Published in final edited form as:

Int J Pharm. 2018 May 30; 543(1-2): 130–138. doi:10.1016/j.ijpharm.2018.03.045.

Complexation of Chol-DsiRNA in place of Chol-siRNA greatly increases the duration of mRNA suppression by polyplexes of PLL(30)-PEG(5K) in primary murine syngeneic breast tumors after i.v. administration

Vishakha V. Ambardekar^f, Rajesh R. Wakaskar^g, Zhen Ye^b, Stephen M. Curran^b, Timothy R. McGuire^c, Don W. Coulter^d, Rakesh K. Singh^{a,e}, and Joseph A. Vetro^{a,b,*}

^aCenter for Drug Delivery and Nanomedicine, College of Pharmacy, University of Nebraska Medical Center, Omaha, Nebraska 68198-6025, USA

^bDepartment of Pharmaceutical Sciences, College of Pharmacy, University of Nebraska Medical Center, Omaha, Nebraska 68198-6025, USA

^cDepartment of Pharmacy Practice, College of Pharmacy, University of Nebraska Medical Center, Omaha, Nebraska 68198-6025, USA

^dDepartment of Pediatrics, Division of Pediatric Hematology/Oncology, Department of Radiation Oncology, J. Bruce Henriksen Cancer Research Laboratories, University of Nebraska Medical Center, Omaha, NE 68198-2168, USA

^eDepartment of Pathology and Microbiology, University of Nebraska Medical Center, Omaha, NE 68198-5900, USA

Abstract

RNA interference has tremendous potential for cancer therapy but is limited by the insufficient potency of RNAi molecules after i.v. administration. We previously found that complexation with PLL(30)-PEG(5K) greatly increases the potency of 3'-cholesterol-modified siRNA [Chol-siRNA] in primary murine syngeneic 4T1 breast tumors after i.v. administration but mRNA suppression decreases 24 hours after the final dose. We hypothesized that complexation of cholesterol-modified Dicer-substrate siRNA (Chol-DsiRNA) in place of Chol-siRNA can increase the potency and duration of suppression by polyplexes of PLL(30)-PEG(5K) in solid tumors. We found that replacing Chol-siRNA with Chol-DsiRNA increased polyplex loading and nuclease protection, suppressed stably expressed luciferase to the same extent in primary murine 4T1-Luc breast tumors under the current dosage regimen, but maintained suppression 72 hours after the final dose.

* Corresponding Author: 1-(402)-559-9359; Fax: 1-(402)-559-9365; jvetro@unmc.edu, Address: 986025 Nebraska Medical Center, WSH 3026A, Omaha, Nebraska 68198-6025.

^fCurrent address: Lupin Ltd, 46/47, A, Village Nande, Taluka Mulshi Dist. Pune 412 115, India

^gCurrent address: INSYS Therapeutics, 444 S Ellis St and 410 S Benson Ln, Chandler, AZ 85224, USA

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Declaration of Competing Interests

JAV is cofounder of a company that currently licenses the described technology.

The kinetics of suppression in 4T1-Luc over 72 hours, however, were similar between DsiLuc and siLuc after electroporation and between polyplexes of Chol-DsiLuc and Chol-siLuc after transfection, suggesting that Chol-DsiRNA polyplexes increase the duration of mRNA suppression through differences in polyplex activities *in vivo*. Thus, replacing Chol-siRNA with Chol-DsiRNA may significantly increase the duration of mRNA suppression by polyplexes of PLL(30)-PEG(5K) and possibly other PEGylated polycationic polymers in primary tumors and metastases after i.v. administration.

Graphical Abstract

Keywords

RNA interference; DsiRNA; drug delivery; Chol-DsiRNA polyplexes; Chol-DsiRNA polymer micelles; Chol-siRNA polyplexes; Chol-siRNA polymer micelles

1. Introduction

RNA interference (RNAi) is a natural, intracellular process that selectively decreases the expression of any specific protein at the mRNA level through the cytosolic localization of gene-specific dsRNA molecules including microRNA (miRNA), small, interfering RNA (siRNA), or longer dicer-substrate siRNA (DsiRNA) (Kim and Rossi, 2007). Thus, RNAi molecules have tremendous potential in the treatment of cancer where the suppression of individual or multiple proteins can produce a therapeutic effect and/or greatly improve the efficacy of current cancer treatments.

