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Effectiveness of siRNA Delivery via Arginine-Rich PEI-based Polyplex in Metastatic and Doxorubicin Resistant Breast Cancer Cells

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Abbreviations

ABCB1: multidrug resistance protein 1; bPEI: branched polyethylenimine; DOX: Doxorubicin; DLS: dynamic light scattering; DMEM: Dulbecco's Modified Eagle's Medium; EDC: 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride; luc-siRNA: anti-luciferase siRNA; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MWCO: molecular weight cutoff; MES: 2-(N-morpholino)ethanesulfonic acid; NHS: N-hydroxysuccinimide; N/P w/w ratio: siRNA/polymer weight/weight ratio; PDI: polydispersity index; PEG: polyethylene glycol; $P(SiDAAr)_5PEG_3$: 3 polyethylene glycol (PEG) modified L-arginine oligo (alkylaminosiloxane) graft poly (ethyleneimine); siDAAr: L-arginine oligo (alkylaminosiloxane); $P(SiDAAr)_5$: 5 L-arginine oligo (alkylaminosiloxane) grafted poly (ethyleneimine); siRNA: small-interfering RNA; TEM: transmission electron microscopy.

Abstract

Poor cellular uptake, rapid degradation in the presence of serum, and inefficient transfection are some of the major barriers in achieving therapeutic efficacy of naked small interfering RNAs (siRNAs). We investigated the efficacy of the polyplex formulated using our synthesized polymer, (P(SiDAAr)$_5$PEG$_3$)—a polyethylene glycol (PEG)-modified L-arginine oligo (-alkylaminosiloxane) that is grafted with poly (ethyleneimine, PEI) for siRNA delivery. We hypothesized that the polyplex formulated using the polymer with a balanced composition of PEI for siRNA condensation and its protection, PEG for polyplex stability and to minimize the PEI-associated toxicity, and with arginine facilitating cellular uptake would overcome the above issues with siRNA delivery. We tested our hypothesis using anti-luciferase siRNA in luciferase-expressing metastatic breast cancer cells (MDA-MB-231-Luc-D3H2LN) and anti-ABCB1 siRNA against an efflux membrane protein, ABCB1, in doxorubicin (DOX) resistant breast cancer cells (MCF-7/Adr). The results demonstrated that the polyplex at an optimal nucleotide/polymer (N/P) ratio is stable in the presence of excess polyanions, has no cellular toxicity, and protects siRNA from RNase degradation. Transfection of MDA-MB-231-Luc-D3H2LN cells with anti-luciferase siRNA polyplex showed almost complete knockdown of luciferase expression. In MCF-7/Adr cells, transfection with anti-ABCB1 siRNA effectively down-regulated its target efflux protein, ABCB1, increased cellular uptake of DOX and enhanced its cytotoxic effect. However, the co-treatment did not completely overcome drug resistance, suggesting that further optimization is needed and/or mechanism(s) other than the efflux protein, ABCB1 may be involved in drug resistance. In conclusion, our polyplex is effective for siRNA delivery and can be explored for different therapeutic applications.

Keywords: Nucleic acid delivery, gene silencing, cancer treatment, polymers, efflux proteins, drug transport

Introduction

Therapeutic promise of small interfering RNAs (siRNA) has gained wide recognition for treating different diseases such as cancer, viral infections, and neurodegenerative diseases (Dykxhoorn et al., 2003; Burnett et al., 2011; Lan et al., 2015). A major advantage of siRNA lies in its function in the cytoplasm rather than in the nucleus, which avoids nuclear penetration, a major barrier for cDNA delivery for gene transfection (Wang et al., 2010a). In cancer, siRNA can repress cancer cell proliferation, metastasis, and reverse drug resistance (Scamuffa et al., 2008; Yang et al., 2015; Zi et al., 2015). Despite these benefits, translation of siRNA-based therapy has been limited due to several factors, including: a) poor cellular uptake of siRNAs because of their anionic charge and hydrophilic nature (Ragelle et al., 2013), b) instability because of their rapid degradation by RNase (Hickerson et al., 2008), and c) delivery vehicle-associated cytotoxicity (de Fougerolles et al., 2007; Whitehead et al., 2009; Shegokar et al., 2011). Cationic lipids (e.g. lipofectamine) (Rietwyk and Peer, 2017) and polymers can both complex siRNA and facilitate transfection (Zhu and Mahato, 2010). The two commonly used polymers for transfection are poly(ethylene imine)s (PEI) and poly(amidoamine)s (Kawakami et al., 2006; Hobel and Aigner, 2010). PEI can spontaneously self-assemble with siRNA to form polyplex (Wang et al., 2010b; Merkel et al., 2011; Yuan et al., 2011; Wu et al., 2012); however, it faces significant toxicity issue such as membrane damage and activation of apoptotic pathways by mitochondria (Moghimi et al., 2005; Hunter and Moghimi, 2010; Alameh et al., 2012). Therefore, a general strategy is to minimize the toxic effect of cationic polymers by modifying them with functional groups such as PEG or polysaccharides (e.g., dextran, pullulan)(Caroline Diana and Rekha, 2017); however, such modifications tend to reduce transfection efficiency (Brissault et al., 2006). Therefore, a critical balance is required for the polymers with a cationic charge that is effective

in condensing nucleic acids but at the same time does not cause cellular toxicity due to cationic charge or aggregation in the presence of negatively charged serum proteins.

