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The Modification of siRNA with 3' Cholesterol to Increase Nuclease Protection and Suppression of Native mRNA by Select siRNA Polyplexes

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Abstract

Polymer-siRNA complexes (siRNA polyplexes) are being actively developed to improve the therapeutic application of siRNA. A major limitation for many siRNA polyplexes, however, is insufficient mRNA suppression. Given that modifying the sense strand of siRNA with 3' cholesterol (chol-siRNA) increases the activity of free nuclease-resistant siRNA *in vitro* and *in vivo*, we hypothesized that complexation of chol-siRNA can increase mRNA suppression by siRNA polyplexes. In this study, the characteristics and siRNA activity of self assembled polyplexes formed with chol-siRNA or unmodified siRNA were compared using three types of conventional, positively charged polymers: (i) biodegradable, cross-linked nanogels (BDNG) (ii) graft copolymers (PEI-PEG), and (iii) linear block copolymers (PLL10-PEG, and PLL50-PEG). Chol-siRNA did not alter complex formation or the resistance of polyplexes to siRNA displacement by heparin but increased nuclease protection by BDNG, PLL10-PEG, and PLL50-PEG polyplexes over polyplexes with unmodified siRNA. Chol-CYPB siRNA increased suppression of native CYPB mRNA in mammary microvascular endothelial cells (MVEC) by BDNG polyplexes (35%) and PLL10-PEG polyplexes (69%) over comparable CYPB siRNA polyplexes but had no effect on PEI-PEG or PLL50-PEG polyplexes. Overall, these results indicate that complexation of chol-siRNA increases nuclease protection and mRNA suppression by select siRNA polyplexes. These results also suggest that polycationic block length is an important factor in increasing mRNA suppression by PLL-PEG chol-siRNA polyplexes in mammary MVEC.

Keywords

Immortomouse; H-2K^b-ts-A58; mammary fat pad; PEGylated cationic polymers; PEGss; PPIB

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1. Introduction

Small interfering RNA (siRNA) is a naturally occurring dsRNA molecule (21–23 nucleotides) processed from longer dsRNA [1] that selectively and persistently suppresses the expression of proteins through the catalytic, sequence-specific degradation of mRNA [2]. As such, siRNA has tremendous potential in therapeutic applications where the suppression or absence of a protein or multiple proteins can produce a clinically beneficial result. Furthermore, many proteins identified as drug targets that cannot be inhibited by conventional low molecular weight drug candidates can potentially be treated by siRNA [3].

siRNA can be administered directly as synthetic siRNA (typically 19 bp dsRNA with two 3' nucleotide overhangs on each strand) that functions upon entry into the cytosol [2] or as pDNA that functions after entry into the nucleus and subsequent expression of short hairpin loops of RNA (shRNA) for cytosolic processing into siRNA [4]. siRNA is incorporated into an RNA-induced silencing complex (RISC) within the cytosol [5] where the sense strand is degraded to reveal the antisense strand [6] and form an activated RISC. Activated RISC then facilitates the degradation of mRNA that is complementary to the loaded antisense strand [7] and remains active [8] for 3–7 days in dividing cells and for several weeks in non-dividing cells [9].

Although pDNA has the potential to deliver higher and more sustained dosages of siRNA, unpredictable and subsequently toxic dosages *in vivo* have been reported [10]. Furthermore, synthetic siRNA, unlike pDNA, does not need to enter and rely on the host nucleus for subsequent function. Thus, from a pharmaceutical perspective, synthetic siRNA is likely safer because dose can be tightly controlled and potentially more effective due to fewer intracellular barriers.

Many clinical applications require the systemic administration of siRNA to achieve a therapeutic effect. The systemic administration of siRNA, however, is limited by several obstacles, including: (i) the extremely short plasma half-life of siRNA due to degradation by nuclease activity and renal clearance (ii) low level cellular uptake of siRNA due to its large size (13 kDa) and negative charge (iii) the inability of siRNA to escape the endosomes/lysosomes into the cytosol, the site of activity [11–13].

Polymer siRNA complexes (siRNA polyplexes) are being actively developed to improve the systemic administration of siRNA [12]. The design of siRNA polyplexes requires balancing many requirements including (i) protecting siRNA molecules against nuclease degradation in the plasma, (ii) increasing plasma half-life and providing a favorable distribution (iii) promoting cellular uptake (iv) facilitating endosome/lysosome escape of siRNA into the cytosol (v) high biocompatibility/low toxicity and (vi) absence of unwanted side effects [11]. The suppression of mRNA, however, is commonly low in many siRNA polyplexes. Thus, identifying designs and/or modifications that can increase mRNA suppression while satisfying the many other requirements for systemic administration is critical to the development of siRNA polyplexes.

Modifying the sense strand of siRNA with 3' cholesterol (chol-siRNA) increases the activity of free nuclease-resistant siRNA in hepatoma cells (Huh-7 and Hep G2) *in vitro* [14,15] and the liver and jejunum *in vivo* [15]. Thus, we hypothesized complexation of chol-siRNA can increase mRNA suppression by siRNA polyplexes and, as such, may potentially be a simple approach to improve siRNA polyplexes for systemic administration. To test this hypothesis, the initial characteristics and siRNA activity of conventional polyplexes of biodegradable Nanogels (BDNG), PEI (2K)-*g*-PEG (10K), PLL10 (1.6K)-*b*-PEG (5K), and PLL50 (8K)-*b*-PEG (5K) formed with chol-siRNA or unmodified siRNA were compared in this study.

