nucleotide wells, 2 µL of DNA or siRNA was used per well. For other wells, 2 µL of DNA was used per well, and the quantities of the siRNA and the polymer were adjusted accordingly. The free nucleotides and polyplexes were run on an electrophoresis system (Bio-Rad, Hercules, CA). The gel was stained using SYBR Green (Thermo Scientific) and visualized under a Carestream Molecular Imaging Gel Logic 112 Imaging System (Carestream Health, Inc., Rochester, NY).

## 2.7. Transmission Electron Microscopy (TEM)

1/1/4 D/S/P polyplexes were prepared in dH<sub>2</sub>O by vigorous pipetting and incubating for 30 min for polyplex self-assembly. Thereafter, 10 µL of the polyplex solution was dropped on TEM grids coated with silicon monoxide stabilized formvar films (Ted Pella Inc., Redding, CA). After drying, the samples were stained with 2% uranyl acetate for 7 minutes and imaged using a Tecnai G2 TEM microscope (FEI, Hillsboro, Oregon).

## 2.8. Polyanion Competition Assay

The relative stability of 1/1/4 D/S/P(SiDAAr)<sub>5</sub>P<sub>3</sub> polyplexes was investigated using a heparin polyanion competition assay, where the nucleotides released from polyplexes were measured using gel electrophoresis. Polyplex solutions were first prepared by mixing 5 µg of DNA, 5 µg of siRNA and 20 µg of polymer and incubating in dH<sub>2</sub>O for 30 min. After that, polyplexes were exposed to heparin sodium (Sigma-Aldrich, St. Louis, MO) solution (10 µL) of varying concentrations for 15 min. Then the samples were run on a 1% Gold Agarose gel (Lonza, Allendale, NJ) in TrisAcetate-EDTA buffer, and the gel stained and visualized by the same method described in section 2.6.

## 2.9. Cell Culture

Cell line MCF-7 was purchased from American Type Culture Collection (Manassas, VA). Luciferase-expressing breast cancer metastatic cell line MDA-MB-231-luc-D3H2LN was purchased from Caliper Life Sciences (Hopkinton, MA). MCF-7/Adr was developed in our laboratory and maintained by culturing in media with 100 ng/mL doxorubicin. MCF-7/Adr was incubated in drug-free medium for two passages before being used in experiments. All cell lines were cultured at 37 °C in humidified air containing 5% CO<sub>2</sub>. DMEM with 15%

FBS and 1% penicillin-streptomycin was used in all cell-culture related studies, including MTT, *in vitro* transfections, flow cytometry and confocal microscopic studies. Cell detachment was done using trypsin/EDTA at 37 °C.

### 2.10. *In Vitro* Cytotoxicity Assay

MTT assay was used to evaluate polymer and polyplex toxicities *in vitro* in MCF-7 and MCF-7/Adr cell lines. For the 72-hr polymer toxicity assay,  $5 \times 10^4$  cells were seeded per well in a 24-well plate (BD Biosciences, San Jose, CA). For the 24-hr polyplex toxicity assay,  $5 \times 10^5$  cells were seeded per well in a 24-well plate and 1  $\mu\text{g}$  of DNA was used per well. The 72-hr polymer MTT was designed to assess the differential growth inhibition effects of the polymers, while the 24-hr polyplex MTT was designed to assess the toxicity of the polyplexes under transfection conditions. After 24 hr of cell attachment, all media was removed, and each well received fresh media containing polymers or polyplexes. After a designated incubation time, media was removed again and replaced with 150  $\mu\text{L}$  of MTT (2 mg/mL in DMEM). Plates were incubated at room temperature overnight according to the standard MTT protocol. On the next day, MTT was removed, wells received 500  $\mu\text{L}$  DMSO, and plates were incubated for 3.5 hr at 37 °C to allow the substrate to dissolve. One hundred and fifty  $\mu\text{L}$  of the dissolved solution was then taken out and absorbance (abs) measured at 575 nm using a clear 96-well plate. Cell viability (as a %) was calculated as  $(\text{abs of sample} - \text{background}) / (\text{abs of control} - \text{background}) \times 100\%$ .

### 2.11. *In Vitro* Luciferase Transfection Assay

Luciferase-DNA (luc-DNA)'s transfection levels in luc-DNA or luc-DNA/s-siRNA polyplexes were measured in MCF-7 and MCF-7/Adr cells. Luc-siRNA's transfection levels in luc-siRNA or luc-siRNA/ $\emptyset$ -pDNA polyplexes were measured in MDA-MB-231-luc-D3H2LN cells. One  $\mu\text{g}$  of luc-DNA/well was used for MCF-7, 2  $\mu\text{g}$  of luc-DNA/well for MCF-7/Adr, and 2  $\mu\text{g}$  of luc-siRNA/well for MDA-MB-231-luc-D3H2LN. MCF-7 and MCF-7/Adr cells were seeded at  $5 \times 10^5$  cells/well and MDA-MB-231-luc-D3H2LN cells at  $2 \times 10^5$  cells/well in 24 well plates. After 24 hr, media was replaced and polyplex suspensions prepared in DMEM were added drop-wise to each well. After 1 day of transfection, the cells were washed twice with PBS and lysed with 100  $\mu\text{L}$  of Reporter Lysis Buffer (Promega). Twenty-five  $\mu\text{L}$  of lysate and 50  $\mu\text{L}$  of Luciferase Assay Reagent was combined, and luciferase activity in relative luminescence units (RLU) was measured in an opaque 96-well plate (Sigma-Aldrich) using a micro plate reader (FLUOstar OPTIMA (BMG Labtech, Durham, NC).

Protein levels were determined using BCA protein assay (Thermo Scientific). Ten  $\mu\text{L}$  of each lysate and 150  $\mu\text{L}$  of BCA reagent were combined in clear 96-well plates and incubated at 37 °C for 30 minutes. Absorbance was measured at a wavelength of 562 nm using an Absorbance Microplate Reader (Molecular Devices, Sunnyvale, CA).

### 2.12. DNA Cellular Uptake/Flow Cytometry

YOYO-pDNA intercalation was carried out by adding 30  $\mu\text{L}$  of 10  $\mu\text{M}$  YOYO-1 to 1  $\mu\text{g}$  of 1 mg/mL DNA and incubating for 30 minutes at room temperature in the dark. MCF-7 and MCF-7/Adr cells were seeded in 6-well plates and allowed to grow to 70% confluence.

Polyplex solutions were added to each well and flow cytometry was carried out after 24 hr. One  $\mu\text{g}$  of DNA per well was used for MCF-7 cells and 2.5  $\mu\text{g}$  of DNA per well were used for MCF-7/Adr cells. Cells were washed twice with PBS, trypsinized and collected for flow cytometry (LSR II System, BD Biosciences). Fluorescence detector  $530 \pm 15$  nm was used for YOYO-1 detection. A population of 10,000 freshly collected cells was used per condition.