Here, we explored the efficacy of the polyplex formulated using our synthesized polymer, L-arginine oligo (alkylaminosiloxane) grafted PEI [P(SiDAAr] and modified with PEG, [$P(SiDAAr)_5PEG_3$] to deliver siRNA to both metastatic (MDA-MB-231-Luc D3H2LN) and doxorubicin (DOX) resistant (MCF-7/Adr) breast cancer cells. Arginine has the ability to facilitate intracellular translocation of macromolecules and nanocarriers, as it has a strong affinity with heparan sulfate expressed on mammalian cell membranes (Morris and Labhasetwar, 2015). Also, the guanidine groups on arginine can form hydrogen bonds with polyhydroxy compounds in cell membrane (Calnan et al., 1991; Richard et al., 2003; Wang et al., 2009). Dendrimers such as amidoamine (PAMAM) and polymer (propylene imine) conjugated to arginine have shown enhanced transfection efficiency (Choi et al., 2004; Kim et al., 2007). We hypothesized that a well-characterized formulation of polyplex would facilitate efficient cellular delivery of siRNA and hence its effect. Our data show that the polyplex at an optimized composition is nontoxic to the cells, and is effective in transfecting anti-luciferase siRNA to metastatic breast cancer cells and therapeutic siRNA against efflux protein, ABCB1 in resistant breast cancer cells.

Materials and methods

Materials. Three-(2-aminoethylamino) propyl-methyl-dimethoxysilane, 25-kDa branched PEI, aspartic acid, L-arginine, O-(2-aminoethyl)-O'-(2-carboxyethyl) polyethylene glycol-3K hydrochloride, 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), heparin

sodium, N-hydroxysuccinimide (NHS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), sodium bicarbonate powder, hydroxylamine hydrochloride, uranyl acetate, and Tris Acetate-EDTA buffer were purchased from Sigma-Aldrich (St. Louis, MO). Fetal bovine serum (FBS) and Trypsin/EDTA were obtained from Gibco (Grand Island, NY). RNase I and Silencer Negative Control No.1 siRNA were purchased from Life Technologies (Carlsbad, CA). Dulbecco's Modified Eagle's Medium (DMEM), DPBS, penicillin, and streptomycin were purchased from the Cell Services' Media Core at the Cleveland Clinic.

Synthesis of $P(SiDAAr)_5PEG_3$. The polymer that consists of 3M (molar) of PEG and 5M of L-arginine oligo (alkylaminosiloxane) grafted poly(ethyleneimine) ($P(SiDAAr)_5PEG_3$) (where numbers in subscript indicate relative molar ratio per mole of polymer) was synthesized as per the **Scheme** described below. In brief, following the synthesis of oligo (-alkylaminosiloxane) (Step A, SiDA) and modification with arginine (Step B, SiDAAr), PEI conjugation to SiDAAr was carried out using aspartic acid as a linker. Specifically, acid groups of aspartic acid were activated using EDC and NHS at room temperature in MES buffer at pH 6. Thereafter, 5M of SiDAAr and 1M of PEI were added to the reaction mixture with stirring for 18 hr at room temperature, followed by dialysis using 12K MWCO dialysis membrane (Spectrum Laboratories, Rancho Dominguez, CA) (Step C). To conjugate PEG to $P(SiDAAr)_5$, free acid groups of $NH_2-PEG-COOH$ were activated using EDC (coupling agent) and NHS (catalyst) in MES buffer at pH 6 for 3.5 hr at room temperature. Thereafter, 3M of activated PEG was added to the 1M equivalent of $P(SiDAAr)_5$ in PBS at pH 7.5 with overnight stirring (Step D). The polymer was dialyzed for 2 days in 2 L of dH_2O using 12K MWCO dialysis membrane to remove unreacted elements and then lyophilized.

TRITC Conjugation to Polymer. To determine cellular uptake of polyplex using flow cytometry, the polymer, $P(SiDAAr)_5PEG_3$ was conjugated to tetramethylrhodamine-6-isothiocyanate (6-TRITC) dye (Invitrogen, Carlsbad, CA). To conjugate, 2 mg of polymer was dissolved in 1 mL of 0.1M sodium bicarbonate buffer (pH =9.5) and 1 mL of 6-TRITC was dissolved in 500 μL of DMSO. While stirring, 100 μL of the above reactive 6-TRITC dye solution was added to the polymer solution dropwise. The reaction was continued with stirring for 1 hr at room temperature and then was stopped by adding 0.1 mL of freshly prepared 1.5M hydroxylamine in distilled water and adjusting the pH to 8.5 with 5M NaOH. The polymer was dialyzed overnight using 12K MWCO dialysis membrane to remove the excess 6-TRITC dye.

Polymer Complexation and Characterization of Polyplex. The polymer, $P(SiDAAr)_5PEG_3$ dissolved in RNase free dH_2O (Qiagen, Valencia, CA) at 1 mg/mL concentration was filtered through a 0.22 μm sterile polyethersulfone filter (Millipore, Darmstadt, Germany). siRNA was also diluted separately to 1 mg/mL in RNase free dH_2O. The formulations of polyplex were prepared by pipetting the siRNA and polymer mixture up and down for ~30 times and incubating it for 1 hr to allow self-assembly. Three μg of siRNA was used per sample and the polymer quantity was changed to form polyplex with different nucleotide/polymer (N/P) w/w ratios. Size and ζ-potential were measured in dH_2O using quasi-elastic dynamic light scattering and zeta potential analyzer (NICOMP 380 ZLS Particle Sizing System, Santa Barbara, CA). Morphology of polyplex was characterized by transmission electron microscopy (TEM) using a Tecnai G2 TEM microscope (FEI, Hillsboro, Oregon). For TEM microscopy, polyplex with 1/4 w/w N/P w/w ratio prepared as above in dH_2O was used. A 10 μL of the polyplex dispersion was dropped on the TEM grids coated with silicon monoxide stabilized formvar films (Electron Microscopy

Sciences, Hatfield, PA). The samples were then air dried and stained with 2% uranyl acetate for 7 min prior to imaging.