2. Materials and methods

2.1 Polymers

BDNG, biodegradable Nanogels (named “NG(PEGss)” in [16]) consisting of biodegradable PEI (28 kDa PEI formed from 2 kDa PEI via disulfide bonds) cross-linked with 8 kDa PEG through carbamate bonds, and **PEI-PEG**, polyethylenimine-*g*-poly(ethylene) glycol graft copolymer with a cationic block consisting of 2 kDa branched PEI (Sigma, St. Louis, MO) and a nonionic hydrophilic block consisting of 10 kDa PEG (Sigma, St. Louis, MO) [17], were synthesized and purified as described. **PLL10-PEG** and **PLL50-PEG**, methoxy-poly(ethylene glycol)-*b*-poly(L-lysine hydrochloride) block copolymers with cationic blocks consisting of 10 (PLL10) or 50 (PLL50) poly-L-lysine groups and a nonionic hydrophilic block consisting of 5 kDa PEG, were purchased from Alamanda Polymers (Huntsville, AL).

2.2. siRNA

Model siRNA: Stock solutions of DNA oligonucleotides (IDT, Coralville, IA) with two 3' AT overhangs (5' - CTC GAT AGA ATA CAC AGG CAT - 3'; 5' - GCC TGT GTA TTC TAT CGA GAT - 3') in 1X IDT duplex annealing buffer (100mM potassium acetate, 30 mM HEPES, pH 7.5) were combined to a final concentration of 20 μ M, incubated at 95°C for 5 min, and cooled at RT. **Chol-model siRNA**: A single RP-HPLC-purified DNA oligonucleotide modified with 3' cholesteryl triethylene glycol (TEG) (5' - CTC GAT AGA ATA CAC AGG CAT/Cholesteryl TEG/- 3') (IDT, Coralville, IA) was annealed to (5' - GCC TGT GTA TTC TAT CGA GAT - 3') (IDT, Coralville, IA) at 20 μ M as described for model siRNA. *siRNA (with UU overhangs)*: Lyophilized **single CYPB siRNA** (Dharmacon D-001820-02: 5' - CAA GUU CCA UCG UGU CAU C - 3'), **single chol-CYPB siRNA** (Dharmacon D-001820-02 modified with cholesterol through a 6 carbon hydroxyproline linker and purified by RP-HPLC), **pooled CYPB siRNA** (Dharmacon D-001820-20: 5' - CAA GUU CCA UCG UGU CAU C - 3'; 5' - CGG CAA AGU UCU AGA GGG C - 3'; 5' - GGA GAA ACC CUU CGC CAU U - 3'; 5' - GAA AGA GCA UCU AUG GUG A - 3') against murine CYPB mRNA (NM_011149), and **single non-targeting siRNA** (Dharmacon D-001810-01: 5' - UGG UUU ACA UGU CGA CUA A - 3') were resuspended according to manufacturer's instructions in 1X Dharmacon Buffer at 20 μ M and stored in aliquots at -80°C.

2.3 Model siRNA loading

Model siRNA polyplexes were formed by adding 10 μ L of model siRNA or chol-model siRNA (20 μ g/mL [1.556 μ M]) in 0.1 M HEPES Buffer [pH 7.4]) to 10 μ L of HEPES buffer alone (N/P = 0) or 10 μ L of HEPES buffer containing polymer at the indicated N/P ratio and incubating at RT for 30 min. N/P ratio was calculated as a ratio between the positively charged groups of a polymer (amines - N) and negatively charged nucleic acids such as siRNA (phosphates - P). N/P molar ratios were calculated using moles PLL-PEG primary amines (PLL10-PEG: 1.5 mmol 1' amine/mol polymer; PLL50-PEG: 3.79 mmol 1' amine/mol polymer) or moles total nitrogen (PEI-PEG: 2.65 mmol N/g polymer; BDNG: 4.4 mmol N/g polymer) to moles siRNA phosphates (42 mol phosphate/mol model siRNA). Polyplexes were loaded (10 μ L) on a 1% TBE agarose gel (UltraPure™ Agarose-1000, Invitrogen/ethidium bromide [0.5 μ g/mL]), run at 120 V for 15 min, and imaged under UV transillumination using a Molecular Imager® ChemiDoc™ XRS (BioRad, Hercules, CA).

2.4 Model siRNA displacement assay

Model siRNA polyplexes were formed as described in 2.3 *Model siRNA loading* at the minimum N/P ratio required for complexation, then incubated with 5 μ L of heparin sodium (196 U/mg Sigma, St. Louis, MO; 10 to 100 μ g heparin sodium/mL in 0.1 M HEPES buffer,

pH 7.4) for 30 minutes at room temperature and analyzed on an agarose gel alongside model siRNA or chol-model siRNA alone.