### 2.13. DNA Intracellular Visualization

MCF-7 and MCF-7/Adr cells were seeded on coverslips placed in 6 well plates and allowed to reach 60% confluence. At 60% confluence, polyplexes formed with YOYO-1 stained DNA were added to each well in FBS enriched media. One  $\mu\text{g}$  of DNA per well was used for MCF-7 cells and 2.5  $\mu\text{g}$  of DNA for MCF-7/Adr cells. After 24 hr incubation, cells were rinsed with DPBS and fixed with 4% paraformaldehyde (Sigma-Aldrich). Afterward, coverslips were mounted on clean glass slides using DAPI ProLong Gold Anti-fade Mounting Media (Invitrogen, Eugene, OR). The cells were then evaluated with a laser scanning confocal microscope (Leica TCS-SP II Spectral Laser Scanning Confocal Microscope, Leica Microsystems GmbH, Wetzlar, Germany).

### 2.14. Statistical Analysis

Data are presented as mean  $\pm$  standard deviation. Statistical analysis of the differences between two mean values was performed by equal variance 1-sided Student's *t*-test using the *t*-test calculator from Graphpad Software. Variance and *t*-values were calculated, and *p*-values generated between two groups in comparison. Confidence level was set to 95%. Statistical significance was defined at *p* 0.05; and highly significant at *p* 0.01 and *p* 0.001.

## 3. Results

### Polymer Characterization

$^1\text{H}$  NMR ( $\text{D}_2\text{O}$ ) confirmed the presence of PEI, arginine, siloxane and PEG groups on the synthesized  $\text{P}(\text{SiDAAr})_5\text{P}_3$  (Fig 1). On the spectrum, the presence of PEG ( $-\text{CH}_2\text{CH}_2\text{O}-$ ) was shown by peaks at 3.2-3.5 ppm. The proton signals of PEI, siloxane and arginine were identified by peaks at 2.3-2.8 ppm: 2.24 for arginine ( $-\text{HCCH}_2\text{CH}_2\text{CH}_2\text{NH}-$ ), 2.86 for arginine ( $-\text{HCCH}_2\text{CH}_2\text{CH}_2\text{NH}-$ ), and 2.4-2.8 for the ethylene groups of PEI ( $-\text{NHCH}_2\text{CH}_2-$ ). The average hydrodynamic diameter ( $D_h$ ) of polymers PEI,  $\text{P}(\text{SiDAAr})_5$  and  $\text{P}(\text{SiDAAr})_5\text{P}_3$  measured by DLS increased after each step of modification, from PEI to  $\text{P}(\text{SiDAAr})_5$  to  $\text{P}(\text{SiDAAr})_5\text{P}_3$  (Table 1). PEI and  $\text{P}(\text{SiDAAr})_5$  have similar polydispersity indices (PDI) (0.3-0.4), whereas  $\text{P}(\text{SiDAAr})_5\text{P}_3$  has a greater PDI (0.62). Zeta potential increased slightly from PEI to  $\text{P}(\text{SiDAAr})_5$  as a result of the amine groups present on arginine. Compared to  $\text{P}(\text{SiDAAr})_5$ , PEG-conjugation reduced  $\text{P}(\text{SiDAAr})_5\text{P}_3$ 's zeta potential.

### Polyplex Characterization

A gel retardation assay showed  $\text{P}(\text{SiDAAr})_5\text{P}_3$ 's nucleotide-binding capacity with different DNA and siRNA weight ratios based on the nucleotides' electrophoretic mobility (Fig 2). Compared to the controls (free luc-DNA and s-siRNA), the nucleotides showed no band on

the gel, dim band in the wells with low P/N polyplexes and no band in the wells with high P/N polyplexes. No DNA migration signifies complete DNA-polymer complexation. No visible DNA in the wells signifies complete DNA masking by the polymer, therefore giving no exposure to the staining dye (Fig 2).

Polyplex morphologies were visualized under TEM in both high and low magnifications (Fig 3). The polyplexes have an almost spherical shape. Furthermore, polyplex hydrodynamic sizes and zeta potentials were measured via dynamic light scattering. Both nucleotide to polymer ratio and the polymeric material play a role in the size and surface charge of the polyplexes. On average, P(SiDAAr)<sub>5</sub>P<sub>3</sub> polyplexes are smaller and have a smaller size range compared to PEI polyplexes (Table 2). Regarding polydispersity, most polyplexes have a PDI ranging from 0.2-0.5. Comparing average zeta potentials, P(SiDAAr)<sub>5</sub>P<sub>3</sub> polyplexes have on average lower zeta potentials than PEI polyplexes. For both PEI and P(SiDAAr)<sub>5</sub>P<sub>3</sub> polyplexes, surface charges increase as the polymer ratio increases in the polyplexes. P(SiDAAr)<sub>5</sub>P<sub>3</sub> polyplexes have both positive and negative surface charges, whereas all PEI polyplexes have positive charges (Table 2). Finally, both siRNA polyplexes and siRNA-co-DNA polyplexes made from P(SiDAAr)<sub>5</sub>P<sub>3</sub> have similar hydrodynamic sizes.

### Polyplexes Stability in the Presence of Competing Polyanions

A heparin displacement assay was used to examine the integrity and stability of DNA/siRNA/ P(SiDAAr)<sub>5</sub>P<sub>3</sub> polyplexes in the presence of heparin sodium (Fig 4). Different concentrations of heparin sodium solutions were used to gradually induce DNA and siRNA decomplexation within the polyplex. When no heparin was added, neither DNA or siRNA was released from the polyplexes. This is an indication of the polyplexes' stability in the absence of competing polyanions. With increasing heparin concentration, polyplexes were sufficiently stable, until reaching 5 mg/mL heparin concentration. At 5 mg/mL, polyplex decomplexation took place, where both DNA and siRNA were replaced by competing polyanions and were released out of the polyplexes.

### Cytotoxicity Evaluation

In the 72-hr dose-dependent polymer toxicity assay of MDA-MB-231-luc-D3H2LN cells, both P(SiDAAr)<sub>5</sub> and P(SiDAAr)<sub>5</sub>P<sub>3</sub> had significantly reduced toxicity compared to PEI [(p < 0.05) at 25 and 50 µg/mL] (Fig 5A). Compared to P(SiDAAr)<sub>5</sub>, P(SiDAAr)<sub>5</sub>P<sub>3</sub> showed further cytotoxicity reduction. Cytotoxicity evaluation following 24 hr of PEI and P(SiDAAr)<sub>5</sub>P<sub>3</sub> polyplex exposure showed that in both MCF-7 and MCF-7/Adr at high polymer concentrations (1/1/4 and 1/2/6 polyplexes), growth inhibition was observed (Fig 5B, C). However, P(SiDAAr)<sub>5</sub>P<sub>3</sub> achieved significantly higher cell viability compared to PEI in MCF-7/Adr in a 1/2/6 D/S/P ratio.