Determination of Optimal siRNA to Polymer Complexation Ratio. Agarose gel electrophoresis was used to determine the optimal siRNA/polymer (N/P) complexation ratio. Polyplex was prepared at the N/P w/w ratios of 1/0.5, 1/1, 1/2, 1/4 and 1/6 w/w, keeping siRNA quantity constant at 1 μg while varying polymer quantity. Using naked luciferase DNA as a reference, naked siRNA and siRNA polyplex were loaded onto a 1% Gold Agarose gel (Lonza, Allendale, NJ) in TrisAcetate-EDTA buffer. The samples were run at 60 V for 60 min on an electrophoresis system (Bio-Rad, Hercules, CA). The gel was stained using SYBR Green (Thermo Scientific, Rockford, IL) and visualized using a UV imager (FUJIFILM FLA-5100, FUJIFILM Life Science, Stamford, CT).

Protection of siRNA from RNase Degradation. The effectiveness of the polyplex to protect siRNA from RNase degradation was determined by incubating naked siRNA and the polyplex prepared at a 1/4 N/P w/w ratio with 100 IU of RNase buffer for 30 min. Absorbance was measured at 260 nm using Nanodrop 1000 (Thermo Fisher Scientific, Waltham, MA)

Polyplex Stability. Stability of the polyplex prepared at a 1/4 N/P w/w ratio was measured in the presence of heparin polyanions using a polyanion competition assay (Danielsen et al., 2005). The polyplex consisting of 5 μg siRNA and 20 μg polymer in 15 μL dH_2O was incubated for 15 min with various concentrations of heparin sodium solution (Sigma-Aldrich). The samples were run on a 1% gold agarose gel (Lonza) in TrisAcetate-EDTA buffer, and the gel was stained and visualized by the same method as described above.

Cell Culture. Resistant MCF-7/Adr cell line was created through a long-term DOX treatment of sensitive MCF-7 cells. Drug resistance was maintained by incubating the cells with 100 ng/mL of DOX (Vijayaraghavalu et al., 2012). Prior to transfection, medium in the wells was changed to a drug-free medium for 2 passages. Both the cell lines, MCF-7/Adr and MDA-MB-231-Luc-D3H2LN (Caliper Life Sciences, Hopkinton, MA) were cultured in 15% FBS enriched DMEM with 1% penicillin-streptomycin under 5% CO_2 and 95% humidity at 37 ºC.

Cytotoxicity of Polyplex. MDA-MB-231-Luc-D3H2LN cells were seeded at 5×10^4 cells/well in a 24 well-plate (BD Biosciences, San Jose, CA) in DMEM with 15% FBS. Polyplex made of 1 μg luciferase siRNA and varying amounts of the polymer to achieve different N/P w/w ratios prepared in 15 μL of DMEM were gently added into each well and mixed with culture medium. After 16 hr of incubation, the medium in the wells was replaced with a fresh medium. The cell viability was determined after 3 days of incubation using MTT assay. For the assay, cell culture medium was removed and replaced with 150 μL of MTT reagent (0.5 mg/mL in colorless DMEM) and incubated for 3.5 hr. Thereafter, the MTT solution in culture plates was replaced with 250 μL of DMSO and incubated for another 30 min. One hundred and fifty μL of the DMSO solution from the culture plates was taken out to a clear 96-well plate to measure absorbance at 595 nm using a plate reader (SpectraMax M2, Molecular Devices Inc., Sunnyvale, CA). Cell viability was calculated as (absorbance of the polyplex treated cells)/(abs of untreated cells)×100%.

Cellular Uptake of Polyplex. Cellular uptake of the polyplex prepared at 1/4 N/P w/w ratio was determined using TRITC-conjugated $P(SiDAAr)_5PEG_3$ in both MDA-MB-231-Luc-D3H2LN and MCF-7/Adr cell lines. Cells were seeded in 6-well plates with 15% DMEM and cultured

until reaching to ~80% confluency. The polyplex made of 3 μg of siRNA and 12 μg of TRITC-conjugated $P(SiDAAr)_5PEG_3$ was added to wells, gently mixed, and incubated for 4 hr. The cells were washed 3 times with PBS and trypsinized prior to analysis by flow cytometry using an LSR II System (BD Biosciences, Franklin Lakes, NJ) with fluorescence detector set at excitation maximum of 530 nm and emission maximum at 569 nm.

siRNA-Mediated Gene Silencing. For ubiquitous gene knockdown, we assayed firefly luciferase gene silencing because it has no apparent effect on cell regulatory machinery. MDA-MB-231-Luc-D3H2LN cells were seeded at a density of 2×10^5 cells/well in 24-well plates and cultured for 24 hr. The medium for all transfection studies contained DMEM with 15% FBS and 1% penicillin-streptomycin. Polyplex containing 2 μg of siRNA was added to each well and incubated for 24 hr. Thereafter, cells were washed with PBS for 3 times and lysed with 100 μL of Reporter Lysis Buffer (Promega, Madison, WI). Twenty μL of cell lysate and 50 μL of Luciferase Assay Reagent (Promega) were added into a Nunc F96-MicroWell White Polystyrene plate (Nunc, Thermo Scientific). Luciferase activity was measured using a Luminescence Plate Reader (Wallace 1420 VICTOR$^{2™}$, PerkinElmer, Waltham, MA). Cell protein levels were analyzed using BCA protein assays (Thermo Fisher Scientific). To perform the assay, 10 μL of cell lysate and 150 μL of freshly prepared BCA reagent (1 part of reagent A and 50 parts of reagent B) were added to a 96 well-plate and incubated at 37 ºC for 30 min. Absorbance was measured at a wavelength of 562 nm using a Microplate Reader (Molecular Devices, Sunnyvale, CA). Luciferase activity was normalized to the cell protein level.