Many clinical applications such as cancer therapy require i.v. administration to achieve a therapeutic effect. The potencies of miRNA, siRNA, or DsiRNA after i.v. administration, however, are extremely low or undetectable due to a relatively short plasma half-life (Morrissey et al., 2005), minimal cellular uptake, and a limited ability to escape the endosomes / lysosomes into the cytosol (Aigner, 2008; Howard, 2009; Whitehead et al., 2009).

Modifying the sense strand of nuclease-resistant siRNA with 3'-cholesterol (Chol-*siRNA) increases the activity of siRNA in the liver and jejunum after i.v. administration but with relatively low potency (50 mg/kg) (Soutschek et al., 2004). We previously found that

complexation with a block copolymer of poly-L-lysine (PLL 30 or PLL 50) and 5 kDa polyethylene glycol [PLL-*b*-PEG(5K)] increases the potency of Chol-*siRNA in primary murine syngeneic 4T1 breast tumors after i.v. administration to BALB/c mice (2.5 mg Chol-siRNA/kg daily for three days) without affecting body weight over the course of the 5-day study (Ambardekar et al., 2013). More recently, complexation with an integrin-targeted PLL(45)-*b*-PEG(12K)-cRGD block copolymer followed by core cross-linking was independently reported to increase the potency of Chol-siRNA (5'-cholesterol on anti-sense strand) in primary human xenogeneic HeLa cervical tumors (ectopic - SQ) after i.v. administration to nude BALB/c mice (three injections of ~1.7 mg Chol-siRNA/kg over two days), although polyplex toxicity during the study or effect of core cross-linking and cRGD modification on the activity of these "Chol-siRNA micelles" was not determined (Oe et al., 2014). Complexation with more hydrophobic p(DMAEMA-co-BMA)-*b*-PEG(5K) copolymers was also recently reported to increase the potency of palmitic acid-modified siRNA (PA-siRNA) in primary human xenogeneic MDA-MB-231 breast tumors after i.v. administration to nude mice (single injection of 1 mg PA-siRNA/kg) without increasing surrogate markers of liver (ALT/AST) or kidney (BUN) toxicity in the blood 48 h after treatment (Sarett et al., 2016). Thus, complexation with variously-modified PEGylated polycationic polymers has great potential to increase the potency of hydrophobically-modified RNAi molecules in solid tumors and metastases.

Although complexation with PLL(30)-*b*-PEG(5K) or PLL(50)-*b*-PEG(5K) greatly increases the potency of Chol-*siRNA in primary murine syngeneic 4T1 breast tumors after i.v. administration, we found that mRNA suppression decreases within 24 hrs after the final dose (Ambardekar et al., 2013), suggesting that high frequency dosing will be required to maintain mRNA suppression. As such, we wanted to identify simple approaches to increase the duration of mRNA suppression by these polyplexes in solid tumors.

Polyplexes of DsiRNA and Lipofectamine® 2000 are ~100-fold more potent and suppress mRNA for a longer duration than polyplexes of siRNA and Lipofectamine® 2000 in a human embryonic kidney cell line (HEK293) (Kim et al., 2005). Given that the increase in the potency and duration of activity with DsiRNA may be due to differences in the activities of the respective DsiRNA and siRNA polyplexes of Lipofectamine® 2000 and not the RNAi molecules themselves, we hypothesized that Chol-DsiRNA polyplexes can increase the duration of mRNA suppression in primary solid tumors over Chol-siRNA polyplexes of PLL(30)-*b*-PEG(5K) after i.v. administration. To test this hypothesis, we compared the extent to which complexation of PLL(30)-PEG(5K) with Chol-DsiRNA in place of Chol-siRNA affects N/P ratio (loading), polyplex diameter, protection from nuclease activity in 90% (v/v) murine serum at 37°C, and mRNA suppression in murine 4T1 breast cancer epithelial cells *in vitro* as well as in primary murine 4T1 breast tumors after i.v. administration.

2. Materials and methods

2.1 Polymer

Block copolymers of methoxy-poly(ethylene glycol)-*b*-poly(L-lysine hydrochloride) with 5 kDa polyethylene glycol (PEG) and poly-L-lysine groups (PLL) blocks of 30 [PLL(30)-

PEG(5K); Avg. MW: 9,900 Da; PDI by GPC: 1 to 1.2; PLL block range: 27 to 33 PLL], or 50 [PLL(50)-PEG(5K); Avg. MW: 13,000 Da; PDI by GPC: 1 to 1.2; PLL block range: 45 to 55 PLL] were obtained from Alamanda Polymers (Huntsville, AL).