### Luciferase DNA Transfection

Luciferase DNA transfection using luc-DNA-co-siRNA polyplexes in MCF-7 and MCF-7/Adr cell lines showed that MCF-7 was more prone to DNA transfection than MCF-7/Adr (Fig 6A, B). With a 2 fold lower dose of DNA, MCF-7 still had higher transfection levels (from 10 to 100-fold) than MCF-7/Adr. In MCF-7, where both P/N ratios

of 1 and 2 were used, polyplexes with P/N of 2 showed a greater transfection level than P/N of 1. In polyplexes with both polymers in both cell lines, s-siRNA incorporation resulted in enhanced transfection expressions. In MCF-7, 1/2/3 (D/S/P ratio) PEI polyplexes achieved 26-fold higher transfections than 1/O/1 PEI polyplexes. In P(SiDAAr)<sub>5</sub>P<sub>3</sub> polyplexes, both 1/1/2 and 1/2/3 polyplexes expressed an over 10 fold higher transfection level compared to 1/O/1 polyplexes. Also, the 2 polymers affected different cell lines differently. In MCF-7, P(SiDAAr)<sub>5</sub>P<sub>3</sub> polyplexes showed significantly higher transfections, whereas in MCF-7/Adr, higher transfections were observed with PEI polyplexes.

Luciferase expression knock-down in MDA-MB-231-luc-D3H2LN cells using luc-siRNA and Ø-pDNA polyplexes showed that ØDNA significantly enhanced siRNA's transfection levels (Fig 7). Consistent with MCF-7, P(SiDAAr)<sub>5</sub>P<sub>3</sub> showed greater transfections than PEI. The maximum transfection level (97% luciferase expression knock-down) was achieved with 1/2/6 D/S/P polyplexes.

#### DNA Intracellular Uptake in MCF-7 and MCF-7/Adr Cell Lines

DNA's intracellular uptake examined in MCF-7 by flow cytometry showed that 1/1/4 polyplexes had a greater DNA uptake level than 1/O/2 polyplexes (Fig 8A). Confocal microscopy showed higher DNA cellular internalization in 1/2/3 polyplexes than in 1/1/2 polyplexes, and both expressed higher DNA cellular internalization than 1/O/1 polyplexes (Fig 8B).

In MCF-7/Adr, both flow cytometry and confocal microscopy showed a higher DNA uptake in 1/1/4 polyplexes compared to 1/O/2 polyplexes. However, 1/2/6 polyplexes showed a reduced DNA uptake, confirmed by both flow cytometry and confocal imaging (Fig 9). In all studies, DNA uptake level is affected by polyplex compositions and the presence of siRNA in the polyplex.

#### 4. Discussion

This study showed that DNA and siRNA enhances each other's transfection levels in PEI-based delivery systems. Luc-DNA was combined with s-siRNA, and luc-siRNA combined with Ø-pDNA. S-siRNA and Ø-pDNA had no genetic interferences with functional nucleotides.

Polymer characterization demonstrated that polymer size increased significantly from P(SiDAAr)<sub>5</sub> to P(SiDAAr)<sub>5</sub>P<sub>3</sub>, partly due to the hydrodynamic size of the long linear PEG chains (Table 1). The reduction in zeta potential from P(SiDAAr)<sub>5</sub> to P(SiDAAr)<sub>5</sub>P<sub>3</sub> indicated the PEG's ability to neutralize polymeric charges. PEI and P(SiDAAr)<sub>5</sub>P<sub>3</sub>'s polyplex demonstrated different characteristics as carrier materials (Table 2). Compare to PEI polyplexes, P(SiDAAr)<sub>5</sub>P<sub>3</sub>'s PEG groups has charge shielding effects. Further, PEI has more positive charges per weight unit compare to P(SiDAAr)<sub>5</sub>P<sub>3</sub>. Both factors resulted in PEI polyplexes having a higher average zeta potential than P(SiDAAr)<sub>5</sub>P<sub>3</sub> polyplexes. Since polyplex formation is a self-assembly process, the nucleotide exposure to the exterior cationic charge results in negative polyplex surface charge due to nucleic acids' anionic nature.



In all complexation ratios, complete nucleotide conjugation was observed, indicated by no nucleotide migration on the electrophoresis gel (Fig 2). Upon complete nucleotide masking by the polymer, SYBR Green dye has no binding with the nucleotide, thus showing no fluorescent band in the well. The 1/1/4 polyplex visualized by TEM showed individual almost spherical shape (Fig 3).

Heparin, a large polyanion, is a glycosaminoglycan, linear and negatively charged with sulfonate groups. It can be found inside cells and on the surface of the cell membrane.<sup>21</sup> Heparin is also a major component of the extracellular matrix of some tissues, including connective tissues.<sup>21</sup> Therefore, we choose heparin as a competition anion to evaluate the polyplex stabilities. 1/1/4 D/S/P polyplexes showed no disintegration at concentrations of up to 5 mg/mL heparin solution (Fig 4), demonstrating the polyplex stability in an anionic environment. The data thus suggest the polyplex would remain stable in the presence of serum or plasma proteins, which is critical for its *in vivo* application. Premature release of the cargo in the presence of serum protein or in circulation could make the polyplex ineffective as a transfecting agent.

Polymer toxicities were determined after 3 days of polymer incubation because 3 days incubation allows a more precise examination of the polymer effect on cell proliferations (Fig 5A). On the other hand, polyplex toxicities were examined in transfection conditions (1 day incubation) to more clearly reflect the polyplexes' growth inhibitory effects during the transfection period (Fig 5B, C). Although they contain equal amounts of DNA, 1/1/4 polyplexes weigh twice as much as 1/0/2 polyplexes, and showed higher toxicity. Although it appears that polyplexes have similar toxic effect in MCF-7 and MCF-7/Adr, the doses of polyplexes used in MCF-7/Adr was twice as much of each polyplex as MCF-7. Therefore MCF-7/Adr is in reality more resistant to polyplex toxicity than MCF-7. Most polyplexes here have a mild growth-inhibitory effect. However, P(SiDAAr)<sub>5</sub>P<sub>3</sub> polyplexes showed less toxicity than PEI polyplexes at higher polyplex concentrations (ex. 1/2/6).