Knockdown of ABCB1 Protein via anti-ABCB1 siRNA, its Effect on Enhancing DOX Uptake and Cytotoxicity in Resistant Cells. To examine the effectiveness of the polyplex in downregulating functional siRNA against its target protein, MCF-7/Adr cells were seeded in a 6-well plate and incubated with anti-ABCB1 siRNA (Dharmacon, Inc., Lafayette, CO) in 15% DMEM for 24, 48 and 72 hr. The initial cell density was adjusted do avoid cells reaching to over-confluency in a long-term study. The day before transfection, the cells were seeded at 7×10^5/well for 24-hr treatment study, 6×10^5/well for 48-hr treatment study and at 3.6×10^5/well for the 72-hr treatment study. Polyplex with two μg of anti-ABCB1 siRNA was used for each condition with no change of media during incubation time. The cells were washed, trypsinized and incubated with 3 μL of phycoerythrin-conjugated anti-ABCB1 antibody (Thermo Fisher Scientific) at 0.1 μg/μL for 30 min in an ice bath. The cells were then washed twice with flow buffer (PBS with 10% FBS and 0.1% sodium azide) and kept in flow buffer on ice for flow cytometric analysis using an LSR II System (BD Biosciences).

To analyze the effect of anti-ABCB1 siRNA on cellular uptake of DOX, MCF-7/Adr cells were seeded in a 6-well plate at 7×10^5 confluency. After 24 hr, the anti-ABCB1 siRNA-polyplex and DOX solution at 2,500 ng/mL was added to the plate and incubated for 4.5 hr. The cells were then washed, trypsinized and collected for flow cytometry at the excitation wavelength of 470 nm and the emission wavelength of 585 nm.

To examine the effect of anti-ABCB1 siRNA on DOX cytotoxicity, MCF-7/Adr cells were seeded in a 96-well plate at 5,000 cells/well. After 24 hr of cell attachment, anti-ABCB1 siRNA-polyplex, and DOX solution were co-treated with 0.5 μg of siRNA/well and different doses of

DOX (100 to 10,000 ng/mL). The cells were incubated for 72 hr prior to MTT assay for cell viability.

Statistical Analysis: Data are expressed as the mean ± s.e.m.. Statistical significance was considered at $p \leq 0.05$.

Results

Polymer and Polyplex Characterization: The polymer, $P(SiDAAr)_5PEG_3$ prepared as per the **Scheme** described above was characterized by ^1H NMR (D_2O) spectrum to confirm the presence of all the components (**Fig. 1A**). Chemical shifts at δ =3.4-2.9 are from SiDAAr (-**H**CCH$_2$CH$_2$C**H$_2$**NH-), and at δ =2.8-2.4 are from (-NHC**H$_2$**C**H$_2$**-, PEI ethylene). Here, the ethylene resonances of the SiDAAr units are clearly visible. The resonances of PEG (-OC**H$_2$**C**H$_2$**-) are also visualized at δ =3.7-3.5. Branched PEI has much more abundant amino groups than the hydroxyl groups on PEG; therefore, from the moles inputted in the reaction mixture, all of the activated PEGs should react with $P(SiDAAr)_5$. The final polymer $P(SiDAAr)_5PEG_3$ has a white sponge-like morphology as a dried polymer.

TEM images of the polyplex formulated at N/P w/w ratio of 1/4 showed positively stained particulate structures with an average size of 78.3 ± 6.2 nm (n=18) **(Fig 1B)**. The hydrodynamic diameter of the same formulation was =349.5 nm (polydispersity index, PI =0.34) and zeta potential of +30 mV. Particle size did not change significantly for the polyplex formulated with the N/P ratios of 1/2 to 1/4 w/w (327 to 350 nm) whereas, at the N/P ratios of 1/5 and 1/6 w/w, the polyplex sizes were smaller (231 to 240 nm), indicating that with increasing the polymer ratio, polyplex acquired smaller size. Zeta potential for the polyplex at N/P ratio was +8.7 mV,

whereas, for all other polyplex combinations, it ranged from +26.2 to +42.5 mV, showing a general trend that the polyplex acquired a more cationic charge with increase in the polymeric ratio (**Table 1**).

Complexation and siRNA Stability: Complete siRNA complexation at 1/4 w/w N/P ratio was evident from no observable migration of free siRNA down the gel **(Fig 2A)**. In the presence of RNase, free siRNA fragmented as evident from the increase in absorbance with the degradation products contributing to this increase; however, no such increase was seen when polyplex was incubated with the nuclease **(Fig 2B)**.

Polyplex Stability in the Presence of Excess Polyanion: Polyanion competition assay showed that the polyplex remained intact up to heparin concentration of 1000 μg/mL; however, at higher concentrations, siRNA was released from the polyplex due to the replacement of siRNA from the polyplex by polyanion heparin molecules. At 5,000 μg/mL heparin concentration, based on band intensity, the amount of free siRNA migrating down the gel is about the same as that of free siRNA **(Fig 3)**.

Cellular Uptake of siRNA Polyplex: Intracellular uptake of polyplex using TRITC-conjugated $P(SiDAAr)_5PEG_3$ polymer showed uptake in both MDA-MB-231-Luc-D3H2LN and MCF-7/Adr cells. This uptake was seen at 4 hr, indicating rapid cellular uptake of the polyplex **(Fig 4)**.

Polyplex Toxicity and Anti-Luciferase Transfection Efficiency: Polyplex showed no significant cytotoxicity at all except for the polyplex prepared at N/P w/w ratio of 1/6 **(Fig 5A)**. Transfection increased with increasing the polymeric ratio in the polyplex as evident from

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increasing luciferase expression knockdown, reaching to 98% at the N/P ratio of 1/4 w/w compared to control, but insignificantly changed thereafter **(Fig 5B)**. The polyplex with 1/4 w/w N/P ratio showed almost complete luciferase gene expression knockdown yet low toxicity; therefore, this ratio was selected for the subsequent experiment.

ABCB1 Protein Downregulation, DOX Uptake and its Cytotoxicity in MCF-7/Adr Cells:

Based on the shift in the fluorescence intensity curve compare to control following 72 hr incubation, there was ~70% downregulation of the ABCB1 protein in MCF-7/Adr cells transfected with anti-ABCB1 siRNA polyplex **(Fig 6A)**. MCF-7/Adr cells transfected with anti-ABCB1 siRNA polyplex also enabled greater intracellular accumulation of DOX as evident from the shift in DOX fluorescence intensity compared to the uptake in un-transfected cells **(Fig 6B)**. Greater uptake of DOX in anti-ABCB1 siRNA polyplex treated cells showed greater drug cytotoxicity than in untreated cells but the effect was not DOX-dose dependent **(Fig 6C)**.

