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Quantitative contributions of processes by which polyanion drugs reduce intracellular bioavailability and transfection efficiency of cationic siRNA lipoplex

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Abstract

RNA Interference (RNAi) is a potentially useful tool to correct the detrimental effects of faulty genes; several RNAi are undergoing clinical evaluation in various diseases. The present study identified the relative contributions of three mechanisms by which polyanion drugs reduced the gene silencing activity of Lipoplex, a complex of small interfering RNA (siRNA) and cationic liposomes. The study used a siRNA against the chemoresistance gene survivin and two model polyanion drugs (suramin, heparin). Products of Lipoplex destabilization were separated, identified, and/or quantified using ultrafiltration, gel electrophoresis, and RT-qPCR (quantitative reverse transcription polymerase chain reaction). Cell binding and endocytosis of fluorescence-labeled Lipoplex and the amount of siRNA at its site of action RISC (RNA-induced silencing complex) were evaluated using endocytosis markers, confocal microscopy, quantitative image analysis, immunoprecipitation, and RT-qPCR. The results show suramin and heparin exerted multiple concentration-dependent effects. First, these agents altered several Lipoplex properties (i.e., reduced particle size, changed surface charge, modified composition of protein biocorona). Second, both caused Lipoplex destabilization to release double- and single-strand siRNA and/or smaller siRNA-lipid complexes with reduced siRNA cargo. Third, both prevented the cell surface binding and internalization of Lipoplex, diminished the siRNA concentration in RISC, and retarded the mRNA knockdown. Suramin and heparin yielded qualitatively and quantitatively different results. Analysis of the experimental results of suramin using quantitative pharmacology (QP) modeling indicated the major cause of gene silencing activity loss depended on drug concentration, changing from inhibition of endocytosis at lower concentration (accounting for

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60% loss at $\sim 9 \mu\text{M}$) to inhibition of cell surface binding and loss of siRNA cargo at higher concentrations (accounting for 64% and 27%, respectively, at $70 \mu\text{M}$). In summary, the present study demonstrates the complex and dynamic interactions between polyanions and Lipoplex, and the use of QP modeling to delineate the contributions of three mechanisms to the eventual loss of gene silencing activity.

Graphical abstract

Keywords

siRNA lipoplex; anionic drug; destabilization; binding; internalization; biocorona

1. Introduction

RNA Interference (RNAi), e.g., small interfering RNA (siRNA), is a potentially useful tool to correct the detrimental effects of faulty genes [1]. Several RNAi are undergoing clinical evaluation in various diseases, e.g., cancer, cystic fibrosis, retinal degeneration, respiratory disorders, and metabolic diseases [2, 3]. Due to its rapid elimination (e.g., degradation by nucleases, renal clearance) and unfavorable physicochemical properties for cellular uptake (e.g., high molecular weight, large size, negative charges), siRNA is delivered in nano-size carriers such as liposomes [4]. Mixing siRNA with cationic liposomes results in a complex (Lipoplex) that shields siRNA from degradation and improves its internalization in cells [5–8].

Surface properties of liposomes play important roles in their binding to cell surface and the subsequent internalization *via* endocytic pathways. For example, binding of cationic liposomes to cell surface is through electrostatic interaction with the anionic lipid head groups on plasma membrane [9–12], and is affected by surface modifications (e.g., with targeting ligands, pegylation, cell penetrating peptides) [13, 14]. Interactions with proteins in the biological milieu result in biocorona coating the liposome; the biocorona formation increases particle size, changes surface charge from positive to negative, and affects liposome transport and disposition [15, 16]. For nucleic acid-Lipoplex, serum proteins destabilize the complex, cause the release of nucleic acid (siRNA, DNA) and reduce its transfection efficiency [17, 18]. Polyanions (dextran, heparin, heparan, chondroitin) have similar effects on DNA-liposome complex as serum proteins [19–21], whereas their effects on RNAi-Lipoplex have not been reported. In addition, while there are multiple possible mechanisms to reduce the siRNA delivery to its molecular site of action RISC (RNA-induced silencing complex), e.g., destabilization and loss of siRNA cargo, diminished internalization or degradation during endocytic transport, the contributions of individual

processes have not been delineated. Such quantitative information is needed to identify the critical areas for research to further improve the delivery and therapeutic potential of siRNA agents.

The present study evaluated the polyanion effects on Lipoplex, including changes in surface properties, siRNA cargo, cell membrane binding, endocytosis, siRNA bioavailability in RISC, and gene silencing activity. The study used siRNA against survivin, a well-established prognostic factor in most human cancers and the target of an anti-sense oligonucleotide undergoing clinical evaluation [22]. The model polyanion drugs were suramin and heparin. Suramin, a hexasulfonated naphthylurea with six negative charges at physiological pH, has been used since 1920s as an anti-parasitic drug and has undergone clinical development first as a cytotoxic and more recently as a chemosensitizer [23–26]. Heparin, a linear, highly sulfated, polydisperse polysaccharide with an average of 2.7 negative charges per disaccharide, is a widely-used anticoagulant [27]. The experimental results were used with quantitative pharmacology (QP) modeling to determine the relative contributions of the three mechanisms by which polyanions reduced the gene silencing activity of Lipoplex.

2. Material and Methods

2.1 Materials

Suramin sodium was provided by the National Cancer Institute (Bethesda, MD). Heparin sodium was purchased from Sigma (St. Louis, MO). The molecular weight of heparin, a mucopolysaccharide, ranges as wide as 6–30 kDa but mainly 17–19 kDa for most of its chains [28]; we used an average MW of 18 kDa in our calculations. Cell culture supplies, including McCoy's 5A medium, penicillin/streptomycin, heat-inactivated fetal bovine serum (FBS, Lot #1743512), non-essential amino acids, and actinomycin D were obtained from Life Technologies (Carlsbad, CA). Lipids (1,2-dioleoyl-3-trimethylammoniumpropane or DOTAP, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine or DOPE, rhodamine-DOPE, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] or DSPE-PEG, cholesterol) were purchased from Avanti Polar Lipids (Alabaster, AL). Primers (with proprietary sequences) for quantitative reverse transcription polymerase chain reaction (RT-qPCR) were purchased from Quanta Biosciences (Gaithersburg, MD). siRNA against survivin (siSurvivin), non-targeting siRNA (siNT), fluorescent AF647-labeled siSurvivin, and RNase-free water were purchased from IDT (Coralville, IA). The siRNA sequences were: siSurvivin: 5'-GGACCACCGCAUCUCUACATT-3' (sense), 5'-UGUAGAGAUGCGGUGGUCCTT-3' (antisense); AF647-siSurvivin: 5'-GGACCACCGCAUCUCUACATT/-3' (sense), 5'-UGUAGAGAUGCGGUGGUCCTT/3AlexaF647N/-3' (antisense); siNT: 5'-AGUACUGCUUACGAUACGGTT-3' (sense), 5'-CCGUAUCGUAAGCAGUACUTT-3' (antisense). All reagents were used as received.

siRNA_{unchanged} is the double-strand (ds) siSurvivin supplied by the manufacturer, and Lipoplex refers to the complex of siSurvivin with pegylated cationic liposomes (PCat) unless noted otherwise. Lipoplex was dispersed in water, serum-free culture medium or serum-containing medium (Lipoplex/Water, SF-Medium or Full-Medium). The three entities appearing in ultrafiltrates are the smaller filterable siRNA-lipid complex with reduced siRNA load, free or degraded siRNA, and/or free or degraded lipids (i.e., not intact PCat).