2.2. RNAi molecules

siRNA (GE Dharmacon) were 19 bp with 3'-UU overhangs on the sense and antisense strands (Ambardekar et al., 2013). **siCtrl** (5'- UGG UUU ACA UGU CGA CUA A - 3'; MW: 13,278 g/mol); **Chol-siCtrl** (siCtrl modified with 3'-cholesterol on the sense strand through a 6 carbon hydroxyproline linker and purified by HPLC; MW: 13,983 g/mol); **siLuc** (Custom anti-luciferase siRNA generated against CpG-free Luc::Sh (InvivoGen) with the Dharmacon siDESIGN center), 5'-AGA AGG AGA UUG UGG ACU A - 3'; MW: 13,293 g/mol). **Chol-siLuc** (siLuc modified with 3'-cholesterol as described for Chol-siCtrl; MW: 13,998 g/mol);

DsiRNA (GE Dharmacon) were an asymmetric, 25-mer dsRNA with a 3' 2-nucleotide overhang on the antisense strand: **DsiCtrl** (sense: 5' – CGU UAA UCG CGU AUA AUA CGC GUA U – 3', antisense: 5' – AUA CGC GUA UUA UAC GCG AUU AAC G(CA) – 3'; MW: 16,545 g/mol); **DsiLuc** (3' extension of siLuc), sense: 5' – AGA AGG AGA UUG UGG ACU AUG UGG C – 3', antisense: 5' – GCC ACA UAG UCC ACA AUC UCC UUC U(UU) – 3'; MW: 16,553 g/mol); **Chol-DsiCtrl** (DsiCtrl modified with 3'-cholesterol on the sense strand through a 6 carbon hydroxyproline linker and purified by HPLC; MW: 17,250 g/mol); **Chol-DsiLuc** (DsiLuc modified with 3'-cholesterol as described for Chol-DsiCtrl; MW: 17,258 g/mol). Constructs were resuspended in manufacturer's buffer per manufacturer's instructions and stored in aliquots at –80°C.

2.3 Minimum N/P ratio for complexation of DsiRNA and Chol-DsiRNA with PLL-PEG(5K)

Minimum N/P molar ratios for complexation of DsiRNA and Chol-DsiRNA by PLL-PEG(5K) were determined with DsiCtrl or Chol-DsiCtrl as previously described for siRNA and Chol-siRNA (Ambardekar et al., 2013) using moles PLL-PEG(5K) primary amines [PLL(30)-PEG(5K): ~3.03 mmol 1' amines / g polymer; PLL(50)-PEG(5K): ~3.85 mmol 1' amines / g polymer] to moles DsiRNA phosphates (52 mol phosphate / mol DsiRNA or Chol-DsiRNA).

2.4 Hydrodynamic diameter of DsiRNA and Chol-DsiRNA polyplexes

Hydrodynamic diameters (Z-average) of DsiCtrl and Chol-DsiCtrl polyplexes in 0.1 M HEPES [pH 7.4] at 1 mg polymer / mL and indicated N/P ratios were determined 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 as previously described (Ambardekar et al., 2013). Average diameters and polydispersity indices ($n=3$ independent measurements $\pm$ SD) of PLL(30)-PEG(5K) and PLL(50)-PEG(5K) polyplexes were compared by unpaired t-test ($P < 0.05$).

2.5 Protection of DsiRNA and Chol-DsiRNA from serum nuclease activity

Protection of DsiRNA or Chol-DsiRNA polyplexes of PLL-PEG(5K) from nuclease activity in 90% murine serum at 37°C was compared by agarose gel electrophoresis as previously

described for siRNA and Chol-siRNA polyplexes (Ambardekar et al., 2013). Percent protected DsiRNA or Chol-DsiRNA [(average density of band from serum-treated polyplexes / average density of band from the corresponding buffer-treated polyplexes) * 100] $\pm$ SD (n=2) were compared by one-way ANOVA with Tukey's post-test.