2.5 Model siRNA nuclease protection assay

Model siRNA polyplexes were formed as described in 2.3 *Model siRNA loading* at minimum N/P ratio required for model siRNA complexation, then incubated with 1 μ L 1U/mL Turbo DNase (Ambion, Austin, TX) or 1 μ L 0.1 M HEPES [pH 7.4] for 1 hr at 37°C. DNase was inactivated by heating the solutions at 90°C for 15 minutes in the presence of 15 mM EDTA. Model siRNA was released by adding 4 μ L of 1000 U heparin/mL (20 IU heparin/ μ g model siRNA) and run on an agarose gel as described in 2.3 *Model siRNA loading*. Model siRNA (dsDNA) bands from treated polyplexes were quantitated by densitometry (Quantity One® software, BioRad) and normalized to bands from untreated polyplexes at the same N/P ratio. Percent protected model siRNA was expressed as [(average density of band from treated complex)/(average density of band from the untreated complex of the same polymer)]*100.

2.6 Polyplex hydrodynamic diameter by dynamic light scattering

The hydrodynamic diameters of model siRNA and chol-model polyplexes in 0.1 M HEPES [pH 7.4] at 1 mg polymer/mL and indicated N/P ratio were measured by Dynamic Light Scattering (DLS) using a ZetaSizer Nano ZS (Malvern Instruments, Malvern, UK) equipped with He-Ne laser ($\lambda = 633$ nm) as the incident beam. Average polyplex diameters ($n=3$ measurement $\pm$ SD) with model siRNA and chol-model siRNA were compared by unpaired t-test ($P < 0.05$).

2.7 Isolation of Immortomouse mammary cells

Isolation procedures were performed with slight modification [18] and approved by IACUC following appropriate guidelines. Surgical instruments were sterilized O/N in Spectra-Soak™, then rinsed with Mince Buffer (HBSS, Amphotericin B [5.0 μ g/mL], Gentamycin [50 μ g/mL], Penicillin G [100 U/mL], Streptomycin Sulfate [100 μ g/mL]) at least 10 minutes before use. Four nulliparous, 6 to 8 week old homozygous female H-2K^b-ts-A58 mice (Immortomouse®, Charles River Labs, Wilmington, MA) were euthanized, then hair from the dorsal side was shortened with an electric shaver and Nair® was applied for 4 minutes before removal with a sterile sponge. Nair®-treated areas were sprayed with 70% ethanol and remaining hair removed with a new sterile sponge. Mammary fat pad was resected and pooled into a 100 $\times$ 20 mm culture dish with 10 mL of Mince Buffer, moved to a fresh 100 $\times$ 20 mm culture dish containing 10 mL of Mince Buffer and cut with new sterile scalpels into 1 mm sized fragments. Fragments were incubated in 10 mL of 0.2% Collagenase Type 1 (Worthington Biochemical Co., Lakewood, NJ) for 60 minutes at 37°C then centrifuged at 400g for 10 min. The pellet was washed 2X with 20% Complete DMEM (DMEM, FBS 20% [Atlanta Biologicals (Atlanta, GA), endotoxin <0.3 EU scale, heat-inactivated by incubation at 56°C for 30 minutes and cooling in an ice bath], 1mM L-glutamine, 2 mM Glutamax™, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 1X vitamins, 5.0 μ g/mL amphotericin B [Fungizone™], 50 μ g/mL gentamycin, 100 U/mL penicillin G, 100 μ g/mL streptomycin sulfate), resuspended in 2 mL Complete DMEM, and cultured at 33°C, 5% CO₂, in a single well of a 6-well plate precoated with 1% gelatin in D-PBS. Unlike previous studies [19–22], IFN- γ was not added to avoid possible long term effects on MVEC surface and function. Colonies were observed 10 to 20 d later. For routine expansion, cells were cultured in 10% FBS Complete DMEM at 33°C, 5% CO₂, in 0.2% gelatin-coated flasks and passaged at no greater than a 1/4 split using Accutase™ (Sigma, St. Louis, MO).

2.8 Isolation of Immortomouse mammary MVEC

Mammary MVEC were isolated from heterogeneous populations of homozygous Immortomouse[®] mammary cells by FACS, double staining for TNF- α -inducible (20 μ g murine TNF- α (Peprotech)/mL at 33°C for 4–6 hours) V-CAM-1/E-Selectin expression [18] using FITC-labeled rat anti-mouse CD106/VCAM1 (1.5 μ g/ 1×10^6 cells; Clone M/K-2: Southern Biotech, Birmingham, AL) and R-PE-conjugated Rat anti-mouse CD62E/E-Selectin (3.0 μ g/ 1×10^6 cells; Clone 10E9.6: BD Biosciences, San Jose, CA). Sorted cells were plated in a single well of a 6-well plate coated with 0.2% gelatin in DPBS and passaged upon confluence as described for mammary cells and not used beyond passage 20.

2.9 Matrigel assay

Matrigel Matrix High concentration (4 mg/mL in DMEM; BD Biosciences) was thawed on ice at 4°C overnight. The next day, 50 μ L of Matrigel was added with ice-cooled tips to each well of an ice-cooled 96-well plate and incubated at 37°C for 1 hour. Mammary MVEC were then added to Matrigel at 2×10^4 cells in 10% Complete DMEM (50 μ L, 4×10^5 cells/mL) and grown at 37°C.