Lipoplex and PCat concentrations are expressed in siRNA-equivalents, e.g., 0.2 μM Lipoplex contains 0.2 μM siRNA whereas 0.2 μM PCat contains the same lipid concentration as in 0.2 μM Lipoplex. *F is fluorescence-labeled moiety.

siRNA solutions were diluted to 10 μM in Duplex Buffer (IDT), aliquoted and stored at -20°C until use. Lipoplex and siNT-Lipoplex were prepared in their desired final concentrations, to which stock solutions of suramin and heparin (prepared and serially diluted with McCoy's 5A medium) were added. All reported concentrations in Lipoplex/polyanion mixtures were corrected for the dilution (ranged from 2 to 20%).

2.2 Preparation of PCat and Lipoplex

PCat, comprising four lipids (DOTAP:Cholesterol:DOPE:DSPE-PEG at a molar ratio of 50:30:19:1), was prepared using the dry film and extrusion method as described previously [29]. *F-PCat was formulated by replacing 1% of DOPE with rhodamine-DOPE. Briefly, lipids were mixed and dissolved in chloroform. Evaporation of chloroform under vacuum yielded a thin lipid film that was subsequently hydrated with RNase-free water (1 mL per 10 mg lipids) at 50°C for 30 min. The resulting PCat suspension was passed through an extruder with a 100 nm membrane (Whatman, Maidstone, UK). Lipoplex was formed by gently mixing PCat with an aqueous solution of siRNA_{unchanged} at room temperature, in a 4:1 DOTAP-to-siRNA charge ratio (21 μL PCat and 100 μL of 10 μM siSurvivin), for at least 20 min before use.

Non-fluorescent siSurvivin (symmetrical 21 nucleotide ds RNA) was used to study the effects of polyanions on Lipoplex physical properties (particle size, surface charge, biocorona), functionality, and siRNA bioavailability in RISC. AF647-siSurvivin and rhodamine-DOPE were used to quantify, respectively, the polyanion destabilization of Lipoplex to release siRNA and lipids. Lipoplex comprising rhodamine-DOPE and non-fluorescent siRNA was used to monitor the cellular binding and intracellular trafficking of siRNA and lipids/Lipoplex.

2.3 Cell culture

Human colorectal adenocarcinoma HT29 cells were purchased from ATCC (Manassas, VA), maintained in Full Medium at 37°C and 5% CO_2 , and routinely checked for mycoplasma contamination. Full medium contained McCoy's 5A medium supplemented with 10% FBS, 1% non-essential amino acids and 1% penicillin/ streptomycin.

2.4 Cytotoxicity of Lipoplex and suramin

Cytotoxicity of Lipoplex and suramin in HT29 cells was determined using the MTT assay [30]. Cells (1×10^4 in 100 μL per well, 96-well plate) were cultured in Full Medium overnight at 37°C in 5% CO_2 , and then with fresh Full Medium containing Lipoplex (0 to 1.8 μM) or suramin (0 to 900 μM) for an additional 24 h. Afterwards, MTT solution (25 μL of 2 mg/mL) was added and, 4 h later, the medium was replaced with solubilizing solution (10% sodium dodecyl sulfate (SDS) plus 0.6% HCL in dimethyl sulfoxide, 10 μL per well), and the absorbance at 570 nm was measured using a microplate reader (Thermo Fisher,

Waltham, MA). Results were expressed relative to their respective controls (i.e., untreated vs. suramin-treated, and siNT-Lipoplex-treated vs. Lipoplex-treated).

2.5 Kinetics of survivin mRNA degradation

We measured the degradation rate of endogenous survivin mRNA. Cells (2×10^6 in 10 mL, 100 mm dish) were cultured in Full Medium overnight, and then with fresh Full Medium containing actinomycin D (5 $\mu\text{g/mL}$, to inhibit RNA synthesis [31]). At predetermined times, cells were harvested, counted using a flow cytometer (Attune[®], Life Technologies), collected (1×10^6) and centrifuged. The resulting cell pellet was stored at -80°C until analysis. mRNA levels were measured using our previously described RT-qPCR method [29], with the following modifications. Briefly, total RNA was extracted from cell pellets using E.Z.N.A.[®] HP Total RNA kit (Omega Bio-Tek, Norcross, GA), and reverse-transcribed to cDNA using qScript[™] cDNA SuperMix. The internal standard was 18s rRNA, which is maintained at relatively constant levels, has a long half-life (e.g., 96 h [32]) and hence would be minimally affected by actinomycin D. RT-qPCR was performed with PerfeCTa[®] MultiPlex qPCR SuperMix in CFX96[™] Real-Time PCR Detection Systems (BioRad, Hercules, CA). The primer probe sets (IDT) were: survivin, forward: 5'-CAACCGGACGAATGCTTTT-3'; reverse: 5'-AAGAACTGGCCCTTCTTGG-3'; probe: 5'-/5HEX/CCAGATGAC/ZEN/GACCCCATAGAGGAA/3IABkFQ/-3'; 18s rRNA, forward: 5'-CTGAGAAACGGCTACCACATC-3'; reverse: 5'-GCCTCGAAAGAGTCCTGTATTG-3'; probe: 5'-/5Cy5/AAATTACCCACTCCCGACCCGG/3IABRQSp/-3'. The multiplex thermal reaction program was: 3 min at 95°C , 40 cycles of 15 sec at 95°C and 1 min at 61°C . Survivin mRNA level relative to 18s rRNA was calculated using the Ct method [33], and reported as % of the initial value without actinomycin D treatment.

2.6 Reduction of survivin mRNA expression by Lipoplex, suramin and/or heparin

Cells (2×10^6 in 10 mL, 100 mm dish) were processed as described above, with the exception that culture medium was replaced with drug-containing Full Medium. At predetermined times, cells were washed twice with ice-cold phosphate-buffered saline (PBS), harvested by trypsinization and centrifugation, followed by PBS wash and centrifugation. Survivin mRNA levels were measured as described above, with the exception that GADPH was the internal standard. The primer probe set (IDT) for GADPH was forward: 5'-AATCCCATCACCATCTTCCAG-3'; reverse: 5'-AAATGAGCCCCAGCCTTC-3'; probe: 5'-/5Cy5/CCAGCATCGCCCCACTTGATTTT/3IABRQSp/-3'. Treatments with single agents (1–500 μM suramin or 0.1–1.2 μM Lipoplex) were for 48 h; results were analyzed with Hill equation to estimate the efficiency of survivin mRNA knockdown by single agents (e.g., $IC_{50,48h,mRNA-KD}$). The combination treatment used suramin and Lipoplex concentrations that were about one-third to three times the values of their concentration producing 50% maximal inhibition ($IC_{50,48h,mRNA-KD}$) and a shorter treatment duration of 4 h. Another anionic compound, heparin, was studied at a single concentration of 70 μM .

2.7 Effects of suramin on particle size, surface charge, pH, and biocorona of Lipoplex

These studies used Lipoplex and suramin at noncytotoxic conditions (either low concentrations or short treatment times), to eliminate ambiguities due to cytotoxicity.

Particle size distribution of Lipoplex/Full-Medium or SF-Medium and zeta potential of Lipoplex/Water were determined using the dynamic light scattering method with a Zetasizer Nano ZS90 (Malvern, Westborough, MA). The biocorona protein composition was determined as previously described [16]. Briefly, a mixture (1 mL) of Lipoplex/Full-Medium (0.2, 0.6 and 1.8 μM final concentration) and suramin (0–662 μM) was incubated at room temperature for 30 min, followed by centrifugation at $20,000 \times g$ for 1 h. After removing the top 900 μL , the bottom fraction containing the pellet was washed three times with ice-cold PBS with centrifugation ($20,000 \times g$ for 10 min) in-between. Proteins were then extracted using 100 μL 1X Bolt® LDS Sample Buffer diluted in M-PER™ Mammalian Protein Extraction Reagent (Life Technologies), and separated using SDS-polyacrylamide gel electrophoresis (SDS-PAGE), as follows. The extracts were heated at 95°C for 5 min and 30 μL was loaded on Bolt® 4–12% Bis-Tris Plus Gels and run at 130 V for ~45 min. A similarly processed FBS sample (Bolt® LDS Sample Buffer containing 10% FBS, 10 μL) was included for comparison. Proteins on the gel were stained with Coomassie SimplyBlue™ SafeStain (Life Technologies) for 1 h, washed by immersion in water for 2 h, and then imaged on a BioRad Molecular Imager ChemiDoc XRS. Densitometry measurements of individual lanes and individual bands were performed using Image Lab (v 3.0, BioRad).