2.6 Cell culture

A murine breast cancer epithelial cell line stably expressing firefly luciferase (4T1-Luc) was grown and maintained as previously described (Ambardekar et al., 2013). The average flux per 4T1-Luc cell was greater than recommended for *in vivo* imaging (3620 vs. 500 photons/sec) (Lim et al., 2009).

2.7 Electroporation and transfection of 4T1-Luc cells

Electroporation and polyplex transfection/cytotoxicity studies in 4T1-Luc cells were performed as described (Ambardekar et al., 2013). Average percent relative luciferase activity was expressed as [(average radiance from treated cells / average radiance from electroporation only or untransfected cells) $\times$ 100] $\pm$ SD (n=2 independent treatments for electroporation; n=3 independent treatments for transfection). Differences in average relative luciferase activities between electroporation treatment groups were compared by unpaired t-test at each time point. Differences in average relative luciferase activities between transfection treatment groups were compared 24 hours post-transfection by one-way ANOVA with Tukey's post-test. Percent viability and total live cell count normalized to untransfected 4T1-Luc was determined at 24 h by trypan blue exclusion (Cellometer Auto T4; Nexcelom Biosciences, Lawrence, MA). Percent live cells were calculated as [(avg. total live cells treated with siRNA polyplexes / avg. total live cells without treatment) $\times$ 100] $\pm$ SD. Differences in the average total percentage of live cells were compared by one-way ANOVA with Dunnett's posttest vs. untreated 4T1-Luc.

2.8 Suppression of luciferase expression in primary murine breast tumors of 4T1-Luc

The suppression of luciferase expression in primary murine 4T1-Luc breast tumors after i.v. administration of DsiRNA or Chol-DsiRNA polyplexes of PLL-PEG(5K) was compared by IVIS as described (Ambardekar et al., 2013). All procedures were approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee. When tumor volumes reached ~50 to 100 mm³ (treatment Day 0), vehicle alone (0.1 mL PBS) or vehicle containing the indicated treatment was injected into the tail-vein on days 0, 1 and 2 (n=5 mice). Average daily radiance from 4T1-Luc tumors within each cohort was normalized to average 4T1-Luc tumor radiance from the same cohort on the first day of treatment (Day 0) and expressed as average percent luminescence from 4T1 tumors [(mean average radiance from cohort on indicated day / mean average radiance from same cohort on treatment Day 0) * 100] $\pm$ SEM. Average daily percent luminescence from 4T1-Luc tumors was compared to the first day of treatment within the same cohort by Friedman nonparametric repeated measures ANOVA with Dunn's post-test. Differences between daily average percent luminescence from 4T1-Luc tumors treated with Chol-DsiLuc polyplexes or Chol-*siLuc polyplexes was compared by two-tailed Mann-Whitney nonparametric unpaired t test.

3. Results

3.1 Effect of PLL block length and modification of DsiRNA with 3'-cholesterol on the minimum N/P ratio required to form neutrally charged polyplexes

Complexes of DsiRNA and polycationic polymers (DsiRNA polyplexes), like siRNA polyplexes, are based on the self-assembly of DsiRNA and polymer at different molar ratios of positively charged groups (amines-N) on the polymer to negatively charged groups (phosphates-P) on the DsiRNA (N/P ratio). An N/P ratio for polyplex formation is typically initially chosen by determining the lowest N/P ratio required to form neutrally-charged polyplexes (minimum N/P ratio). A low minimum N/P ratio that still results in an active polyplex is ideal because it requires less polymer and, consequently, increases RNAi molecule loading in the polyplex. This increases the amount of RNAi molecules delivered to target cells by individual polyplexes and decreases the potential for polymer-associated toxicity.

We previously found that increasing the PLL block length of PLL-PEG(5K) from 10 to 50 lysines and modifying the sense strand of siRNA with 3'-cholesterol decreases the minimum N/P ratio for PLL-PEG(5K) to form neutral polyplexes (Ambardekar et al., 2013). To determine whether PLL block length and/or modifying DsiRNA with 3'-cholesterol also affects the minimum N/P ratio for DsiRNA with PLL-PEG(5K), we compared the minimum N/P ratios required to neutralize DsiRNA or Chol-DsiRNA by PLL-PEG(5K) with PLL block lengths of 30 or 50 by agarose gel electrophoresis (Table 1). We started with a PLL block length of 30 instead of 10 because PLL(10)-PEG(5K) does not protect relatively shorter Chol-siRNA from degradation in 90% (v/v) murine serum at 37°C for at least 24 hrs (Ambardekar et al., 2013) and compared whole number N/P ratios because it was experimentally convenient and provided a reasonable estimation of the lowest N/P ratio for complexation.