2.10 Suppression of CYPB mRNA in mammary MVEC by siRNA polyplexes

Homozygous Immortomouse mammary MVEC were seeded in a 24-well plate at 20,000 cells/well and incubated at 33°C 24 h before transfection. On the day of transfection, polymers were sterilized under vacuum for 2 hrs in a desiccator containing a glass dish of 95% alcohol, resuspended in 0.1 M HEPES buffer (pH 7.4), sonicated for 10 min., and spun at 16,100g for 10 min. A stock solution of siRNA (20 μ M) was added to different stock solutions of polymer to form siRNA polyplexes at the appropriate N/P ratio and mixed. The mixture was incubated at RT for 30 min., then diluted 1/100 in 10% Complete DMEM to give a final concentration of 100 nM siRNA. Mammary MVEC were incubated with 500 μ L of polymer-siRNA complexes (2.5×10^{-15} mol siRNA/cell) for 4 hours and media replaced with Complete DMEM. After 44 hrs (48 hrs from starting treatment), total RNA was isolated using the FastLane cDNA kit (Qiagen, Valencia, CA) according to manufacturer's instructions. RNA from two independent treatments was combined and quantitated by measuring A_{260} using a BioPhotometer (Eppendorf, Hamburg, Germany). All samples were normalized to the lowest concentration of RNA obtained. Complementary DNA (cDNA) was synthesized from 4 μ L of the normalized RNA samples using FastLane cDNA kit according to manufacturer's instructions. Relative levels of CYPB mRNA were determined from cDNA by real time PCR (iCycler iQ[™] Real Time PCR Detection System, BioRad) using murine GAPDH and CYPB primer assays (Qiagen) and QuantiFast[™] SYBR[®] Green PCR kit (Qiagen) according to manufacturer's instructions. Amplified bands were verified by T_m (melting curves) and size (agarose gel). Levels of CYPB in cells treated with polyplexes containing active siRNA were normalized to the levels of CYPB in cells treated with polyplexes containing single non-targeting siRNA and calculated using the $2^{-\Delta\Delta C_t}$ method [23] and expressed as percent relative suppression of CYPB mRNA. Differences between CYPB siRNA polyplexes and chol-CYPB siRNA polyplexes (n=2) mRNA suppression were determined by one way ANOVA and the Tukey-Kramer multiple comparison test.

2.11 Relative suppression of CYPB mRNA in mammary MVEC by electroporation

Homozygous Immortomouse mammary MVEC (70–80% confluent) were electroporated with single CYPB siRNA, pooled CYPB siRNA, chol-CYPB siRNA or single non-targeting siRNA using a Nucleofector (Lonza AG, Basel, Switzerland) on setting T-023 and a Basic Nucleofector Kit for Mammalian Endothelial Cells (VPI-1001) with 300 nM siRNA and 5×10^6 cells/mL (6×10^{-17} mol siRNA/cell) according to manufacturer's instructions. Relative

levels of CYPB mRNA were determined after 48 hours as described in *2.10 Suppression of CYPB mRNA in mammary MVEC by siRNA polyplexes*.

2.12 Cytotoxicity of siRNA polyplexes in mammary MVEC

Cells were treated in the same manner as described under *2.10 Relative suppression of CYPB mRNA in mammary MVEC by polyplexes* using polyplexes of single non-targeting siRNA except that total live cell count and percent viability was determined 48 hrs after transfection with Trypan Blue using a Cellometer Auto T4 (Nexcelom Bioscience, Lawrence, MA) according to manufacturer's instructions. Differences in cell count and percent cells excluding trypan blue were assessed by one way ANOVA and Dunnett's post test vs. cells only.

3. Results

3.1 Formation of chol-siRNA polyplexes

siRNA polyplex designs are commonly based on the self assembly of positively charged polymers (amines - N) and negatively charged nucleic acids such as siRNA (phosphates - P) formed at different ratios of polymer (N) to nucleic acid (P) (N/P ratios) [12]. Nucleic acids frequently have higher activity in polyplexes formed at N/P ratios that produce neutralized/electropositive polyplexes. As such, any change in the N/P ratio that forms neutralized/electropositive siRNA polyplexes may affect subsequent mRNA suppression.

To determine whether chol-siRNA significantly alters self assembly, the minimum N/P ratios required to form neutral/electropositive polyplexes of model siRNA and chol-model siRNA were compared (Fig. 1). Polyplexes were formed from three general types of conventional, positively charged polymer constructs: (i.) biodegradable, cross-linked nanogels (ii.) graft copolymers and (iii.) linear block copolymers (Table 1). The specific polymers were chosen because they were expected to minimally form stable polyplexes in solution (PLL10-PEG and PLL50-PEG) or form stable polyplexes that may potentially increase mRNA suppression (BDNG and PEI-PEG) through the presence of PEI [24,25]. Given that dsDNA is comparatively inexpensive, less susceptible to background nuclease activity, and has an equivalent charge density and similar structure to siRNA (dsRNA), a model siRNA composed of 19 bp dsDNA with two 3' nucleotide overhangs on each strand and a model siRNA modified with 3' cholesterol on one of the DNA strands (chol-model siRNA) were used as analogs of siRNA and chol-siRNA, respectively, for all characterization studies.

BDNG and PEI-PEG neutralized model siRNA at N/P ratios ≥ 6 (BDNG: 18.37 wt% and PEI-PEG: 11.9 wt% siRNA), whereas both PLL-PEG polymers neutralized model siRNA at N/P ratios ≥ 2 (PLL10-PEG: 18.7 wt% and PLL50-PEG: 36.7 wt% siRNA) (Fig. 1A) as polyplex migration was observed at N/P ratios < 2 (data not shown). The same patterns were observed with polyplexes of chol-model siRNA (Fig. 1B). Thus, chol-model siRNA does not increase the minimum N/P ratio to form neutral/electropositive siRNA polyplexes.