2.8 Separation of siRNA and lipid from Lipoplex by ultrafiltration

We used ultrafiltration to separate the larger Lipoplex from the smaller destabilization products. Three studies were performed. The first study determined the extents of destabilization by suramin under the same conditions used for the siRNA gene knockdown study; in this case the siRNA:suramin molar ratios were kept constant (i.e., 0.2, 0.6 or 1.8 μM Lipoplex plus 24, 70 or 178 μM suramin) to eliminate potential ambiguity introduced by changing ratios. The second study compared the contribution of destabilization to the suramin dose-dependent inhibition of gene knockdown, relative to the two other suramin effects (inhibition of Lipoplex binding to cell membrane and internalization); in this case the suramin concentration was increased from 3 to 70 μM while the Lipoplex concentration was kept constant at 0.6 μM . The third study was performed using heparin as in the first suramin study (same molar concentrations). Briefly, aliquots (135 μL) of AF647-Lipoplex were incubated in Full Medium without or with polyanions at 37°C for 30 min, followed by cooling on ice for 3 min. Afterwards, 75 μL was placed in a cold ultrafiltration column with a 1000 kDa membrane (pore size of ~100 nm [34], Vivaspine 6, Vivaproducts, Littleton, MA) and centrifuged at $2,500 \times g$ at 4°C for 20 min. Aliquots of filtrates and pre-ultrafiltration samples (50 μL each) were mixed with 100 μL of Triton X-100 (2% v/v in nuclease free water, to solubilize Lipoplex) in a 96-well plate precooled on ice, and their fluorescence intensities measured using SpectraMAX Gemini EM microplate reader (Molecular Devices, Sunnyvale, CA) at excitation/emission wavelengths of 640/680 nm for AF647-siRNA and 543/594 nm for rhodamine-lipid. We determined the extent of fluorescence quenching; the average fluorescence intensity of siRNA in Lipoplex was $86 \pm 7\%$ ($n=9$) of the intensity of equal concentration of siRNA_{free}, indicating a 14% quenching due to complexation; this value was used to convert *F-siRNA in Lipoplex to that in the released siRNA_{free}. There was no difference in the lipid fluorescence in PCat or in Lipoplex, indicating no quenching. We also determined the mass balance; a comparison of

the sum of the fluorescence in the ultrafiltrate and the upper chamber of the filtration unit (including the residual volume and filter) to the fluorescence in pre-filtration samples indicated complete recovery ($103 \pm 9\%$, $n=21$ from 12 suramin concentrations). The amounts of *F-siRNA and *F-lipid in the ultrafiltrate were compared to the amounts in pre-filtration samples, to calculate the % released from Lipoplex. A pilot study indicated the release of siRNA from Lipoplex due to FBS reached its maximum level within 10 min. Hence, the remaining experiments study used 30 min treatments.

The release of siRNA from Lipoplex was further confirmed with PAGE. Solutions containing siRNA_{unchanged} or Lipoplex (both containing $0.6 \mu\text{M}$ siRNA-equivalents) were incubated in $0\text{--}70 \mu\text{M}$ suramin or heparin at 37°C for 30 min, then mixed with loading buffer (Novex® TBE-Urea Sample Buffer, Invitrogen, Carlsbad, CA) and heat-denatured at 70°C for 3 min. An aliquot ($10 \mu\text{L}$) was loaded on a denaturing gel (Novex® TBE-Urea Gels 15%, Invitrogen), and nucleic acids on the gel were stained with SYBR® Green II (Invitrogen) for 40 min and then imaged. The two markers were single-strand (ss) RNA oligonucleotides ranging from 50 to 1000 nucleotides and from 17 to 25 nucleotides.

2.9 Cellular binding and internalization of Lipoplex: Confocal microscopy and quantitative image analysis

Confocal microscopy was used to visualize the binding to cell membrane and trafficking in endocytic organelles, of Lipoplex (rhodamine-DOPE-labeled). We found in a separate study that internalization of Lipoplex in MCF7 cells within the first 6 h used mainly receptor-mediated endocytosis that involved Rab5-expressing organelles including early endosomes and macropinosomes [35–37]. Cells (1.6×10^4 cells/1 mL) were transfected with an early endosome marker (fusion construct of Rab5 with green fluorescent protein (GFP); CellLight® Early Endosomes-GFP, $45 \mu\text{L}$, Life Technologies), per manufacturer's protocol. Transfected cells (2,000 in $100 \mu\text{L}$ Full Medium) were seeded on a $\mu\text{-Slide VI}^{0.4}$ (Ibidi, Madison, WI), allowed to attach to the growth surface overnight, and then incubated with fresh Full Medium containing Lipoplex ($0.6 \mu\text{M}$) and suramin or heparin ($0\text{--}70 \mu\text{M}$) for 1 h. Afterwards, slides were moved to the environmental chamber in a confocal microscope (SP8, Leica, Wetzlar, Germany), visually scanned to select 5 cells (per treatment group) with highest Rab5 signals, and microscopic images were obtained using a 63X oil immersion objective (with 2.25X digital zoom) at 1000 Hz and 1024×1024 resolution and the 552 and 488 nm laser lines to detect rhodamine-DOPE/PCat and GFP-Rab5, respectively. Z stack images were obtained by first determining the top and bottom of each cell and then using the built-in computer software to acquire fluorescence signals from each stack ($n=3$ per treatment condition). As endosomal vesicles move rapidly and typically do not stay in the same microscopic plane over time, we used the total signals of three Z-planes, located near the mid-height of z-stack images, for each cell. The optical sections were $1 \mu\text{m}$ -thick, to ensure the measurements of different vesicles.

Analysis of microscopic images was performed using CoLocalizer Pro 2.7 (CoLocalization Research Software, Japan). The background fluorescence was established using randomly selected fields devoid of fluorescence signals. We defined the cell boundary, and then measured, in a z-plane image, the areas (i.e., number of pixels) containing only *F-lipid

signals (red) or co-localized *F-lipid/*F-Rab5 signals (yellow) inside the boundary. We assumed the former entity represented the membrane-bound *F-lipid/Lipoplex, the latter the endocytosed entity (accounting for nearly all of the cytosolic *F-lipid), and their sum the amount of total cell-associated Lipoplex.