In contrast to siRNA and Chol-siRNA, increasing PLL block length from 30 to 50 lysines did not affect the minimum N/P ratio required for complexation of DsiRNA or Chol-DsiRNA with PLL-PEG(5K) (N/P 1), whereas modifying DsiRNA with 3'-cholesterol decreased the minimum N/P ratio required for complexation to the same extent at each PLL block length (N/P 3 to N/P 1) (Table 1). Agarose gel band intensities of DsiRNA and Chol-DsiRNA in the absence of PLL-PEG(5K) were statistically similar, indicating that differences in DsiRNA and Chol-DsiRNA loading were not due to differences in respective stock solution concentrations (not shown). Furthermore, Chol-DsiRNA required a lower minimum N/P ratio than Chol-siRNA for complexation with PLL(30)-PEG(5K) (N/P 1 vs. N/P 3) or PLL(50)-PEG(5K) (N/P 1 vs. N/P 2) (Table 1). Thus, with the current N/P ratios and range of PLL block lengths, (i.) modifying DsiRNA with 3'-cholesterol increases DsiRNA loading by decreasing the minimum N/P ratio required for complexation with PLL-PEG(5K), whereas increasing PLL block length has no effect in the presence or absence of 3'-cholesterol and (ii.) replacing Chol-siRNA with Chol-DsiRNA increases loading with PLL(30)-PEG(5K) and PLL(50)-PEG(5K) by decreasing the minimum N/P ratio required for complexation.

3.2 Effect of PLL block length and modification of DsiRNA with 3'-cholesterol on the hydrodynamic diameter and polydispersity of DsiRNA polyplexes of PLL-PEG(5K)

We previously found that increasing PLL block length from 30 to 50 lysines has no effect on the hydrodynamic diameter of Chol-siRNA polyplexes of PLL-PEG(5K) at minimum N/P ratios that form neutral polyplexes (Ambardekar et al., 2013). To determine whether PLL block length and/or modifying DsiRNA with 3'-cholesterol affects the diameter and/or diameter distribution of DsiRNA polyplexes of PLL-PEG(5K), the average hydrodynamic diameters and polydispersity indices of DsiRNA and Chol-DsiRNA polyplexes of PLL(30)-PEG(5K) or PLL(50)-PEG(5K) at the minimum N/P ratios required to form neutral polyplexes (Table 1) were compared by DLS (Table 2). Unlike Chol-siRNA polyplexes (Ambardekar et al., 2013), increasing PLL block length from 30 to 50 lysines increased the hydrodynamic diameter of Chol-DsiRNA polyplexes ~ 34 nm [46 ± 1 (SD) vs. 80 ± 2 nm, $P = 0.000012$] but decreased the polydispersity index ~ 2 -fold [0.26 ± 0.03 (SD) vs. 0.13 ± 0.01 , $P < 0.001$] (Table 2). Furthermore, the polydispersity indices of DsiRNA polyplexes were too high (>0.6) to calculate hydrodynamic diameters (Table 2). Thus, modifying DsiRNA with 3'-cholesterol decreases the polydispersity of PLL-PEG(5K) polyplexes and increasing PLL block length increases the hydrodynamic diameter and decreases the polydispersity of Chol-DsiRNA PLL-PEG(5K) polyplexes with the current N/P ratios and range of PLL block lengths.

3.3 Effect of PLL block length and modification of DsiRNA with 3'-cholesterol on the ability of PLL-PEG(5K) to protect complexed DsiRNA from nuclease degradation in high concentrations of serum

DsiRNA, like siRNA, is susceptible to nuclease degradation. Thus, it is important to determine the extent to which complexation with PLL-PEG(5K) is likely to protect complexed DsiRNA from nuclease degradation in the bloodstream after i.v. administration to allow enough time for a sufficient proportion of the DsiRNA dose to accumulate in target cells within primary tumors and metastases.