Concerning DNA transfections, PEI and P(SiDAAr)<sub>5</sub>P<sub>3</sub> polyplexes show cell line dependent differential transfection levels. For example, P(SiDAAr)<sub>5</sub>P<sub>3</sub> shows higher transfection in MCF-7 and PEI in MCF-7/Adr (Fig 6, 7). In the process of acquiring drug resistance, MCF-7/Adr's membrane lipid composition undergoes changes, forming more compact and rigid cell membranes and increased lysosomes compared to MCF-7. This change affects its cellular endocytosis, membrane trafficking and intracellular processing/transport, which contribute to decreased DNA transfection.<sup>2223</sup> Thus MCF-7/Adr could require a highly cationic delivery vehicle for interaction with cell membrane to trigger cellular uptake process. On the other hand, drug sensitive cell lines (MCF-7 and MDA-MB-231-luc-D3H2LN) have relatively easy membrane transport of polyplexes due to fluid nature of the membrane to cause endocytosis. In drug sensitive cells, P(SiDAAr)<sub>5</sub>P<sub>3</sub> may better facilitate intracellular nucleotide release and nuclear localization due to arginine and siloxane's aforementioned properties.

Significantly enhanced luciferase protein knock-down was observed in siRNA-pDNA polyplexes compare to siRNA polyplexes (Fig 7), yet their average sizes and zeta potentials do not differ significantly from the siRNA polyplexes. Therefore, there may be other

mechanisms that assist in the transfections, such as the polyplexes' endosomal escape and intracellular trafficking. DNA uptake studies confirmed that high nucleic acid internalization and nuclear localization rates are not the only influences on transfection rates. For example, free YOYO-DNA's cellular uptake was significantly higher than that of 1/0/2 polyplexes in MCF-7 (Fig 8), yet free DNA showed insignificant transfections compared to polyplexes (Fig 6A). Also, 1/1/4 P(SiDAAr)<sub>5</sub>P<sub>3</sub> polyplexes have approximately 2-fold higher DNA uptake than 1/0/2 polyplexes, yet showed over 6-fold DNA transfection (Fig 6A). All these data indicate that the polymers not only play a role in DNA's intracellular uptake, they also affect other parameters that influence gene transfections. In all gene therapies, transfection level is heavily dependent upon the carrier's ability to overcome extracellular and intracellular barriers, including (1) cellular uptake, (2) endosomal escape and protection from lysosomal degradation, and (3) intracellular polyplex de-complexation for effective gene functioning.<sup>24</sup> Therefore, further studies are needed to understand their intracellular trafficking and sorting into different intracellular compartments that could impact transfection.

One observation to note is that 1/2/6 polyplexes showed a lower intracellular DNA uptake compare to both 1/0/2 and 1/1/4 polyplexes, as detected by both flow cytometry and confocal microscopy (Fig 9). This may due to YOYO-1-DNA's fluorescence quenching as a result of polymer and siRNA masking. When the overall ratio of (siRNA and polymer) to DNA increases, DNA masking is likely to result, thus lowering the detected DNA signal.

One possibility for co-delivery to enhance DNA and siRNA's transfection levels is by alterations in polyplex structures. DNA, which has long and winding strands, and siRNA, which has short and rigid strands, have different PEI binding behaviors. Study has shown DNA-PEI and siRNA-PEI polyplexes to have different hierarchical mechanism of formation.<sup>25</sup> Further, in siRNA polyplexes, siRNA undergoes biphasic binding with the polycations and molecular reorganization at low N/P ratios, which is not present with DNA polyplexes.<sup>25</sup> When combined, they may result in different polyplex properties from those formed by single nucleotides, thus potentially affecting the nucleotides' intracellular release, localization, and interaction with intracellular molecules.

Co-delivery is a promising yet challenging field. siRNA and paclitaxel co-delivery has been achieved by cationic core-shell nanoparticles with enhanced transfection efficiencies.<sup>26</sup> Poly(2-ethyl-2-oxazoline)-PLA-g-PEI,<sup>27</sup> multifunctional chitosan nanoparticles<sup>28</sup> and PEI-functionalized lipid nanocapsules<sup>29</sup> have all been used to explore nucleotide and drug co-delivery to enhance transfection outcomes. In this study, DNA and siRNA have been co-delivered in PEI-based polyplexes as helper polyanions to enhance each other's transfection levels. Mechanistically, both DNA and siRNA are complementary to one other. For example, when siRNA targets one gene and pDNA targets its suppressor gene, the combination may elongate the silencing effects.

## 5. Conclusions

This study demonstrated that long, supercoiled pDNA and short, rigid siRNA enhances each other's gene transfection levels in a cell. We found that the 1/1/4 D/S/P polyplex is a better

composition for co-delivery of DNA and siRNA as it resulted in higher DNA and siRNA transfection with reduced toxicity than other polyplex compositions tested. Co-delivery of siRNA and pDNA using PEI-based carrier is a promising approach to maximize gene transfection efficiency. Such an approach has the potential to achieve synergistic therapeutic effects by simultaneously targeting multiple genetic levels. This study showed that certain synergistic effects observed in co-delivery are due to the presence of a helper polyanion (DNA or siRNA). The study also gives incentive for future explorations in designing and implementing multi-nucleic acid delivery strategies. A better understanding of co-delivery's physicochemical, biological and therapeutic characteristics will be essential to future implementation of co-delivery strategies in clinical settings.

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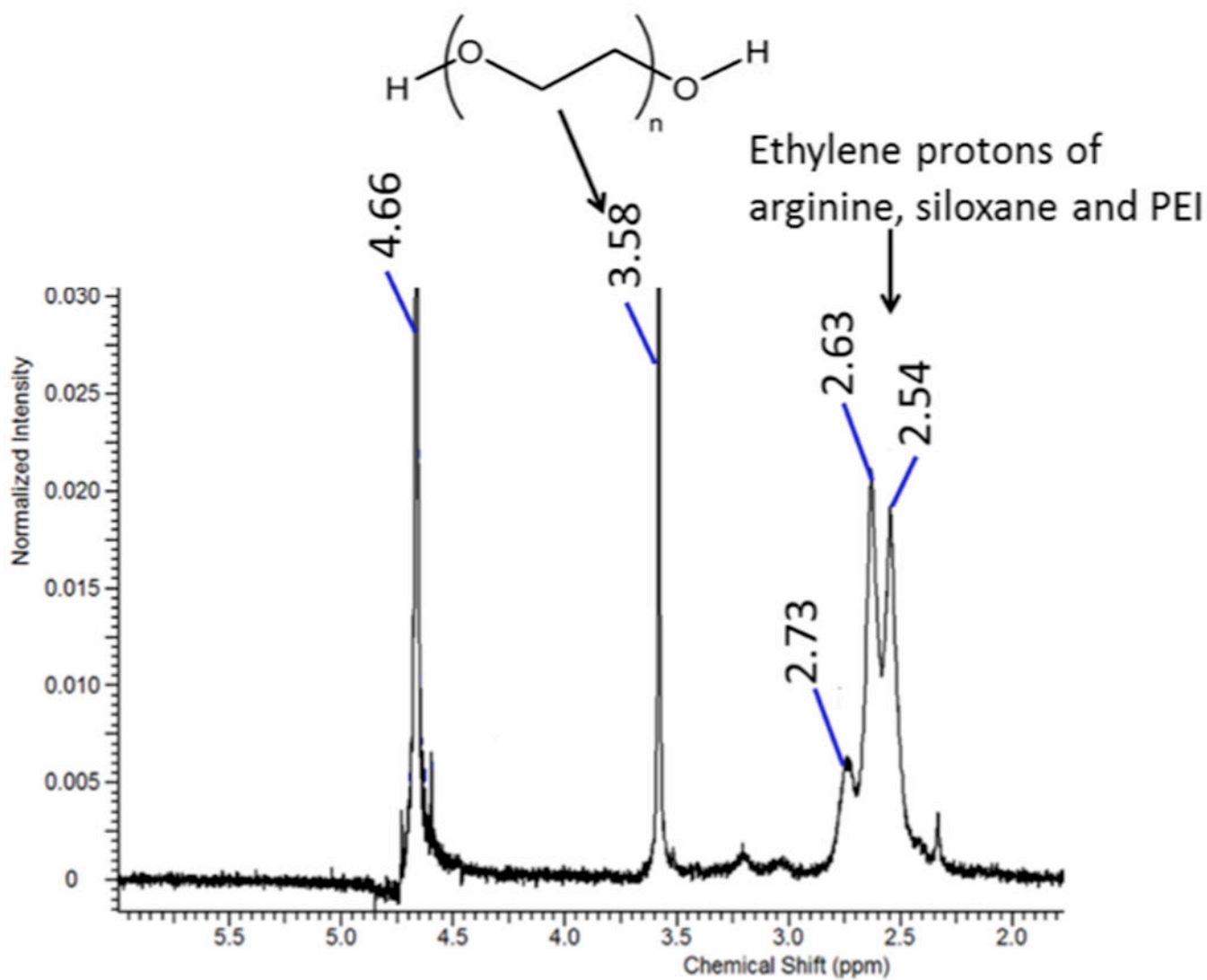
## Abbreviations