2.10 Quantification of siRNA level in RISC

Quantification of siSurvivin in RISC is based on its binding to Argonaute 2 (Ago2), the catalytic unit of RISC [38]. The endogenous miR16, which is present at relatively constant level in various cell lines and found in RISC [39, 40], was used for normalization. The first step was Ago2 immunoprecipitation (Dynabeads® Protein A Immunoprecipitation Kit; Thermo Fisher), to separate the Ago2-bound siRNA from other siRNA molecules. Cell pellets (2×10^6 cells) were lysed in 500 μ L of Pierce™ IP lysis buffer containing RNaseOUT (1:20) and protease/phosphatase inhibitor cocktail (1:100, Pierce), mixed by up-and-down pipetting and incubated for 30 min, followed by centrifugation at $20,000 \times g$ for 10 min. The supernatant was transferred to a fresh tube and incubated with 10 μ L anti-Ago2 antibody (Abcam, Cambridge, UK) with rotation overnight at 4°C, to allow the formation of antibody-Ago2-siRNA complex. The sample was then incubated, with rotation for 2 h at 4°C, with Dynabeads® Protein A beads (magnetic beads covalently bound to Protein A, which binds the Fc region of the antibody). A magnetic bar was placed outside the tube to separate the beads from the solution. After three washes with Washing Buffer (removed by pipetting), the pellet, which contained bead-bound Ago2-siRNA, was resuspended in 100 μ L Washing Buffer and transferred to a fresh Eppendorf tube. Next, siRNA was extracted from the beads using a commercial kit (mirVana™ PARIS™ RNA and Native Protein Purification Kit, Thermo Fisher). Briefly, beads were incubated with 600 μ L Lysis/Binding solution and 60 μ L miRNA Homogenate Additive on ice for 10 min, followed by mixing with 600 μ L acid-phenol:chloroform and then centrifugation at $10,000 \times g$ for 5 min. An aliquot of the supernatant (600 μ L) was transferred to a fresh tube and mixed with 200 μ L of ethanol. The mixture was passed through Filter Cartridge to remove large RNA molecules. The filtrate, which contained the smaller RNA (siRNA, miR16), was collected, mixed with 500 μ L of ethanol, and loaded on a second Filter Cartridge. After washing the Cartridge with miRNA Wash Solution-1/-2/3, the smaller RNA was eluted with 100 μ L of pre-heated (95°C) Elution Solution.

The extracted siRNA was quantified using our previously published RT-qPCR method [41]. Briefly, siRNA was extended using poly-A polymerase to add a poly-A tail onto the 3'-end of either strand of siRNA (qScript™ microRNA cDNA Synthesis Kit, Quanta Biosciences). The siRNA-poly-A tail sequence was then reverse transcribed using an Oligo-dT adapter primer with a universal PCR primer sequence. The resulting cDNA template was diluted in RNase-free water and an aliquot (5 μ L) was mixed with 15 μ L of a master-mix containing PerfeCTa® Universal PCR Primer, primers for siSurvivin and miR16 (respective sequence was 5'-UGUAGAGAUGCGGUGGUCCTT-3' and 5'-CCAGUAUUAACUGUGCUGCUGA-3', and PerfeCTa® SYBR® Green SuperMix (Quanta Biosciences). qPCR used the following program on the above-described PCR Detection System: 3 min at 95°C, 40 cycles of 15 s at 95°C and 1 min at 61°C. Results that were not significantly higher than zero (one-tailed t test) were reported as undetectable.

2.11 Statistical Analysis

All experiments were performed in triplicates, and results are expressed as mean±SD of 3 experiments. Statistical analyses, including unpaired Student's t-test, one way ANOVA with Tukey's post-test (for comparing all treatment groups to each other), and Dunnett's post-test (for comparing all treatment groups to a single control group), were performed using Prism 5 (GraphPad Software Inc., San Diego, CA). Statistical significance was defined as $p < 0.05$.

2.12 QP modeling to determine contributions of three mechanisms by which polyanions reduce intracellular siRNA bioavailability

We used QP modeling to delineate the contributions of three mechanisms by which polyanions reduced the intracellular siRNA bioavailability. The calculation used the suramin effects on the destabilization, cell membrane binding and internalization of Lipoplex. For the modeling, we first obtained the numerical values of model parameters, as follows.

The results of the siRNA cargo release study, using a fixed Lipoplex concentration (0.6 μM), show insignificant release (<1%) at low suramin concentrations (3 – 14 μM) but a higher and near constant release (average of 26.6%, range of 25% to 28%) at higher concentrations (19–140 μM). Hence, we used % $F_{\text{destabilization}}$ of 0% at 14 μM and 26.6% at 19 μM .

$$\% \text{ siRNA remained in Lipoplex} = 100\% - \%F_{\text{destabilization}} \quad \text{Eq. 1}$$

The suramin inhibition of Lipoplex binding to cell membrane and endocytosis showed sigmoidal concentration-effect relationships; these data were analyzed using nonlinear regression (Phoenix[®] WinNonlin[®] version 6.4; Certara USA, Princeton, NJ) in accordance to the Hill equation, to solve for maximal effect (I_{max}), drug concentration that caused 50% maximal effect (IC_{50}), and the Hill equation curve shape parameter n . For example, the results in Figure 5B were used to calculate the inhibition of Lipoplex binding to cell surface; analysis of the plot of membrane-bound *F-lipid for the Lipoplex+suramin combination vs. suramin concentration using Eq. 2 yielded the best-fit values of $I_{\text{max, binding}}$, $IC_{50, \text{binding}}$, and n_{binding} . Similarly, the results of Figure 5C were used to calculate the inhibition of Lipoplex internalization; analysis of the plot of Rab5-co-localizing *F-lipid vs. suramin concentration using Eq. 3 yielded the best-fit values of $I_{\text{max, internalization}}$, $IC_{50, \text{internalization}}$, and $n_{\text{internalization}}$. Note that the extent of internalization represents the net of input (e.g., endocytosis) and efflux (e.g., via exosomes).

$$\% \text{ Membrane bound Lipoplex} = 100\% \times \left(1 - \frac{I_{\text{max, binding}} \times [\text{Suramin}]^{n_{\text{binding}}}}{IC_{50, \text{binding}}^{n_{\text{binding}}} + [\text{Suramin}]^{n_{\text{binding}}}} \right) \quad \text{Eq. 2}$$

$$\% \text{ Internalized Lipoplex} = 100\% \times \left(1 - \frac{I_{\text{max, internalization}} \times [\text{Suramin}]^{n_{\text{internalization}}}}{IC_{50, \text{internalization}}^{n_{\text{internalization}}} + [\text{Suramin}]^{n_{\text{internalization}}}} \right) \quad \text{Eq. 3}$$

Substituting the best-fit model parameter values and a suramin concentration into Eq. 1–3 yielded the % siRNA remained in Lipoplex, % membrane-bound Lipoplex, and % internalized Lipoplex at the selected suramin concentration; these values were then used to calculate the loss of intracellular siRNA bioavailability ($F_{intracellular, siRNA}$) due to each mechanism. The calculation accounted for the sequential nature of the three effects, e.g., total loss of $F_{intracellular, siRNA}$ equaled the multiplication product of (loss from destabilization) $\times$ (loss from reduced binding) $\times$ (loss from reduced internalization). The relative contributions of individual mechanisms to the overall loss (equaled [loss by individual mechanism] divided by [total loss]) at various suramin concentrations were calculated.

3 Results

3.1 Defining treatment conditions

The degradation of endogenous survivin mRNA in HT29 cells exhibited first order kinetics, with a half-life of ~27 h (Figure 1A), indicating its relative slow turnover.

Figure 1B shows the concentration-response curves of survivin mRNA knockdown by 48 h treatment of Lipoplex or suramin. While both agents yielded concentration-dependent knockdown, neither was complete at the highest concentrations tested. The maximal inhibition was 67% (achieved at 1.2 μ M) and the $IC_{50, 48h, mRNA-KD}$ was 0.6 μ M for Lipoplex, and 78% (achieved at 500 μ M) and 75 μ M for suramin, respectively. Lipoplex showed a steeper dose-response curve with a 3-times higher n value compared to suramin (2.1 vs. 0.7).

Neither Lipoplex and suramin, at their $IC_{50, 48h, mRNA-KD}$ for 24 h, caused cytotoxicity (Figure 1C). Hence, we used three drug concentrations there were approximately one-third to three times the $IC_{50, 48h, mRNA-KD}$ value (0.2, 0.6 and 1.8 μ M for Lipoplex, and 24, 70 or 178 μ M for suramin) and the 4-h treatment duration to study the polyanion effects. Under these conditions, the degradation of endogenous mRNA was minimal (10% based on a half-life of 27 h), suramin had negligible effect on survivin mRNA (which occurred at a later time of 48 h, see additional results in 3.5) and neither suramin or Lipoplex showed appreciable cytotoxicity (hence no change in cell numbers).