Discussion

Critical issues in siRNA delivery include its effective incorporation into a delivery vector, able to protect siRNA from nuclease degradation, and achieving effective transfection even in the presence of serum proteins. In this study, we demonstrated that the polyplex formulated using the polymer, $P(SiDAAr)_5PEG_3$ meets the above criteria due to its balanced composition of PEI to condense and protect siRNA, PEG to stabilize polyplex and reduce toxicity, and arginine to facilitate cellular uptake. The spectral characterization confirms the presence of all the above components in the polymer **(Fig 1A)**. The hydrodynamic diameter of the polyplex was significantly greater than the TEM diameter **(Fig 1B)** but this is expected considering that the

TEM diameter is in a dry state of the polyplex whereas the hydrodynamic diameter is measured in a hydrated state, and this hydration effect is expected to be greater with our polyplex because of the branched nature of the polymer with several hydrophilic hydroxy and amino groups. At an optimal 1/4 w/w N/P ratio, the polyplex demonstrated complete complexation of siRNA **(Fig 2A)** and stabilize it from degradation due to nucleases **(Fig 2B)**. The formed polyplex showed stability in the presence of excess polyanion-heparin **(Fig 3**), indicating interaction of siRNA and polymer, and stability of the polyplex in the presence of anions such as serum proteins, which is also evident from the transfection data since it was carried out in culture medium containing serum. Although PEI is one of the most widely used transfection reagents, it has limited cellular uptake in the presence of serum, which translates to its limited *in vivo* efficacy (Wang et al., 2016). Critical for transfection is the release of RNA from the polyplex following its cellular uptake. Our data show the uptake of polyplex inside cells (**Fig 4**) but also it is effective in transfecting cells, an indication that siRNA is released from the polyplex following its cellular uptake **(Fig 5)**.

Cytocompatibility of polyplex is an equally important issue (Douglas et al., 2008). Our data show that the polyplex at an optimized 1/4 w/w N/P ratio is quite cytocompatible, suggesting that this composition provides the right balance between PEI and PEG in masking cationic charge of PEI without influencing the polyplex cellular uptake (**Fig 4)** or its transfection (**Fig 5**). Previously, we have shown that PEI alone is quite toxic to cells (Lu et al., 2015).

ABCB1 is a major contributor to multi-drug resistance as it actively pumps out chemotherapeutic agents, thus lowers drug concentration inside cells (Susa et al., 2010). MCF-7/Adr cells are

known to overexpress ABCB1 efflux pump (Jiang et al., 2016). It evident from our data that MCF-7/Adr incubated with anti-ABCB1 siRNA polyplex resulted in a time-dependent increased downregulation of ABCB1 protein (**Fig 6A**). This effect can be explained considering half-life of degradation ABCB1 protein from cell membrane ranges between 15–72 hr (Richert et al., 1988; Mickley et al., 1989; Chin et al., 1990; Aleman et al., 2003). Anti-ABCB1 siRNA transfection resulted in enhanced DOX cellar uptake **(Fig 6B)**, hence increased drug cytotoxicity **(Fig 6C)**, further confirming the efficacy of anti-ABCB1 siRNA polyplex. However, the cytotoxic effect of DOX was not dose dependent, suggesting that further optimization of the treatment protocol may be needed which could involve pre-treating resistant cells with anti-ABCB1 siRNA polyplex to optimize the dose and time required to maximize downregulation of the efflux protein prior to treatment with DOX. Others have also tested this approach and have shown that sustained delivery for siRNA is better for drug efficacy than its transient effect (Susa et al., 2010).

In addition to overexpression of membrane-bound drug transporter proteins, such as P-glycoprotein (P-gp, ABCB1), there are other multidrug resistance-associated proteins (MRP1, ABCC1, and MRP2, ABCC2), and breast cancer resistance protein (BCRP, ABCG2) (Housman et al., 2014; Issa et al., 2017) which may not have been affected by treating resistant cells with anti-ABCB1 siRNA alone. Thus, a cocktail of siRNA against all the efflux proteins and/or other cellular targets responsible for drug resistance may be needed to optimize the treatment effect to reverse drug resistance. We have previously demonstrated that the similarly formulated polyplex is effective in delivering DNA alone or DNA and siRNA together, achieving enhanced transfections of both the moieties (Morris and Sharma, 2010; Lu et al., 2015). Thus with our polyplex, both cytoplasmic and nuclear factors (e.g. DNA damage repair mechanism,

transcription factors, epigenetic modifications, etc.) responsible for drug resistance can be targeted simultaneously (Housman et al., 2014). We have also reported epigenetic modifications in MCF-7/Adr cells as a cause of drug resistance (Vijayaraghavalu and Labhasetwar, 2013), thus a combination treatment that targets multiple mechanisms may be needed to address the complex nature of drug resistance.

Although we have not determined the effect of functional siRNA in metastatic breast cancer cells using our polyplex, there are several genes (e.g. CDK8, a cyclin-dependent kinase member of the mediator complex, Rho-associated coiled-coil-containing protein kinase, etc.) that can be targeted to inhibit cancer cell proliferation (Lee et al., 2016; Ahmadzada et al., 2018). In addition to cancer cells, our studies with similarly formulated DNA-polyplex demonstrated gene transfection in neuronal cells, both *in vitro* and *in vivo* (Joshi et al., 2018), indicating broader applications of our polyplex for delivery of nucleic acids for potentially treating different disease conditions.