<b>abs</b>	absorbance
<b>bPEI</b>	branched polyethylenimine
<b>D<sub>h</sub></b>	hydrodynamic diameters
<b>DLS</b>	dynamic light scattering
<b>DMEM</b>	Dulbecco' s Modified Eagle's Medium
<b>D/S/P ratio</b>	DNA/siRNA/polymer weight/weight ratio
<b>luc-DNA</b>	luciferase-DNA
<b>luc-siRNA</b>	Anti-luciferase siRNA
<b>Ø-pDNA</b>	non-coding plasmid DNA
<b>PAMAM</b>	poly(amidoamine)
<b>PDI</b>	polydispersity Index
<b>PLGA</b>	poly(lactide <i>co</i> -glycolic acid)
<b>PEG</b>	polyethylene glycol
<b>PLL</b>	poly (L-lysine)
<b>P/N</b>	polymer/nucleotide weight/weight ratio
<b>P(SiDAAr)<sub>5</sub>P<sub>3</sub></b>	3 PEG-conjugated 5 siloxane-arginine modified bPEI polymer
<b>siRNA</b>	small-interfering RNA
<b>s-siRNA</b>	scramble siRNA
<b>TEM</b>	transmission electron microscopy

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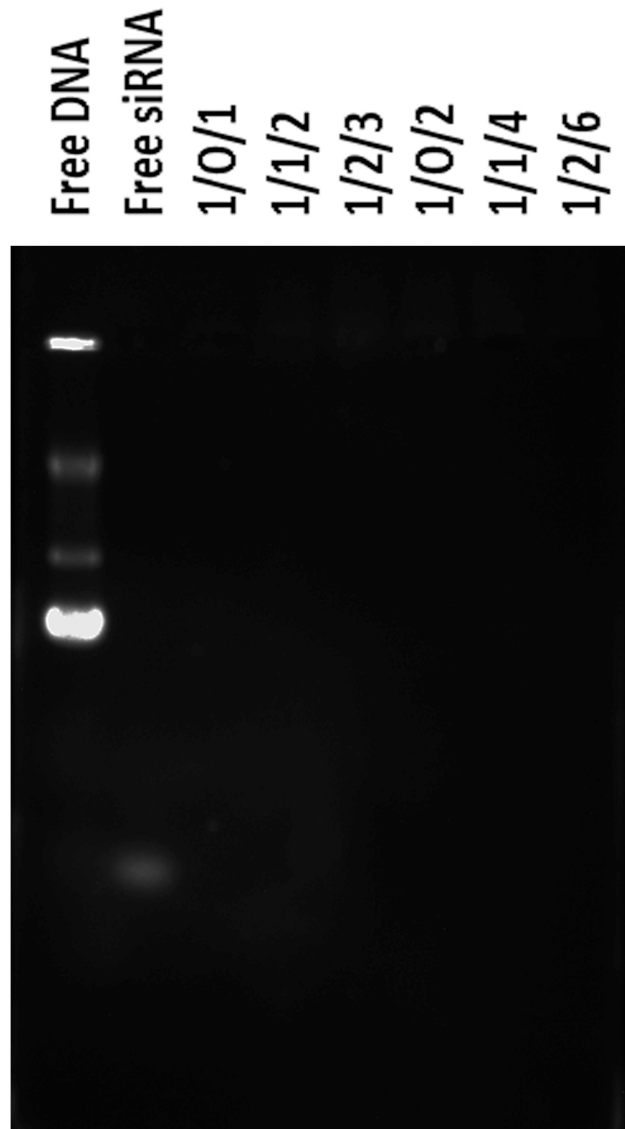
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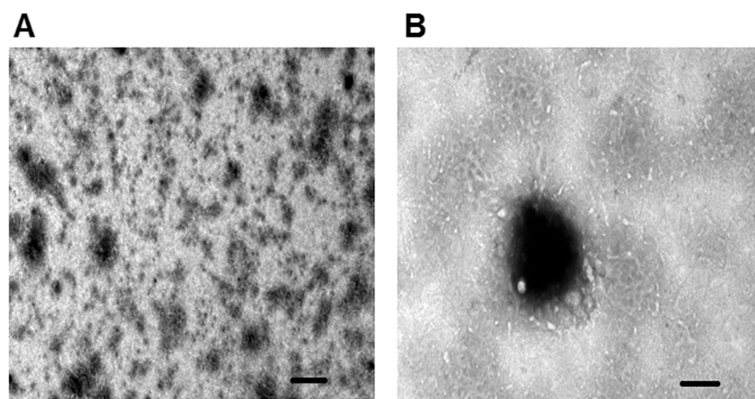


**Figure 1. Spectral characterization of  $\text{P}(\text{SiDAAr})_5\text{P}_3$**

$^1\text{H}$  NMR spectra showed the polymer's PEG ( $-\text{CH}_2-\text{CH}_2-\text{O}-$ ) groups, as well as arginine, siloxane and PEI's ethylene protons ( $-\text{CH}_2-$ ).