It remains possible that chol-model siRNA decreases the minimum N/P ratio for neutralizing siRNA by less than the 1 molar N/P increment used in this assay. As such, chol-siRNA may decrease the N/P ratio to form neutral/electropositive siRNA polyplexes but cannot be determined from these results. The same minimum N/P ratios for siRNA and chol-siRNA, however, formed neutral/electropositive polyplexes and, as such, were suitable for use in subsequent studies.

3.2 Resistance of chol-siRNA polyplexes to displacement by heparin

Polyanions disassemble DNA polyplexes *in vitro* [26,27]. Thus, polyanions attached to cell surface proteoglycans, such as heparan sulfate, have the potential to disassemble siRNA polyplexes, leading to the release of siRNA upon interactions with the interior of blood vessels or other cell surfaces.

To determine whether modification of siRNA with 3' cholesterol increases the resistance of siRNA polyplexes to disassembly by polyanions, the concentration-dependent displacement of model siRNA and chol-model siRNA from polyplexes by a model of heparan sulfate, heparin, was compared at the minimum N/P ratios to form neutral/electropositive siRNA polyplexes (Fig. 2). The pattern of heparin displacement of model siRNA from each polyplex (Fig. 2A) was similar to the pattern of heparin displacement of chol-model siRNA (Fig. 2B). Furthermore, the weight ratio of heparin to siRNA that completely released model siRNA and chol-model siRNA from each polyplex was the same: 1.25:1 for BDNG, PLL10-PEG, and PLL50-PEG (Fig. 2A and 2B) and 2:1 for PEI-PEG (data not shown). Thus, chol-model siRNA does not increase the resistance of these polyplexes to the displacement of siRNA by heparin.

It remains possible that chol-model siRNA decreases the weight ratio of heparin to siRNA for complete siRNA release by less than the heparin increment used in this assay. Thus, chol-siRNA may decrease the resistance of polyplexes to siRNA release by heparin but cannot be determined from these results.

3.3 Chol-siRNA polyplex protection against nuclease degradation

Systemic nucleases greatly decrease the plasma half life of siRNA [28]. Thus, it is important to form polyplexes that provide long term protection from nuclease degradation to allow time for the polyplexes to accumulate in the target cells after systemic administration. Given that the hydrophobic 3' cholesterol moieties are likely to interact, it is possible that the arrangement of siRNA within chol-siRNA polyplexes will be different and change polyplex protection against nuclease activity.

To determine whether modifying siRNA with 3' cholesterol changes polymer protection of siRNA from nuclease degradation under maximal loading conditions, polyplexes were formed with model siRNA or chol-model siRNA at the minimum N/P ratio required to form neutral/electropositive polyplexes and resistance to nuclease activity over 24 hours was compared (Fig. 3). Model siRNA and chol-model siRNA were completely degraded within an hour under these conditions (data not shown) and were used to more easily identify differences in nuclease protection by each polyplex.

Chol-model siRNA increased the duration of nuclease protection by PLL10-PEG polyplexes the most (at least 8 hours) as model siRNA was undetectable after 4 hours (Fig. 3A), whereas chol-model siRNA was undetectable after sometime between 12 and 24 hours (Fig. 3B). The duration of nuclease protection by BDNG also increased (at least 6 hours), as model siRNA was undetectable after 6 hours, whereas chol-model siRNA was undetectable after sometime between 12 and 24 hours. The duration of nuclease protection by PLL50-PEG was unchanged over the course of the study due to high levels of protection for both unmodified and chol-model siRNA, but the extent of chol-model siRNA protected after 24 hours increased 19 % ($71 \pm 2\%$ model siRNA vs. $90 \pm 4\%$ chol-model siRNA, $n=2$, $P < 0.01$). In contrast, chol-model siRNA decreased the duration of nuclease protection by PEI-PEG (Fig. 3).

Considering that the amount of protected model siRNA or chol-model siRNA did not change over 24 hours, it is unclear whether increased nuclease protection by chol-siRNA

PLL50-PEG polyplexes is due to a slower rate of siRNA degradation or an increase in siRNA that is inaccessible to nuclease activity. Thus, chol-siRNA selectively increases the duration and/or, in the case of PLL50-PEG, the extent of nuclease protection by these polyplexes at the minimum N/P ratio to form neutral/electropositive polyplexes.

3.4 Chol-siRNA effect on polyplex hydrodynamic diameters

Given that the hydrophobic 3' cholesterol moieties of chol-siRNA are likely to interact within chol-siRNA polyplexes, the arrangement of “soft nanomaterials” such as siRNA polyplexes may be altered and reflected as a change in the hydrodynamic diameter. Chol-model siRNA decreased the hydrodynamic diameters of PEI-PEG, PLL10-PEG, and PLL50-PEG polyplexes but increased the hydrodynamic diameter of BDNG siRNA polyplexes (Table 2). Thus, chol-siRNA changes the hydrodynamic diameters of these siRNA polyplexes.