Conclusion

The formulated polyplex at an optimal composition is cytocompatible and effective in delivering siRNA to both resistant and metastatic breast cancer cells. With functional siRNA against anti-ABCB1, the treatment was partially effective in overcoming DOX resistance. Since polyplex showed stability in the presence of excess anion and the transfection studies were carried out in the presence of serum, it is likely that our polyplex formulation could be effective *in vivo* and potentially can be explored in treating different types of cancers and other diseases.

Authorship Contributions

Participated in research design: Shan Lu, Viola B. Morris, Vinod Labhasetwar

Conducted experiments: Shan Lu, Viola B. Morris

Performed data analysis: Shan Lu, Viola B. Morris, Vinod Labhasetwar

Wrote or contributed to the writing of the manuscript: Shan Lu, Viola B. Morris, Vinod Labhasetwar

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Figure Legends

Scheme for Polymer Synthesis: Steps involved in synthesis of polymer (**A)** oligo(-alkylaminosiloxanes) (SiDA), **(B)** L-arginine modified oligo (-alkylaminosiloxanes) ($SiDAAr_5$), **(C)** L-arginine oligo (-alkylaminosiloxane) grafted poly(ethylenimine) ($P(SiDAAr)_5$), and **(D)** Conjugation to PEG ($P(SiDAAr)_5PEG_3$).

Figure 1. Spectral characterization of polymer and morphological analysis of siRNA polyplex. (A) ^1H NMR spectra $P(SiDAAr)_5PEG_3$ shows the polymer's PEG ($-CH_2-CH_2-O-$) groups, as well as arginine, and PEI's ethylene protons ($-CH_2-$). **(B)** TEM images of 1/4 siRNA/$P(SiDAAr)_5PEG_3$ polyplex at (left) low (×23000) and (right) high (×68000) magnifications.

Figure 2. Complexation and protection of siRNA from RNase. (A) Agarose gel electrophoresis of polyplex with different N/P w/w ratios. Un-complexed siRNA is shown as white bands on the gel. Complete complexation took place at N/P w/w ratio of 1/4 and 1/6 w/w. **(B)** siRNA degradation assay showed no increase in absorbance (a measurement of free siRNA) in 1/4 $P(SiDAAr)_5PEG_3$ polyplex, indicating polymer protecting siRNA from RNase degradation. Data as mean ± s.e.m.; n=2-3. $p<0.01$ between siRNA, siRNA-polyplex + Nuclease vs. siRNA + nuclease.

Figure 3. Stability of polyplex in the presence of polyanion. Polyanion competition assay revealed siRNA polyplex stability in the presence of varying concentrations of heparin sodium. **(A)** Gel image **(B)** Quantification of band intensity. Partial siRNA release took place at 2500

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μg/mL of heparin sodium whereas at 5000 μg/mL of heparin sodium nearly all the siRNA is released from the polyplex.

Figure 4. Polyplex uptake in metastatic and resistant breast cancer cells. Uptake in **(A)** MDA-MB-231-Luc-D3H2LN and **(B)** MCF-7/Adr cell lines by flow cytometry and the respective mean fluorescence intensity following incubation of cells with polyplex for 4 hr. No treatment: Cells that are not treated with polyplex. Luc-siRNA PP: Cells that are treated with polyplex prepared with TRITC-conjugated polymer. Y-axis represents the data normalized to the maximum number of cells counted whereas X-axis represents the channel used for measuring fluorescence signal. Untreated n=1, treated n = 8-9 repeats of the same sample measurements.

Figure 5. Cytotoxicity and transfection efficiency of siRNA/P(SiDAAr)$_5$PEG$_3$ polyplex in the MDA-MB-231-Luc-D3H2LN cell line. (A) Polyplex toxicity. *: $p<0.05$ compared to the control group, n=6. **(B)** Luc-siRNA transfection with polyplex at varying N/P w/w ratios. The polymer composition of the polyplex increases and becomes highly effective at N/P ratio of 1/4 w/w, where there is a complete complexation of siRNA. *$p<0.01$ compared to the control group, n=2.

Figure 6. Knock-down ABCB1 with anti-ABCB1 siRNA polyplex and its effect on DOX accumulation and cytotoxicity in MCF-7/Adr cells. (Aa) Flow cytometric analysis of suppression of ABCB1 protein expression following incubation with anti-ABCB1 siRNA polyplex; **(Ab)** the suppression was seen to increase with incubation time. **(Ba)** Co-treatment of cells with anti-ABCB1 siRNA polyplex significantly enhanced DOX uptake; **(Bb)** change in

mean floreence intensity of cells co-treated with anti-ABCB1 siRNA polyplex and DOX. Cells were treated with anti-ABCB1 siRNA polyplex and DOX solution at 2,500 ng/mL for 4.5 hr prior to flow cytometric analysis of cells for DOX uptake. **C)** Cell viability following treating cells with DOX solution alone or co-treatment with anti-ABCB1 siRNA polyplex at different doses DOX (numbers indicates ng/ml dose) for 72 hr prior to measuring cell viability using MTT assay. n= 6. *$p<0.05$, **$p=0.053$ compare to DOX alone. Abbreviation PP = Polyplex. Flow cytometry: Y-axis represents the data normalized to the maximum number of cells counted whereas X-axis represents the channel used for measuring fluorescence signal.