3.2 Effects of suramin on surface charge, particle size, and biocorona of Lipoplex

Lipoplex/Water (0.2 μ M) showed a positive surface charge of $+33.9 \pm 0.02$ mV. Addition of FBS (10%), suramin (24 μ M), or their combination, changed the charge to negative (-19.1 ± 0.7 , -24.3 ± 0.3 , and -20.5 ± 0.52 mV, respectively). Note that the zeta potential of Lipoplex/Medium was indeterminate.

The pH of the water used in the experiments was 6.1 ± 0.13 , and was not altered by addition of 75 μ M suramin (5.9 ± 0.03).

Figure 2 shows the average particle size and size distribution of PCat or Lipoplex, and the effects of FBS and suramin. PCat/SF-Medium and Lipoplex/SF-Medium showed similar sizes at all concentrations (152–179 and 187–208 nm, respectively). Addition of 10% FBS

(i.e., Full Medium), which causes the formation of protein biocorona on Lipoplex [15, 18], broadened the size distribution and increased the average size with greatest increases at 1.8 μ M Lipoplex. In contrast, addition of suramin partially reversed the protein-induced size increase and narrowed the size distribution. PCat and Lipoplex responded similarly to FBS and suramin, indicating the interaction and size change were related to their common property of cationic surface charge.

A potential mechanism by which suramin reversed the protein-mediated particle size increase is removal of protein biocorona. We first determined if adding suramin (70 μ M) before or after 30 min incubation of serum proteins (10% FBS) with Lipoplex (0.6 μ M), i.e., during or after biocorona formation, affected the outcome; both conditions yielded comparable particle sizes and polydispersity indices (~210 nm and 0.11, respectively), and similar total protein amounts (i.e., comparable intensities of all PAGE protein bands of $9.2 \pm 0.7 \times 10^7$ vs. $1.1 \pm 0.1 \times 10^8$ arbitrary unit). These results indicate similar suramin effects on biocorona formation and preformed biocorona. Subsequent studies used simultaneous incubation of suramin, Lipoplex and FBS for 30 min.

We next compared the total amounts of proteins in Lipoplex-biocorona, in the absence and presence of suramin. Figure 3A shows the SDS-PAGE results; both FBS and Lipoplex-biocorona contained several major protein bands and several minor bands between 10–200 kDa. Changes in biocorona protein levels depended on both suramin and Lipoplex concentrations (Figure 3B). In the absence of suramin, the amount of total biocorona proteins, as they depended on the amount of the available Lipoplex, increased with Lipoplex concentration (from 1.5×10^8 at 0.2 μ M to 6.7×10^8 at 1.8 μ M). However, after normalization to Lipoplex concentration, the amount of biocorona proteins per μ M Lipoplex was ~50% lower at 1.8 μ M Lipoplex compared to 0.2 μ M Lipoplex, suggesting protein depletion at the higher Lipoplex concentration. Addition of suramin caused biphasic changes, with initial decrease in the total biocorona protein amount followed by an increase at higher suramin concentrations. While equal maximal decline of ~90% was attained at all Lipoplex concentrations, higher suramin concentrations were required to achieve this effect at the higher Lipoplex concentrations (i.e., 10, 23, and 59 μ M suramin for 0.2, 0.6 and 1.8 μ M Lipoplex, respectively), suggesting concentration-dependent interactions.

The protein compositions of Lipoplex biocorona were also compared. There are several distinct changes; the protein bands located between 14–62 kDa disappeared or diminished whereas the bands between 98–200 kDa became more intense, with increasing suramin concentrations (Figure 3C).

3.3 Polyanion drugs destabilize Lipoplex

Figure 4 shows the Lipoplex destabilization by polyanions at constant siRNA:polyanion concentration ratios, similar to the conditions of the gene knockdown study. No *F-siRNA or *F-lipid were released from Lipoplex/Water into the ultrafiltrates (Figure 4A). In contrast, the addition of FBS (10%) yielded significant *F-siRNA/lipid release; the release was greatest at 0.2 μ M Lipoplex and became negligible at higher Lipoplex concentrations (Figure 4B). Based on the total protein concentration in 100% FBS (36 μ g/ μ L per product certificate of analysis) and the siRNA concentration of 13.3 ng/ μ L at 1.0 μ M for AF647-siSurvivin

(MW of 13330), the protein:siRNA concentration ratios at 0.2, 0.6 and 1.8 μM Lipoplex were calculated to be 1300, 420 and 120; these results indicate decreasing destabilization with decreasing protein:siRNA ratios, a finding similar to our observation with another Lipoplex containing a different siRNA [42]. Compared to FBS, the addition of suramin and heparin generally resulted in more extensive $^*\text{F}$ -siRNA/lipid release that also fluctuated with Lipoplex and polyanion concentrations. We calculated the net increase in release due to polyanions by subtracting the % release of $^*\text{F}$ -siRNA/lipid in FBS, and observed qualitatively and quantitatively different results for the two polyanions (Figure 4C). The net $^*\text{F}$ -siRNA release increased with suramin concentration whereas the net $^*\text{F}$ -lipid release did not. In contrast, heparin showed biphasic effects on both (reduced release at 24 μM and enhanced release at higher concentrations). The reduced net release indicates heparin, at low concentration, protected Lipoplex against FBS-induced destabilization. Heparin, on a molar basis, was slightly less effective compared to suramin in destabilizing Lipoplex.

Next, we evaluated the siRNA entities in various mixtures using denaturing gel-PAGE (Figure 4D); these results are semi-quantitative in view of potential siRNA degradation by the heating during sample preparation. Comparison of the band locations of the water control and the various mixtures indicate the bottom band below 17 ss nucleotides corresponded to suramin (due to binding with the positively charged Sybr® Green). The siRNA_{unchanged}/Water mixture showed two bands between 21 and 50 nucleotides, corresponding to intact siRNA (42 nucleotides for ds siRNA and 21 nucleotides for its two ss components). These ds/ss siRNA bands were absent in the Lipoplex/Water mixture, indicating no release and thus confirming the ultrafiltration results in Figure 4A. Suramin or heparin did not alter the bands derived from siRNA_{unchanged}/Water, indicating no effects on siRNA stability. In contrast, addition of suramin or heparin to Lipoplex/Water caused the release of the 21–50 nucleotide bands, with greater release for suramin than for heparin, again confirming the ultrafiltration results in Figure 4B.

3.4 Polyanion drugs reduce cell binding, endocytosis and intracellular bioavailability of Lipoplex

Figure 5A shows the confocal microscopic images of cells treated with Lipoplex (0.6 μM for 1 h). The green $^*\text{F}$ -Rab5 resided in cytoplasm, whereas the red $^*\text{F}$ -lipid resided on cell surface and in cytoplasm. Results of quantitative Image analysis indicate the surface-bound $^*\text{F}$ -lipid accounted for 88% of the total cell-associated $^*\text{F}$ -lipid whereas the $^*\text{F}$ -Rab5-colocalizing $^*\text{F}$ -lipid accounted for 12%. Suramin (3–70 μM) did not alter the total $^*\text{F}$ -Rab5 pixel ($1.33 \pm 0.44\%$ of total cell area with suramin vs. $1.31 \pm 0.69\%$ without suramin), but significantly reduced both surface-bound and internalized $^*\text{F}$ -lipid (Figure 5B). Comparison of the latter two entities further showed that suramin reduced the internalization of surface-bound $^*\text{F}$ -lipid (Figure 5C); the reduction increased with suramin concentration such that no $^*\text{F}$ -lipid was observed on cell surface or in cytoplasm at 70 μM suramin. Similar effects were observed for heparin, which at the same concentration also completely eliminated the $^*\text{F}$ -lipid binding and internalization.