3.4 Cytotoxicity and suppression of native mRNA in mammary MVEC by chol-siRNA polyplexes

Unlike free siRNA, free chol-siRNA is active *in vitro* [14] and *in vivo* [17]. Thus, we hypothesized that complexation of chol-siRNA can increase the suppression of mRNA by siRNA polyplexes. To provide a model target cell that is similar to primary cells and potentially decrease variability between studies *in vitro* and future studies with murine models *in vivo*, murine MVEC were isolated from the mammary fat pad of the Immortomouse strain [19]. The Immortomouse strain carries a temperature-sensitive SV40 Large T antigen under the control of the H-2K^b promoter (activated by interferon- γ [IFN- γ]), which can be used to conditionally immortalize isolated primary cells and decrease the number of primary cell isolations when grown at permissive temperature (33°C). MVEC [18] and other cell types [20,21] isolated from various Immortomouse[®] tissues have also been shown to retain the functional phenotype of the original primary cells after at least 30 passages.

Mammary MVEC were isolated from the female homozygous Immortomouse[®] strain by FACS, double selecting for the TNF- α induced expression of E-Selectin (CD62E) and VCAM-1 (CD106) [18]. IFN- γ was not added during any stage of isolation to avoid possible long term effects on mammary MVEC surface and function. A cobblestone morphology (Fig. 4A) and capillary-like structure in Matrigel (Fig. 4B) characteristic of vascular endothelial cells [18] were observed. Cells were not used beyond passage 20 at 33°C as a decrease in growth rate was consistently observed after this passage (data not shown).

To determine whether chol-siRNA increases suppression of native mRNA, Cyclophilin B (CYPB) mRNA levels in mammary MVEC treated with polyplexes of CYPB siRNA (single siRNA or pool of 4 siRNA constructs) or chol-CYPB siRNA (same sequence as single CYPB siRNA but with 3' cholesterol on sense strand) were compared to CYPB mRNA levels in mammary MVEC treated with polyplexes containing single non-targeting siRNA (Fig. 5A). CYPB was chosen as a model of native mRNA because it is expressed at high levels and knockout does not affect cell viability [29].

Varied levels of CYPB mRNA suppression were observed with single or pooled CYPB siRNA polyplexes that did not exceed 40% (Fig. 5A), whereas single and pooled CYPB siRNA alone suppressed 88–90% CYPB mRNA by electroporation (Fig. 5B). Chol-CYPB siRNA increased suppression of CYPB mRNA by BDNG polyplexes (35%, $P < 0.05$, $n=2$) and PLL10-PEG (69%, $P < 0.001$, $n=2$) over equivalent CYPB siRNA polyplexes but had no statistical effect on PEI-PEG or PLL50-PEG polyplexes (Fig. 5A). In contrast, chol-CYPB siRNA alone suppressed 30% CYPB mRNA by transfection (Fig. 5A) and 10% by

electroporation (Fig. 5B). Each chol-CYPB siRNA polyplex had low level cytostatic effects but little or no cytotoxicity compared to mammary MVEC alone or mammary MVEC treated with chol-CYPB siRNA under the same transfection conditions (Fig. 6). Thus, chol-siRNA increases suppression of native mRNA by select siRNA polyplexes in mammary MVEC without increasing cytotoxicity.

4. Discussion

This study provides evidence that chol-siRNA increases the suppression of native mRNA by select siRNA polyplexes. Chol-siRNA increased the suppression of CYPB mRNA in conditionally immortalized mammary MVEC by PLL10-PEG and BDNG siRNA polyplexes but did not change suppression by PEI-PEG or PLL50-PEG siRNA polyplexes (Fig. 5A). Differences in mRNA suppression between PLL10-PEG (88% suppression) and PLL50-PEG (30% suppression) further suggest that polycationic block length is an important factor in mRNA suppression by PLL-PEG chol-siRNA polyplexes. This study also shows that chol-siRNA selectively increases nuclease protection by siRNA polyplexes. The duration and/or extent of nuclease protection was increased for BDNG, PLL10-PEG and PLL50-PEG chol-siRNA polyplexes but decreased for PEI-PEG chol-siRNA polyplexes (Fig. 3).

Changes in mRNA suppression by chol-siRNA polyplexes

There are at least three possible mechanisms that may singly or collectively explain why select chol-siRNA polyplexes suppress more mRNA than siRNA polyplexes within 48 hours. A first possibility is that, unlike unmodified CYPB siRNA, chol-CYPB siRNA is active after being released from siRNA polyplexes by serum [30] or by polyanions attached to proteoglycans on the surface of mammary MVEC. This would suggest that chol-siRNA is completely released from BDNG, PEI-PEG, and PLL50-PEG chol-CYPB siRNA polyplexes which had similar levels of CYPB mRNA suppression as free chol-CYPB siRNA (30 to 40% suppression) (Fig. 5A). PLL10-PEG chol-siRNA polyplexes, however, suppressed more CYPB mRNA (88% suppression) than free chol-CYPB siRNA or the other chol-siRNA polyplexes (Fig. 5A) but had similar resistance to heparin displacement of chol-siRNA as BDNG and PLL50-PEG polyplexes (Fig. 2). Although the relationship between the release of chol-siRNA by heparin and release by serum or the surface of mammary MVEC has not been established, this suggests that the same amount of chol-siRNA will be released by PLL10-PEG polyplexes during transfection as the BDNG and PLL50-PEG polyplexes. Thus, a mechanism where chol-siRNA is only active after being completely released by serum or by cell surface proteoglycans seems unlikely to fully explain differences with chol-siRNA polyplexes.