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Table 1. Physical characteristics of polyplex formulated at different siRNA to polymer (N/P w/w) ratios.

siRNA/P(SiDAAr)$_5$P$_3$ (N/P)	Mean Hydrodynamic Diameter (nm)	Polydispersity Index (PDI)	Zeta Potential (mV)
1/2	326.8	0.21	+8.7
1/3	294.7	0.64	+26.2
1/4	349.5	0.34	+30.0
1/5	230.6	0.52	+42.5
1/6	240.1	0.46	+36.3

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Scheme for polymer synthesis

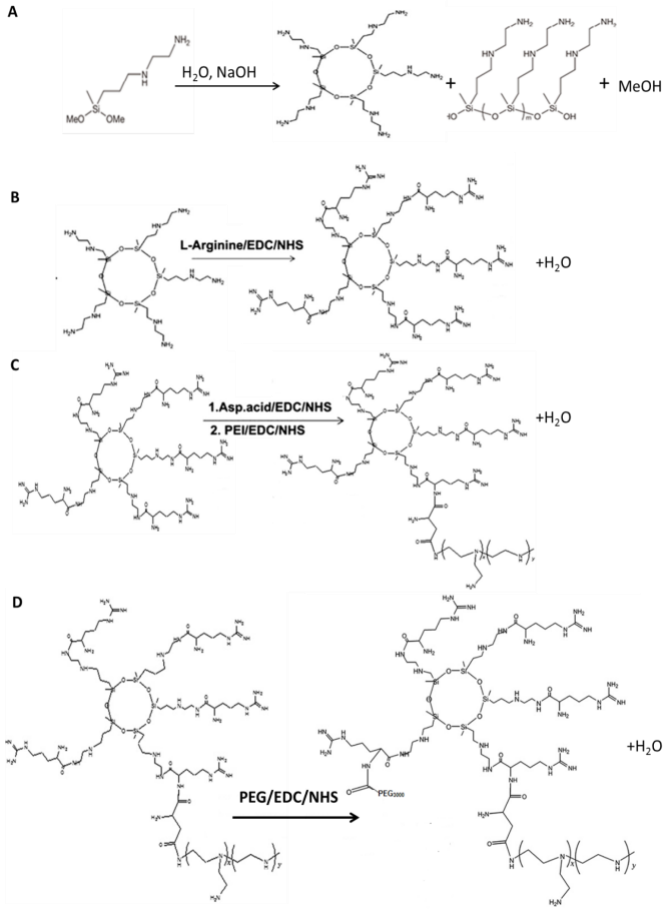

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Figure 1