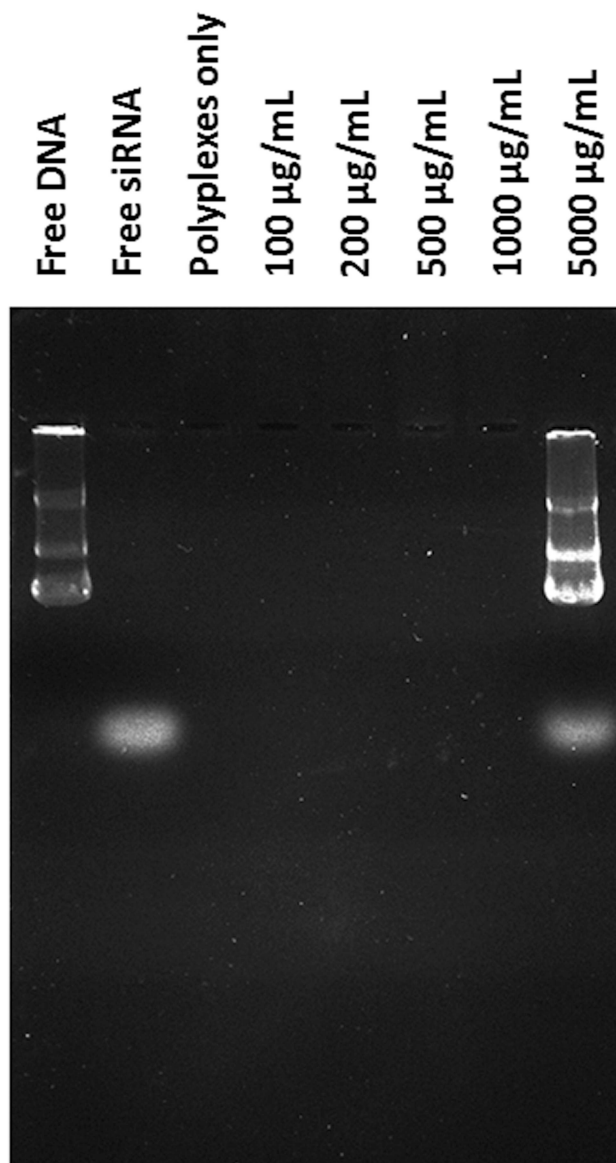


**Figure 2. Polyplex agarose gel electrophoresis identifies the un-complexed nucleotides** Complete nucleotide ( $\emptyset$ -pDNA and anti-luc siRNA) and polymer ( $P(\text{SiDAAr})_5\text{P}_3$ ) complexation was shown in all D/S/P w/w ratios.



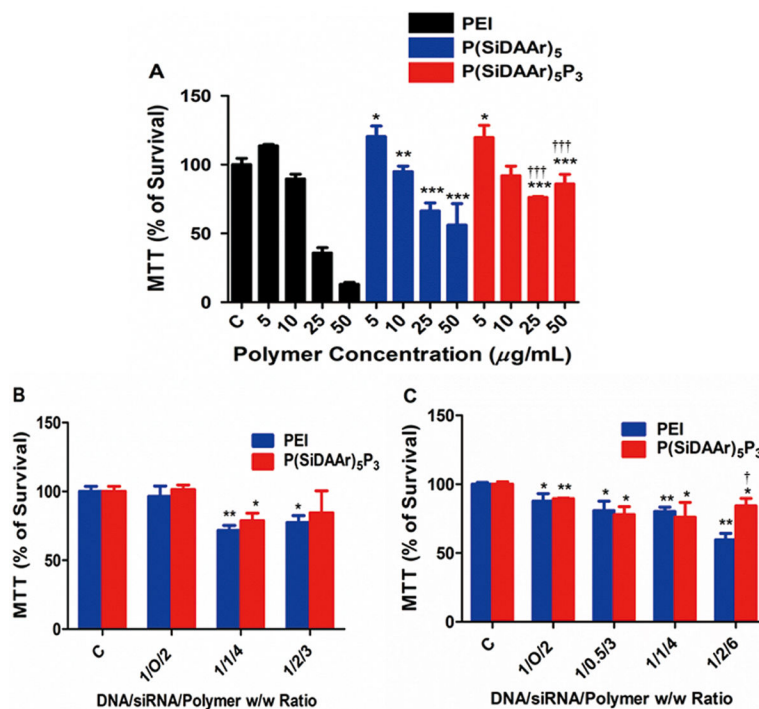
**Figure 3. Transmission electron microscopic (TEM) images of polyplex**  
Representative image shown is that of 1/1/4 DNA/siRNA/P(SiDAAr)<sub>5</sub>P<sub>3</sub> polyplexes in low (A,  $\times 13000$ , bar= $1 \mu\text{m}$ ) and high (B,  $\times 30000$ , bar= $0.5 \mu\text{m}$ ) magnifications.