A second possibility is that chol-siRNA increases the rate of polyplex uptake by mammary MVEC. Given that mRNA suppression is a kinetic phenomenon and was assessed at only a single time point, differences in the kinetics of uptake cannot be ruled out.

A third possibility is that, in addition to possible differences in release and uptake of free chol-siRNA, there are differences in the release rates of chol-siRNA from polyplexes within the endosomes. We found evidence that suggests chol-siRNA requires polymer complexation for maximal mRNA suppression within the 48 hour time frame as free chol-CYPB siRNA suppressed 30% of CYPB mRNA by transfection (Fig. 5A) and 10% by electroporation (Fig. 5B) but 88% when transfected in PLL10-PEG polyplexes (Fig. 5A). These results suggest that the 3' cholesterol moieties of chol-siRNA stick to the cell surface and limit the amount of free chol-siRNA that is endocytosed (transfection) or diffused through transient holes in the cell membrane (electroporation). In contrast, assuming chol-siRNA is not displaced by serum or at the cell surface (or, at least, not to a large extent), polyplexes can potentially limit the interaction of chol-siRNA with the cell surface and carry

a greater amount of chol-siRNA into the endosomes. Endocytosed chol-siRNA, however, must be released from the polyplexes or able to interact with the endosomal membrane and/or, possibly, intracellular transporters that traffic cholesterol from the endosomes to the endoplasmic reticulum [31] before it can escape the endosomes. Consistent with this possibility, PLL10-PEG suppressed more mRNA than PLL50-PEG chol-siRNA polyplexes (Fig. 5) and was more accessible to nuclease activity than PLL50-PEG (Fig. 3). This suggests that chol-siRNA in PLL10-PEG polyplexes is better able to interact with and be released from the endosomes than chol-siRNA in PLL50-PEG polyplexes. Thus, increased activity by chol-CYPB siRNA polyplexes may be through a combination of released and endocytosed chol-siRNA, but is potentially the most active when endocytosed by polyplexes. These results further suggest that the cationic block length is an important factor for mRNA suppression by PLL-PEG chol-siRNA polyplexes.

Changes in nuclease protection by chol-siRNA polyplexes

The duration and/or extent of nuclease protection was increased for BDNG, PLL10-PEG and PLL50-PEG chol-siRNA polyplexes but decreased for PEI-PEG chol-siRNA polyplexes (Fig. 3). Differences in nuclease protection by siRNA and chol-siRNA polyplexes were not due to differences in nuclease activity against model siRNA and chol-model siRNA as both were completely degraded within an hour under the same assay conditions. The most likely explanation is that that chol-siRNA changes the arrangement of siRNA polyplexes which, in turn, changes in the accessibility of complexed siRNA to nuclease activity. This is supported by changes between the hydrodynamic diameters of siRNA and chol-siRNA polyplexes (Table 2) which suggest changes in the arrangement of siRNA polyplexes by chol-siRNA. Thus, interactions between 3' cholesterol moieties of complexed chol-siRNA change the arrangement of siRNA polyplexes to make siRNA less (increased protection by BDNG, PLL10-PEG, and PLL50-PEG chol-siRNA polyplexes) or more (decreased protection by PEI-PEG chol-siRNA polyplexes) accessible to nuclease activity.

For the PLL-PEG block copolymers, it is possible that chol-siRNA forms siRNA polyplexes with better defined core-shell morphologies. Thus, a better defined shell of PEG may more effectively interfere with nuclease accessibility to siRNA within the core of the polyplex. A similar formation of core-shell polyplexes is observed with complexes of cholesterol-modified polycationic polymers and DNA [32]. For the PEI-PEG graft copolymer, it is possible that chol-siRNA has the opposite effect and interferes with the formation of well defined core-shell morphologies, leading to decreased protection against nuclease activity. Thus, for some polymers like PLL10-PEG, chol-siRNA may form better defined core shell polyplexes but with the added advantage of increased mRNA suppression.

CONCLUSIONS

In summary, our results indicate that chol-siRNA increases nuclease protection and mRNA suppression by select siRNA polyplexes. Thus, complexation of chol-siRNA may, ultimately, be a simple approach to improve the systemic administration of siRNA by select siRNA polyplexes through increased plasma protection and mRNA suppression at the target tissue.

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Figure 1. Minimum N/P ratio for polymer complexation of model siRNA and chol-model siRNA (A) Model siRNA (dsDNA analog of siRNA) or (B) model siRNA modified with 3' cholesterol on a single DNA strand (chol-model siRNA) were incubated with the indicated N/P ratio of polymer in 0.1 M HEPES [pH 7.4] for 30 minutes at room temperature and run on a 1% agarose/ethidium bromide gel alongside model siRNA or chol-model siRNA alone (Lane 0). The first N/P ratio where polyplexes are retained in the well indicates charge neutralization of siRNA and is defined as the minimum N/P ratio required for complexation. Data are representative of two independent experiments.