3.5 Polyanion drugs abolishes siRNA occupancy in RISC and gene silencing activity of Lipoplex

In the absence of suramin, the amount of siSurvivin in RISC relative to miR16 at 4 h increased with Lipoplex doses, from 0.18 relative units at 0.2 μM Lipoplex to 2.5 units at 1.8 μM (Figure 6A). In contrast, addition of suramin at a constant Lipoplex:suramin concentration ratio completely eliminated the siRNA in RISC.

Treatment with Lipoplex for 4 h yielded significant mRNA knockdown in a concentration-dependent manner (Figure 6B), with knockdown and IC_{50} comparable to the effects of 48 h-treatments shown in Figure 1B. On the contrary, treatment with suramin for 4 h at 225 μM , which was 3-times its $IC_{50,48h,mRNA-KD}$, had no effect. The rapid gene silencing by Lipoplex is consistent with the siRNA action mechanism, i.e., causing mRNA degradation by Ago2 in RISC, whereas the absence of suramin effect at 4 h may be due to its indirect effect (e.g., lag time from inhibiting the β -catenin-mediated regulation of survivin gene transcription [43, 44]).

Figure 6B further shows suramin completely abolished the gene silencing by Lipoplex. Similarly, heparin (70 μM for 4 h), which has no known effects on survivin signaling, also completely abolished the Lipoplex gene silencing (data not shown).

Taken together, these results indicate polyanion drugs reduced $F_{intracellular,siRNA}$ and thereby abolished the gene silencing activity of Lipoplex.

3.6 QP modeling to determine the concentration-dependent contributions of polyanion-Lipoplex interactions to reducing intracellular siRNA bioavailability

The above data show that polyanion drugs reduced $F_{intracellular,siRNA}$ through three mechanisms, i.e., destabilization leading to loss of siRNA cargo, inhibition of Lipoplex binding to cell membrane and the subsequent internalization (summarized in Figure 7A). Because these three effects occurred simultaneously and in a suramin concentration-manner, their relative contributions to the overall $F_{intracellular,siRNA}$ reduction changed with suramin concentration.

$F_{destabilization}$ was negligible at 14 μM , and equaled 26.6% at 19–70 μM . Figure 7B shows the inhibition of Lipoplex binding to cell membrane and inhibition of Lipoplex internalization, as function of suramin concentration (Left panel). Analyses of these plots using Eq. 2 and 3, respectively, yielded the model parameters (see Figure Legends). The $IC_{50,internalization}$ value was about one-half the $IC_{50,binding}$ value.

Figure 7B also shows the overall reduction of $F_{intracellular,siRNA}$ (for 0.6 μM Lipoplex) by suramin, approaching 100% at 46 μM suramin (Middle panel). The model-simulated results indicate the relative contributions of the three mechanisms changed with suramin concentration (Right panel). Inhibition of Lipoplex internalization was the major cause at lower suramin concentration (50% reduction of $F_{intracellular,siRNA}$ at 9 μM), after which point inhibition of Lipoplex binding became more significant such that it accounted for ~60% of the total reduction at 70 μM suramin. Destabilization or loss of siRNA cargo was negligible at low suramin concentrations (<1% at 14 μM), but jumped to 26.6% at 19–70

μM . This data indicates no destabilization at high Lipoplex:suramin molar concentration ratio (1:23), whereas about one-fourth of the siRNA cargo was released at a slightly lower ratio (1:31) with no further increases as the ratio decreased by another ~4-fold, suggesting an all-or-none destabilization effect.

4 Discussion

Results of the present study show polyanions such as suramin and heparin interacted with, and altered the properties and functionality of, Lipoplex in several ways. First, polyanions modified the physicochemical properties of Lipoplex (i.e., reduced size, changed surface charge, altered composition of Lipoplex protein biocorona). These physical and structural changes are associated with Lipoplex destabilization, causing a substantial release of the siRNA cargo. Third, polyanions prevented the cell membrane binding and internalization of Lipoplex, diminished the intracellular bioavailability of siRNA and the siRNA-RISC occupancy, and reduced the mRNA knockdown by Lipoplex. In contrast, a separate study in our laboratory showed that treatments with a monoanion, sodium phosphate (140–1255 μM), did not cause release of another siRNA (siMetadherin, an asymmetrical ds siRNA of 25 and 27 nucleotides) or lipid from Lipoplex. As discussed below, these various effects were dependent on polyanion concentrations.

Changes in structures or surface properties of Lipoplex are likely the results of physicochemical interactions between Lipoplex and polyanions. For example, suramin is a polyanion with six negative charges ($\text{pK}_a < 1$), and its binding to cationic sites on Lipoplex changed the surface charge from positive to negative (e.g., from +34 mV to –24 mV for Lipoplex/Water), similar to the finding that binding of serum proteins to siRNA-Lipoplex resulted in negative surface charge (e.g., data in the present study and earlier studies [18, 45]). On the other hand, for situations where serum proteins already reduced the Lipoplex surface charge to –19 mV, addition of suramin only had a minor effect (reduction to –21 mV). This finding, together with the finding that suramin, in a dose-dependent manner, reduced the amount and modified the composition of the Lipoplex protein biocorona, indicate suramin competed with and/or displaced serum proteins from cationic binding sites on Lipoplex. For both serum proteins and suramin, the positive-to-negative change in surface charge was associated with Lipoplex destabilization to release siRNA and lipids. Another polyanion, heparin, similarly caused release of siRNA and lipids from Lipoplex. These findings collectively indicate negatively charged molecules such as proteins and polyanion drugs destabilized Lipoplex to release its siRNA cargo.

The suramin data further indicated an all-or-none destabilization, with no release at 14 μM but reaching the maximum release at the next higher concentration used in the experiment (19 μM) with no further increases up to 70 μM . The cation:anion ratios at these suramin concentrations (in combination with 0.6 μM siRNA) were calculated to be 1:1.1 at 14 μM and 1:1.4 at 19 μM , suggesting a threshold ratio of between 1.1 and 1.4 for destabilization. Previous reports have shown that for nucleic acid-lipoplex/polyplex formation, a cation:anion (N/P) ratio near unity is when the lipoplex/polyplex is the least stable and the hydrophobic forces between the smaller units cause their aggregation to form larger complexes [46, 47].

The two polyanions, suramin and heparin, yielded qualitatively and quantitatively different outcomes. First, suramin and heparin, at equal molar concentrations, yielded up to ~30% difference in the extents of siRNA and lipid release, suggesting differences in their interactions with Lipoplex. Second, heparin at low concentration (24 μM) retarded the serum protein-mediated Lipoplex destabilization whereas suramin did not. We speculate this heparin protection effect, in view of the binding of heparin to a wide variety of proteins [48], is because the binding reduces the amount of serum proteins available to interact with Lipoplex. Note that this protection effect was reversed at higher heparin concentrations, suggesting the direct destabilization at higher concentration exceeded the indirect protection against serum proteins.