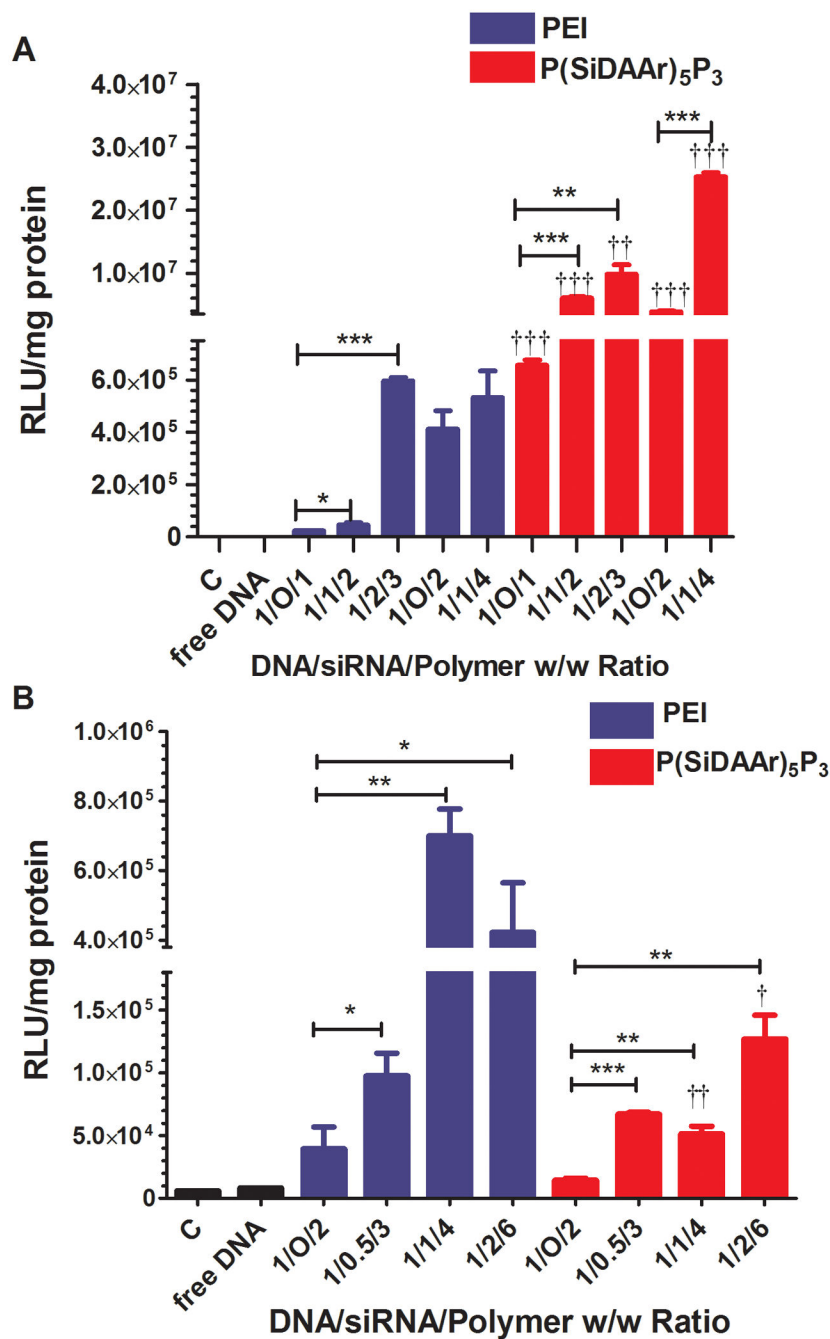


**Figure 4. Stability of polyplexes in the presence of polyanions**

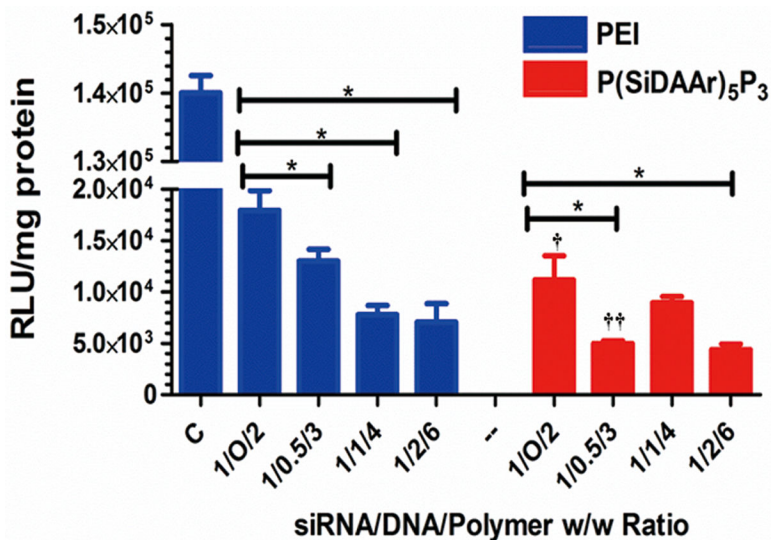
The stability of polyplex-nucleotides was measured using the polyanion competition assay. 1/1/4 P(SiDAAr)<sub>5</sub>P<sub>3</sub> polyplexes were incubated with heparin solution of varying concentrations, then separated using gel electrophoresis. Polyplexes incubated in 5 mg/mL of heparin released their nucleotides.



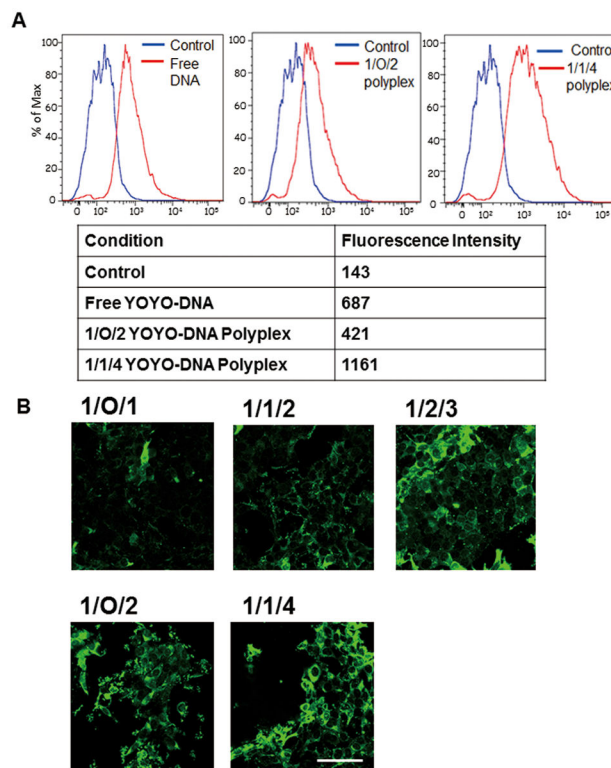
**Figure 5. Polymer and polyplex cytotoxicity assays in drug-sensitive and resistant cell lines** (A) Polymer solution MTT assay after 72 hr of polymer incubation in MDA-MB-231-luc-D3H2LN cells. \*: statistically significant compared to PEI polymer at the same concentration. †: statistically significant compared to P(SiDAAr)<sub>5</sub> polymer at the same concentration. (B) Polyplex cytotoxicity in MCF-7 after 24 hr polyplex incubation. (C) Polyplex cytotoxicity in MCF-7/Adr after 24 hr of polyplex incubation. All data are represented as mean ± standard deviation (n=2). \*: statistically significant compared to the control group; †: statistically significant compared to PEI polyplexes of the same composition. \*, † *p* 0.05; \*\*, †† *p* 0.01; \*\*\*, ††† *p* 0.001. Data as mean ± standard deviation.



**Figure 6. DNA transfection levels of both PEI and P(SiDAAr)<sub>5</sub>P<sub>3</sub> luc-DNA-co-s-siRNA polyplexes in MCF-7 and MCF-7/Adr cells**  
 (A) In MCF-7 cell line, P(SiDAAr)<sub>5</sub>P<sub>3</sub> polyplexes achieved a significantly higher transfection level than PEI polyplexes with the same composition. (B) In MCF-7/Adr cell line, PEI polyplexes achieved significantly higher transfection levels than P(SiDAAr)<sub>5</sub>P<sub>3</sub> polyplexes with the same composition. siRNA incorporation significantly enhanced DNA transfection levels in both cell lines. †: statistically significant compared to PEI polyplexes of the same composition. Data as mean ± standard deviation (n=2). \*, † *p* 0.05; \*\*, †† *p* 0.01; \*\*\*, ††† *p* 0.001.