Figure 2. Heparin displacement of model siRNA and chol-model siRNA from polyplexes (A) Model siRNA (dsDNA analog of siRNA) or (B) model siRNA modified with 3' cholesterol on a single DNA strand were incubated with polymer in 0.1 M HEPES, pH 7.4 for 30 minutes at room temperature at the indicated minimum N/P ratio required for complexation. Complexes were then incubated with the indicated weight ratio of heparin to siRNA for 30 minutes at room temperature and run on a 1% agarose gel/ethidium bromide gel with model siRNA or chol-model siRNA alone (lane C). Data are representative of two independent experiments.

Figure 3. Polyplex protection of model siRNA and chol-model siRNA from nuclease activity (A) Model siRNA (dsDNA analog of siRNA) or (B) model siRNA modified with 3' cholesterol on a single DNA strand was incubated with polymer for 30 minutes at room temperature at the indicated minimum N/P ratio required for complexation then incubated in the absence or presence of DNase for the indicated time. Model siRNA and chol-model siRNA were completely degraded within one hour under the same conditions (data not shown). DNase was inactivated and remaining model siRNA was released from the polyplexes by heparin and separated on a 1% agarose/ethidium bromide gel. Bands from DNase-treated polyplexes were quantitated by densitometry and normalized to bands from untreated polyplexes at the same N/P ratio. Percent protected model siRNA $\pm$ SD (n=2) is an average of two independent experiments.

Figure 4. Homozygous Immortomouse mammary MVEC
Phase contrast images of mammary MVEC grown at 33°C (A) in 2D (0.2% gelatin) and (B) in 3D (Matrigel) culture.

Figure 5. Suppression of native CYPB mRNA in homozygous Immortomouse mammary MVEC by CYPB siRNA and chol-CYPB siRNA polyplexes 48 hours after transfection

(A) *Transfection*: Conditionally immortalized mammary MVEC (grown at 33°C) were transfected in 10% Complete DMEM for 4 hours with polymer-siRNA complexes (BDNG N/P 6, PEI-PEG N/P 6; PLL-PEG N/P 2) containing single, pooled (4 CYPB siRNA constructs), or cholesterol CYPB siRNA (3' sense strand) at 100 nM siRNA (2.5×10^{-15} mol siRNA/cell) then media was replaced with 10% Complete DMEM. CYPB mRNA levels $\pm$ SD (n=2) in treated cells were compared to CYPB mRNA from cells treated with the same polyplexes containing single non-targeting siRNA after 48 hours by QRT-PCR ($2^{-\Delta\Delta C_t}$ method [23] normalized to GAPDH). "No polymer" is chol-CYPB siRNA alone vs. CYPB siRNA alone. Differences between CYPB siRNA polyplexes and chol-CYPB siRNA polyplexes were determined by one way ANOVA and the Tukey-Kramer multiple comparison test for (* $P < 0.05$; *** $P < 0.001$). (B) *Electroporation*: mammary MVEC were electroporated with 300 nM of siRNA (6×10^{-17} mol siRNA/cell) and CYPB mRNA levels $\pm$ SD (n=2) in CYPB siRNA treated cells were compared to CYPB mRNA from cells treated with single non-targeting siRNA after 48 hours by Q-RT-PCR ($2^{-\Delta\Delta C_t}$ method normalized to GAPDH). Results are representative of two independent experiments.

Figure 6. Cytostatic and cytotoxic effects of chol-siRNA polyplexes in homozygous Immortomouse mammary MVEC 48 hours after transfection

Conditionally immortalized mammary MVEC (grown at 33°C) were transfected in 10 % Complete DMEM for 4 hours with or without chol-CYPB siRNA alone (Chol-CYPB) or polymer-siRNA complexes at 100 nM chol-CYPB siRNA (2.5×10^{-15} mol siRNA/cell) then media was replaced with 10% Complete DMEM. Average ($n=2 \pm SD$) total (black bars) and percent viable (white bars) cells were determined by cell counting with trypan blue exclusion after 48 hours. One way ANOVA and Dunnett's post test vs. cells only (* $P < 0.05$).

Table 1
Polymer constructs in this study

Polymer	Description	Ref
BDNG	Biodegradable PEI (28 kDa PEI: 2 kDa PEI linked by disulfide bonds) cross-linked with 8 kDa PEG through carbamate bonds Graft copolymer of branched polyethylenimine (2 kDa) and	[16]
PEI-PEG	poly(ethylene)glycol (10 kDa)	[17]
pLL10-PEG	Block copolymer of poly-L-lysine (10 Lys) and PEG (5 kDa)	Alamanda Polymers
pLL50-PEG	Block copolymer of poly-L-lysine (50 Lys) and PEG (5 kDa)	Alamanda Polymers

Table 2
Hydrodynamic diameters of model siRNA and chol-model siRNA polyplexes

Values are an average $\pm$ SD (n=3).

Polyplex	Hydrodynamic Diameter (nm $\pm$ SD)	
	<i>Model siRNA</i>	<i>Chol-model siRNA^a</i>
BDNG (N/P 6)	114 $\pm$ 2	169 $\pm$ 2
PEI-PEG (N/P 6)	41 $\pm$ 1	34 $\pm$ 2
pLL10-PEG (N/P 2)	81 $\pm$ 1	54 $\pm$ 0.4
pLL50-PEG (N/P 2)	120 $\pm$ 2	66 $\pm$ 4

^a $P < 0.05$ vs. polyplex diameter with model siRNA by unpaired t-test