The potential mechanisms by which suramin and heparin block the cellular binding and internalization of Lipoplex are several. First, these polyanions may bind to the cell surface and thereby limit the access of Lipoplex to binding sites. Suramin, other polysulfonated-polyaromatic-planar compounds (e.g. trypan blue, Evans blue) and heparin have been shown to block the binding of ligands (e.g., interleukin-2, urokinase-type plasminogen activator, $\alpha 2$ -macroglobulin) to their cell-surface receptors [49–51]. Second, the binding of polyanions to Lipoplex functions as a decoy to reduce the Lipoplex available for cellular binding, similar to the situation by which suramin inhibits binding of growth factors to their receptors [52]. Third, in view of the binding of DNA-lipoplex or DNA-polylysine complexes to cell membrane through nonspecific ionic interactions [53, 54], polyanions by reducing the positive surface charges of Lipoplex would reduce the ionic interactions between Lipoplex and cell membrane. However, this latter effect is likely to be minor because addition of suramin in the presence of FBS only slightly altered the surface charge of Lipoplex. Fourth, changes in biocorona of Lipoplex by polyanions, as indicated by changes in particle size and protein composition, can affect the Lipoplex binding to cell membrane [55, 56].

Results of the present study indicate polyanions reduced $F_{intracellular, siRNA}$ and consequently reduced the gene silencing activity of Lipoplex. We considered several other potential mechanisms. First, our results showed suramin did not change the extracellular pH, which rules out the possibility of pH lowering that can inhibit the movement of clathrin- and nonclathrin-coated vesicles/pits into the cytoplasm [57]. A second possibility is suramin inhibition of siRNA binding to Ago2, as previously reported for the loading of miR21 in reconstituted RISC (IC_{50} of 0.69 μM) and the de-repression of siRNA-mediated gene silencing in human cells (~40% de-repression at 25 μM suramin) [58]. However, this mechanism is unlikely to have a major role since the three mechanisms (destabilization, inhibition of binding and internalization) accounted for the 94–100% loss of $F_{intracellular, siRNA}$ at 23–70 μM suramin (Figure 7B, Right panel). Third, as endocytic recycling and limited endosomal escape reduce the bioavailability of siRNA in the cytosol and RISC [59–61], an increase in endocytic recycling can reduce $F_{intracellular, siRNA}$. Based on the lower IC_{50} for internalization compared to IC_{50} for binding (9.1 vs. 18.9 μM), suramin was more efficient in decreasing uptake and/or increasing efflux than in inhibiting binding. As we did not monitor Lipoplex recycling, the possibility that suramin enhanced efflux cannot be ruled out. On the other hand, because the QP model depicts internalization as the net of input (e.g., endocytosis) and output (e.g., *via* exosomes), the modeling results and the conclusion on the relative contribution of the three kinetic processes to the overall loss of

$F_{intracellular, siRNA}$ have accounted for the recycling. Finally, suramin-enhanced Lipoplex efflux *via* exosomes is not likely because (a) suramin abolished the internalization and hence abolished the generation of exosomes, and (b) the relative contribution of internalization decreased with increasing suramin concentrations, which is opposite to what would be expected for a positive cause-and-effect relationship.

To our knowledge, this is the first report on the multiple interactions between polyanion drugs and Lipoplex. The results indicate interactions on several scales, i.e., cellular level (binding and internalization) and system level (Lipoplex degradation in extracellular fluid). Cationic lipoplex and polyplex are the two major nonviral carriers of gene therapeutics, as they (a) form complex with DNA and siRNA and thereby protect them from enzymatic degradation, and (b) facilitate nanoparticle binding to cell membrane and the subsequent endocytosis [6, 62–65]. These properties derive from their electrostatic interactions with the negatively charged nucleic acids and cell membrane [66, 67] and therefore can be abolished by agents that interfere with these interactions. In addition to the findings of the current study that polyanions reduced the gene silencing by siRNA-lipoplex, other examples include the destabilization of siRNA-lipoplexes and DNA-polyplexes by negatively charged serum proteins and polyanions [42, 67]. Taken together, we propose that polyanions or other ionized agents capable of disrupting the electrostatic interactions between cationic liposome/lipoplex, polymer/polyplex, nucleic acid, and/or cell membrane proteins/receptors would perturb delivery systems that depend on such interactions for drug loading, binding and endocytosis.

Finally, the current study used a new approach, i.e., QP modeling, to delineate the relative contributions of the competing polyanion effects on three kinetic processes (Lipoplex destabilization to release siRNA, inhibition of Lipoplex binding to cell membrane and internalization) to the loss of $F_{intracellular, siRNA}$ and siRNA-RISC occupancy. Accounting for these simultaneous, polyanion concentration-dependent processes enabled us to address several questions. First, in the nucleic acid-lipoplex literature, the loss of DNA or siRNA cargo is generally assumed to be the major cause of loss of therapeutic efficacy. The present study indicates this assumption was partially correct; Figure 7B shows the major mechanism of diminished $F_{intracellular, siRNA}$ changed from inhibition of Lipoplex internalization at low suramin concentrations to inhibition of membrane binding at medium to high concentrations, whereas the contribution from the loss of siRNA cargo was comparatively minor and did not exceed 30%. Second, the QP modeling results identified the dynamic nature of the major barriers to delivering siRNA to RISC and achieving gene silencing by siRNA-Lipoplex (e.g., the major barrier changes with the properties of the polyanions and their concentrations). In view of the well-recognized difficulties in translating nanotherapeutics into clinically useful entities [68], our findings highlight the need of (a) defining how and how much the environment in which the Lipoplex resides affect the Lipoplex stability and cellular processing (e.g., types and concentrations of proteins or other negatively charged molecules that can interact with Lipoplex), and (b) relating the interactions on one scale to another (e.g., from molecule, cell, tissue to whole organism). For example, as the different polyanion-lipoplex interactions are highly dependent on polyanion concentrations, it is conceivable that their relative importance may change in the presence of 100% serum or other protein-rich matrices under *in vivo* conditions. Additional studies to

elucidate how such changes will affect the siRNA-RISC occupancy will be useful in guiding the *in vitro-to-in vivo* translation of nucleic-acid-lipoplex/polyplex. Finally, the QP modeling results of the current study, by establishing the quantitative impacts of direct physical interactions between polyanions and Lipoplex, identified elimination of such interactions as a means to improve the silencing efficacy of siRNA-lipoplex; this prediction was confirmed in a separate study where siRNA-lipoplex was administered 6 h prior to suramin [69].

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Abbreviations

Ago-2	Argonaute 2
Ct	cycle threshold
CV	coefficient of variation
DOPE	1,2-dioleoyl-sn-glycero-3-phosphoethanolamine
DOTAP	1,2-dioleoyl-3-trimethylammoniumpropane
ds	double-strand
DSPE-PEG	1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000]
EDTA	ethylenediaminetetraacetic acid
*F	fluorescence-labeled moiety
F_{IntracellularsiRNA}	intracellular siRNA bioavailability
Full Medium	McCoy's 5A medium supplemented with 10% FBS, 1% non-essential amino acids and 1% penicillin/streptomycin
GFP	green fluorescent protein
I_{max}	maximal inhibitory effect
IC₅₀	drug concentration that caused 50% maximal effect
Lipoplex	complex of siSurvivin and PCat
n	Hill equation curve shape parameter
NT	non-targeting siRNA
PAGE	polyacrylamide gel electrophoresis
PCat	pegylated cationic liposomes

PBS	phosphate buffered saline
QP	quantitative pharmacology
RISC	RNA-induced silencing complex
RT-qPCR	quantitative reverse transcription polymerase chain reaction
SD	standard deviation
SDS	sodium dodecyl sulfate
SF-Medium	serum-free medium
siSurvivin	Survivin siRNA
ss	single-strand

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Figure 1. Defining treatment conditions

(A) Kinetics of endogenous survivin mRNA degradation in HT29 cells. Cells were cultured in Full Medium, treated with 5 μg/ml actinomycin D, collected at predetermined intervals and analyzed using RT-qPCR (5×10^5 cells per sample); the mRNA level of survivin was normalized to that of the internal standard 18S rRNA, and expressed as % of the initial survivin level at time 0 before actinomycin D treatment. The best-fit line was $y = 94.56 \cdot e^{-0.0257 \cdot x}$ ($r^2=0.905$). (B) Effects of Lipoplex and suramin on survivin mRNA level. Cells were incubated in Full Medium and treated with Lipoplex or suramin for 48 h. mRNA levels were measured using RT-qPCR and the best-fit curves by the Hill equation are shown. (C) Cytotoxicity measured using MTT assay (24 h treatment).