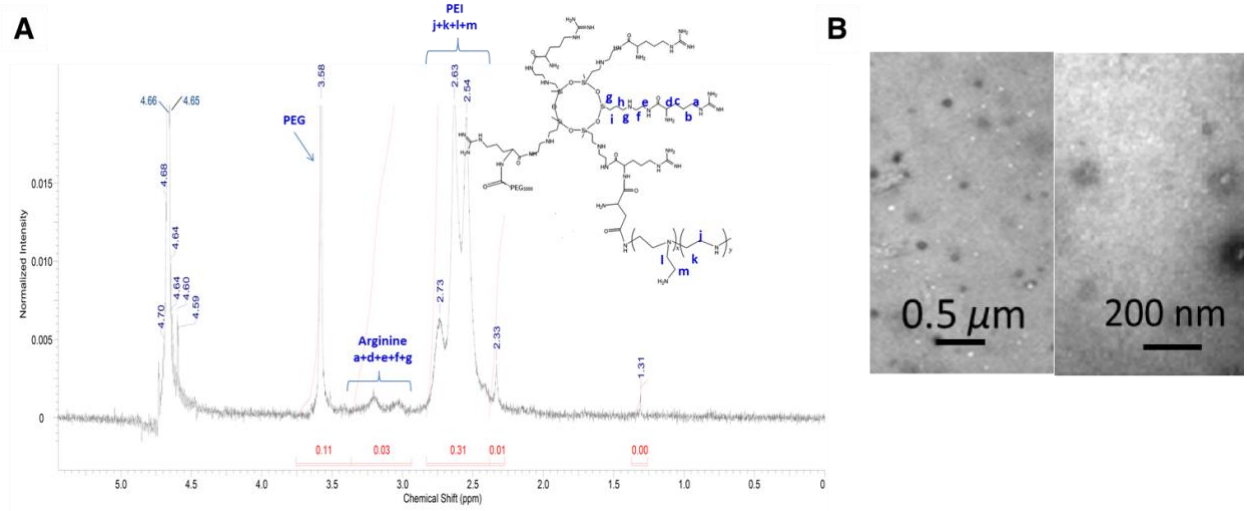

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Figure 2

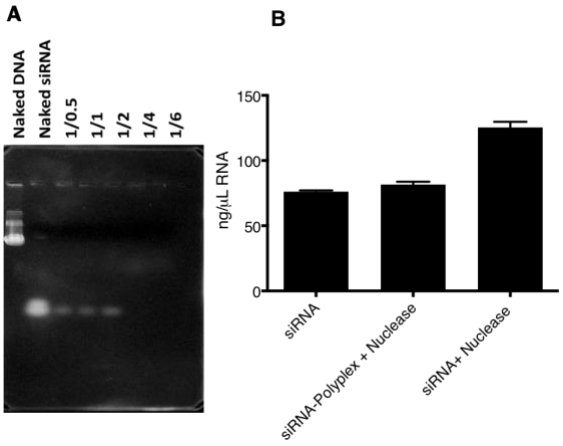

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Figure 3

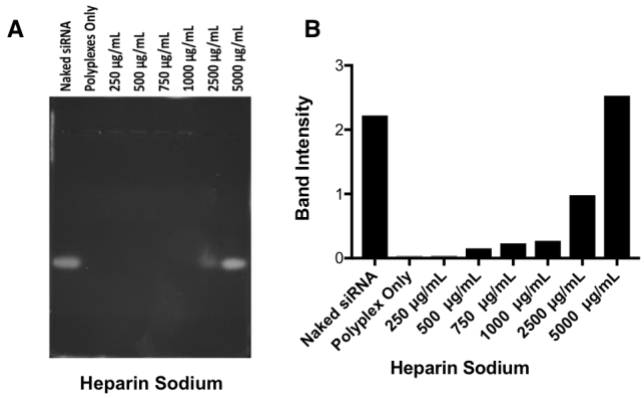

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Figure 4

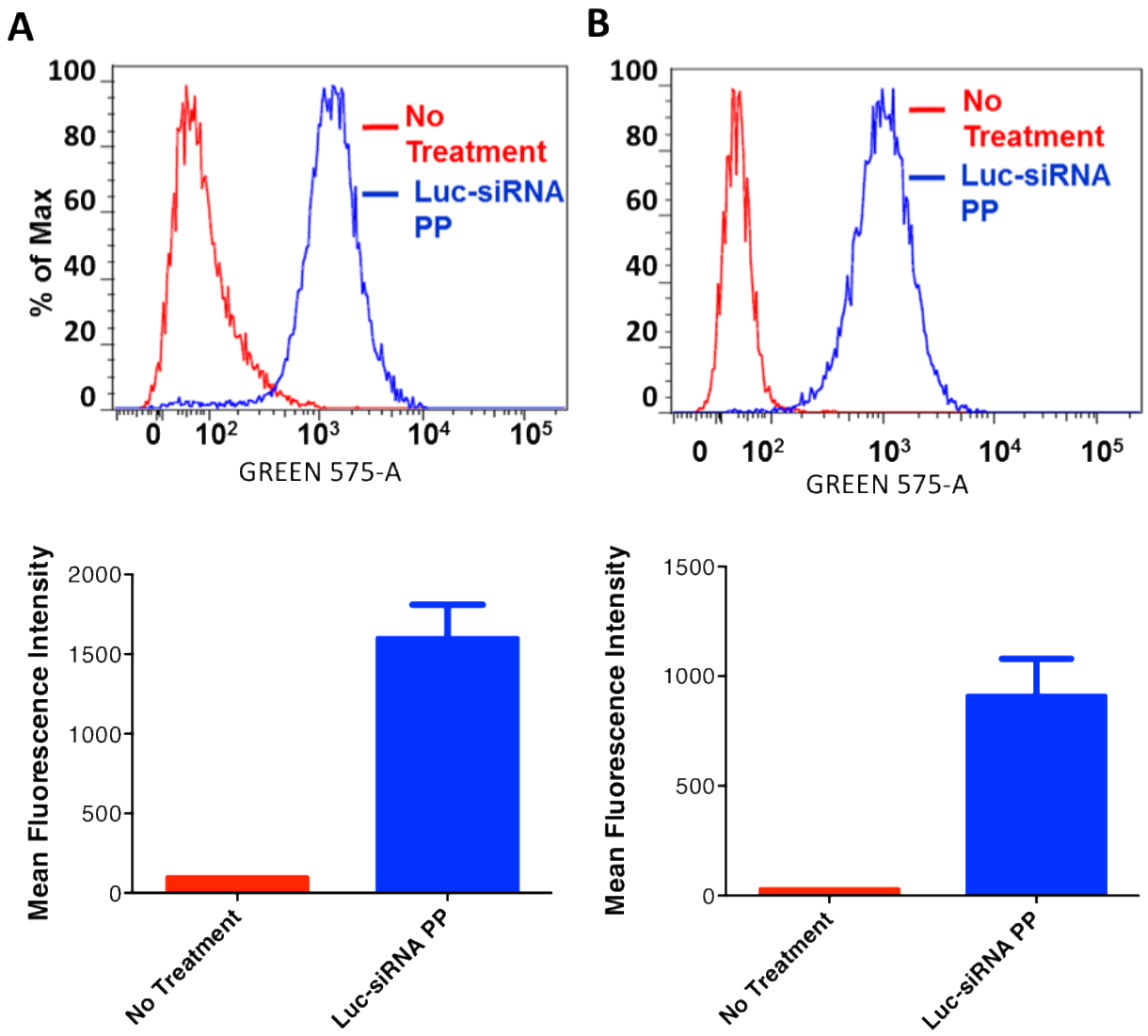

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Figure 5

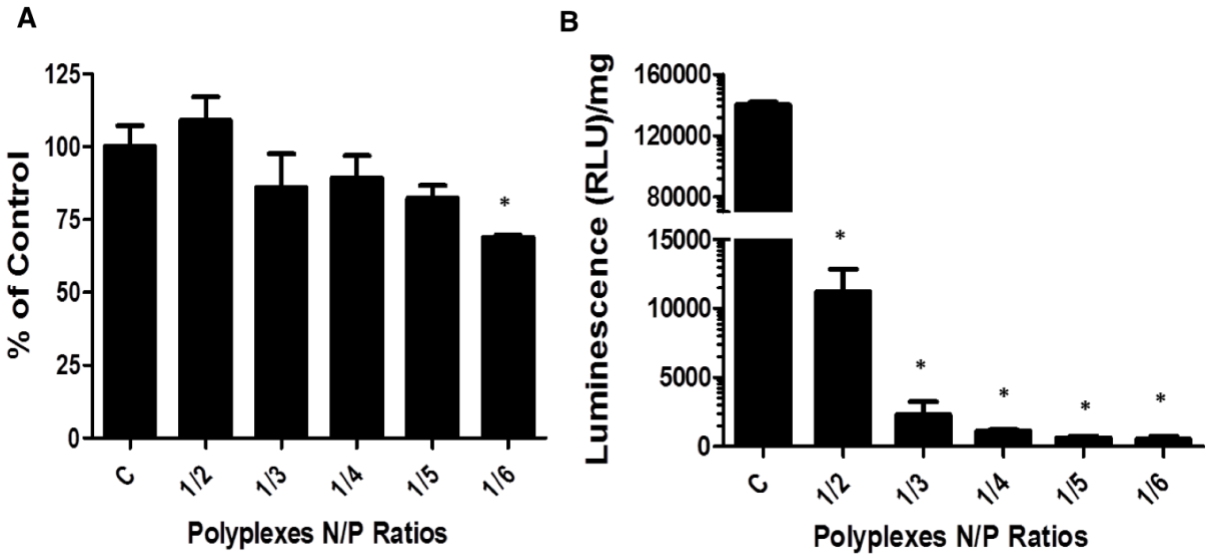

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Figure 6