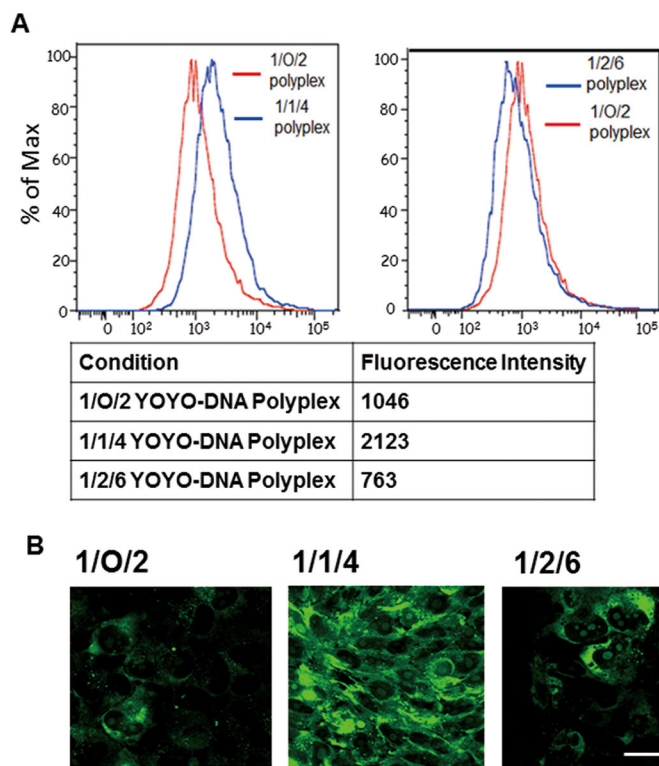


**Figure 7. Transfection of siRNA-polyplex in MDA-MB-231-luc-D3H2LN cell line**  
 In siRNA/DNA/polymer (PEI or P(SiDAAr)<sub>5</sub>P<sub>3</sub>) polyplexes, Ø-pDNA significantly enhanced luc-siRNA's transfection levels in MDA-MB-231-luc-D3H2LN cell line. In this condition, P(SiDAAr)<sub>5</sub>P<sub>3</sub> is a more effective transfection agent compared to PEI. Data as mean ± standard deviation (n=2). †: statistically significant compared to PEI polyplexes of the same weight ratio. \*, † *p* 0.05; \*\*, †† *p* 0.01; \*\*\*, ††† *p* 0.001.



**Figure 8. Quantification of DNA uptake in MCF-7 cell line**

(A) Flow cytometry showed that DNA-siRNA polyplexes have a greater DNA cellular uptake than DNA polyplexes. (B) Intracellular DNA was visualized by confocal microscopy showed polyplexes with siRNA (1/2/3 and 1/1/4) have a stronger DNA internalization than those without siRNA (1/O/1 and 1/O/2). Images were captured using a 40× objective lens. Bar = 100 μm.



**Figure 9. Quantification of DNA uptake in MCF-7/Adr cell line**

(A) Flow cytometry quantified P(SiDAAr)<sub>5</sub>P<sub>3</sub> polyplexes' DNA uptake levels in MCF-7/Adr cell line. 1/1/4 polyplexes showed over 2-fold higher DNA uptake than 1/0/2 polyplexes. The level dropped in 1/2/6 polyplexes due to polymer masking. (B) Confocal microscopy visualized YOYO-1-DNA's cellular internalization. 1/1/4 polyplexes showed a stronger DNA internalization than 1/0/2 polyplexes. Fluorescence intensity dropped in 1/2/6 polyplexes, confirming the flow cytometry results. Images were captured using 80× objective lens. Bar=50  $\mu$ m.

**Table 1**  
**Mean hydrodynamic diameter ( $D_h$ ) (nm) and zeta potential (mV) of polymer solutions in dH<sub>2</sub>O**

Polymer	Size, mean (SD)	PDI, mean (SD)	Zeta, mean (SD)
PEI	8.67 (0.78)	0.40 (0.10)	8.11 (6.29)
P(SiDAAr) <sub>5</sub>	10.47 (1.05)	0.38 (0.11)	12.11 (3.67)
P(SiDAAr) <sub>5</sub> P <sub>3</sub>	41.43 (11.30)	0.62 (0.38)	10.43 (5.72)

PDI: polydispersity index.

**Table 2**  
**Average hydrodynamic diameter ( $D_h$ ) (nm) and zeta potential (mV) of PEI and P(SiDAAr)<sub>5</sub>P<sub>3</sub> polyplexes at different weight ratios**

DNA/siRNA/PEI	Size, mean (SD)	PDI, mean (SD)	Zeta, mean (SD)
1/O/1	276.33 (156.16)	0.44 (0.21)	40.58 (5.99)
1/1/2002	301.33 (148.34)	0.34 (0.25)	39.05 (8.72)
1/0.5/1.5	454.2 (159.56)	0.29 (0.10)	38.3 (8.95)
1/2/2003	385.77 (142.15)	0.34 (0.21)	38.83 (3.20)
1/O/2	313.4 (80.77)	0.37 (0.17)	48.19 (9.39)
1/1/2004	438.57 (127.17)	0.23 (0.18)	36.93 (10.10)
1/0.5/3	312.83 (6.34)	0.28 (0.05)	53.56 (23.09)
1/2/2006	355.5 (84.76)	0.29 (0.15)	67.15 (18.04)
DNA/siRNA/ P(SiDAAr) <sub>5</sub> P <sub>3</sub>	Size, mean (SD)	PDI, mean (SD)	Zeta, mean (SD)
1/O/1	289.1 (22.89)	0.24 (0.04)	3.90 (2.50)
1/1/2002	386.3 (47.34)	0.37 (0.15)	-2.44 (18.09)
1/0.5/1.5	307.17 (65.69)	0.24 (0.14)	14.30 (15.80)
1/2/2003	302.07 (55.46)	0.29 (0.35)	20.17 (7.25)
1/O/2	280.47 (17.12)	0.28 (0.04)	21.17 (14.51)
1/1/2004	283.93 (29.41)	0.26 (0.10)	22.69 (19.38)
1/0.5/3	306.5 (14.35)	0.29 (0.06)	22.59 (5.95)
1/2/2006	318.5 (44.29)	0.31 (0.07)	17.87 (8.83)
siRNA/DNA/ P(SiDAAr) <sub>5</sub> P <sub>3</sub>	Size, mean (SD)	PDI, mean (SD)	Zeta, mean (SD)
1/O/2	233.43 (17.96)	0.25 (0.07)	20.44 (15.04)
1/0.5/3	228.13 (10.86)	0.19 (0.06)	10.96 (1.03)
1/1/2004	271.97 (86.71)	0.31 (0.23)	29.08 (15.92)
1/2/2006	296.67 (85.45)	0.30 (0.14)	18.25 (7.54)

PDI: Polydispersity Index, D/S/P: DNA/siRNA/polymer, S/D/P: siRNA/DNA/polymer.