Lipoplex-equivalents μM	Particle size (nm), mean±SD (Polydispersity index)					
	PCat/SF-Medium	Lipoplex/SF-Medium	PCat/Full Medium		Lipoplex/Full Medium	
	No Suramin	No Suramin	No Suramin	With Suramin	No Suramin	With Suramin
0.2	179.6 ± 10.0 (0.08 ± 0.01)	207.5 ± 7.7 (0.09 ± 0.03)	276 ± 9.6 (0.11 ± 0.02)	232 ± 3.9 (0.09 ± 0.02)	272 ± 5.9 (0.08 ± 0.04)	216 ± 5.1 (0.09 ± 0.05)
0.6	163.1 ± 8.5 (0.05 ± 0.01)	192.2 ± 6.1 (0.08 ± 0.01)	283 ± 9.2 (0.12 ± 0.02)	205 ± 3.6 (0.11 ± 0.01)	273 ± 10.6 (0.10 ± 0.04)	219 ± 3.4 (0.10 ± 0.06)
1.8	152.1 ± 3.3 (0.08 ± 0.01)	186.5 ± 10.5 (0.08 ± 0.01)	862 ± 59 (0.24 ± 0.06)	270 ± 6.2 (0.23 ± 0.04)	470 ± 37.2 (0.14 ± 0.07)	247 ± 10.1 (0.19 ± 0.12)

Figure 2. Suramin reverses the Lipoplex particle size increase due to biocorona formation
Particle sizes of PCat and Lipoplex were measured using the dynamic light scattering method. Dotted, dashed and solid lines are, respectively, 0.2, 0.6, and 1.8 μM Lipoplex or PCat containing equivalent lipid concentration. The respective suramin concentrations at these Lipoplex concentrations were 24, 70, and 178 μM. Data are expressed as percentage of distribution intensity. (A) Results in SF-Medium. (B) Results in Full Medium without suramin. (C) Results in Full Medium with suramin. Table shows mean ± SD of 3 experiments (triplicate samples per experiment).

A. SDS-PAGE of protein biocorona

B. Total protein band intensity

C. Individual protein level

Figure 3. Effect of suramin on Lipoplex-biocorona

(A) SDS-PAGE results. Lipoplex (0.2, 0.6 and 1.8 μM) were dispersed in Full Medium and incubated without or with suramin (0–662 μM). Biocorona proteins were stripped from Lipoplex, separated using SDS-PAGE, and the band intensity measured. Sur, suramin. (B) Changes in total proteins. Total protein band intensities of Lipoplex biocorona, in the absence of suramin, were analyzed without (Left panel) or with normalization (Middle panel) to initial Lipoplex concentration. Right panel shows the suramin concentration-dependent changes in total Lipoplex biocorona proteins (normalized to the values for Lipoplex without suramin). Data of 0.2, 0.6 and 1.8 μM siRNA are represented by circles, squares and triangles. (C) Changes of intensities of individual biocorona protein bands

located near 14–28, 62, 98 and 100–200 kDa with suramin concentrations. Symbols are as in (B). Some SD are smaller than the symbols.

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Figure 4. Destabilization of Lipoplex by suramin and heparin

*F-Lipoplex comprising *F-siRNA and *F-PCat was dispersed in water or Full Medium (which contained 10% FBS), in the absence or presence of suramin or heparin. (A-C) *F-siRNA (top panel) and *F-lipid (bottom panel) recovered in the 1000 kDa ultrafiltrates were determined as % of the fluorescence in the pre-filtration samples. Mean $\pm$ SD (n=3). (D) PAGE analysis of destabilization products. Solution of siRNA_{unchanged} or Lipoplex (both containing 0.6 μM of siRNA) was incubated in water with increasing concentrations of suramin and heparin for 30 min at 37°C, followed by analysis with denaturing urea gel (stained by SYBR® Green).

Figure 5. Suramin and heparin inhibit cellular binding and internalization of siRNA-lipoplex
Cells transduced with GFP-Rab5 (green) were seeded on a glass slide, treated with Lipoplex (containing *F-PCat (red) and 0.6 μM siRNA) and different concentrations of suramin (0–70 μM), at 37°C for 1 h. (A) Confocal microscopy results (bar, 7.5 μm). (B) Results of quantitative image analysis. Rab5-colocalized intracellular *F-lipid (black bars) and cell surface-bound *F-lipid (gray bars) *p<0.05 (one-way ANOVA, Dunnett's post-test comparing all treatment groups to the single control group). (C) Rab5-colocalized intracellular *F-lipid is shown as fraction of cell surface-bound *F-lipid to show the kinetics of internalization.

Figure 6. Suramin reduces siRNA occupancy in RISC and abolishes siRNA-mediated mRNA knockdown

Cells were treated with siRNA-Lipoplex (0.2, 0.6 and 1.8 μM), without or with suramin (25, 75, 225 μM for single agent suramin and 24, 70 and 178 μM for combination) for 4 h. (A) RISC/Ago-2-associated siRNA was isolated from cell extracts using immunoprecipitation and quantified with RT-qPCR using miR16 as the internal standard. siRNA level was expressed relative to miR16 level. (B) Suramin abolished mRNA knockdown by Lipoplex. mRNA expression was determined using RT-qPCR and expressed as % of control (i.e., without Lipoplex or suramin). * $p < 0.05$ (one-way ANOVA, Tukey post-hoc comparing all treatment groups to each other).

A. Reduction of intracellular siRNA bioavailability by polyanions

B. QP modeling: Contributions of individual mechanisms

Figure 7. QP modeling to determine contributions of individual mechanisms of polyanions to reduce $F_{\text{intracellular, siRNA}}$

(A) Schematic depiction of interactions between polyanions with Lipoplex on two scales. Left panel: Interactions between polyanions and Lipoplex resulting in changes in Lipoplex properties (particle size, surface charge) plus destabilization (i.e., loss of siRNA cargo) (systems level). Right panel: Polyanions inhibit Lipoplex binding to cell membrane and the subsequent Lipoplex internalization (cellular level). (B) QP modeling to determine relative contributions of individual mechanisms. Left panel: Obtaining model parameters for the polyanion-mediated changes in Lipoplex binding and internalization by fitting Eq. 2 and 3 to the experimental results (symbols). The best-fit Hill equation curves are shown and the corresponding best-fit parameter values were $IC_{50, \text{binding}} = 18.9 \mu\text{M}$ suramin, $IC_{50, \text{internalization}} = 9.1 \mu\text{M}$, $I_{\text{max, binding}} = 1.0$, $I_{\text{max, internalization}} = 1.0$, $n_{\text{binding}} = 1.5$ and $n_{\text{internalization}} = 1.7$ (corresponding standard errors were 31, 8, 12, 3, 37, and 13% of the mean). Middle panel: Total $F_{\text{intracellular, siRNA}}$ reduction due to destabilization, inhibition of membrane binding and internalization. Note that destabilization occurred only at higher suramin concentrations ($>19 \mu\text{M}$). Results are the sum of the losses due to individual mechanisms (calculated using Eq. 1–3). Right panel: Model-simulated contributions of individual mechanisms to the overall reduction of $F_{\text{intracellular, siRNA}}$. In B and C, red arrow corresponds to the suramin concentration that resulted in the all-or-none destabilization (26.6% at $19 \mu\text{M}$ and higher).