We previously found that modifying siRNA with 3'-cholesterol is required for PLL-PEG(5K) with PLL block lengths up to at least 50 lysines to protect full-length siRNA from nuclease degradation in 90% (v/v) murine serum at 37°C (Ambardekar et al., 2013). Furthermore, increasing PLL block length from 10 to 50 lysines and increasing the nuclease resistance of siRNA through base modifications increases the extent of Chol-siRNA protection by PLL-PEG(5K) under the same conditions (Ambardekar et al., 2013).

To determine whether PLL block length and/or modifying DsiRNA with 3'-cholesterol also affects the ability of PLL-PEG(5K) to protect DsiRNA from nuclease degradation in high concentrations of serum, DsiRNA or Chol-DsiRNA was complexed with PLL-PEG(5K) at the minimum N/P ratios that form neutral polyplexes (Table 1). Protection against nuclease degradation in 90% (v/v) murine serum at 37°C after 24 hrs was then compared by polyacrylamide gel electrophoresis (Fig.1). DsiRNA or Chol-DsiRNA alone were undetectable after 1 hr under these conditions (not shown).

Increasing PLL block length from 30 to 50 lysines increased the protection of DsiRNA (Fig. 1, white bars) by 16% [61 ± 2 (SD) vs. $77 \pm 3\%$, $P = 0.0402$] but had no effect on the already complete protection of Chol-DsiRNA (Fig.1, black bars) by PLL(30)-PEG(5K) [$P = 0.9895$]. Furthermore, modifying DsiRNA with 3'-cholesterol increased protection 43% by PLL(30)-PEG(5K) [61 ± 3 (SD) vs. $104 \pm 5\%$, $P = 0.0011$] and 25% by PLL(50)-PEG(5K) [78 ± 5 (SD) vs. $103 \pm 3\%$, $P = 0.0078$] over comparable DsiRNA polyplexes (Fig.1, black bars vs. white bars) and, consequently, fully protected DsiRNA at both PLL block lengths under these conditions. Thus, unlike siRNA, modifying DsiRNA with 3'-cholesterol is sufficient for PLL-PEG(5K) to fully protect DsiRNA from nuclease degradation in high concentrations of murine serum at 37°C for at least 24 hrs with the current N/P ratios and PLL block lengths.

3.4 Kinetics of siRNA and DsiRNA activity after electroporation of murine breast cancer epithelial cells

Complexes of DsiRNA and lipofectamine® 2000 significantly have significantly greater potency and duration of mRNA suppression than complexes of siRNA and lipofectamine® 2000 after transfection of a human embryonic kidney cell line (Hek293) (Kim et al., 2005). To first determine whether DsiRNA alone increases the potency and/or duration of mRNA suppression over siRNA in 4T1 cells, the extent that equimolar amounts of siLuc or DsiLuc (3' extension of siLuc sequence by 6 nucleotides) suppressed luciferase activity in 4T1 cells that stably express firefly luciferase (4T1-Luc) was compared by luminescence imaging 24, 48, and 72 hrs after electroporation (Fig.2). Luciferase activity is directly proportional to the intracellular concentration of luciferase protein and, consequently, proportional to luciferase mRNA levels due to the short intracellular half-life of the firefly luciferase protein in mammalian cells (~2 to 3 hrs) (Ignowski and Schaffer, 2004; Thompson et al., 1991). Furthermore, in contrast to transfection reagents such as Lipofectamine® 2000, electroporation is expected to deliver similar amounts of active siRNA and DsiRNA into the cytosol. Electroporation of 4T1-Luc with siLuc or DsiLuc decreased luciferase activity below electroporated 4T1-Luc to the same extent after 24 ($P = 0.4461$), 48 ($P = 0.5323$), and 72 hrs ($P = 0.1940$) (Fig.2). Thus, DsiRNA does not increase the potency or duration of mRNA suppression over siRNA in murine 4T1 breast cancer epithelial cells.

3.5 Effect of PLL block length and modifying DsiRNA with 3'-cholesterol on the activity of DsiRNA polyplexes of PLL-PEG(5K) in murine 4T1 breast cancer epithelial cells

We previously found that modifying siRNA with 3'-cholesterol increases the potency of siRNA polyplexes of PLL-PEG(5K) in murine breast microvascular endothelial cells (Ambardekar et al., 2011) and murine 4T1 breast cancer epithelial cells (Ambardekar et al., 2013) and changing PLL block length between 10 and 50 affects the potency of Chol-siRNA polyplexes of PLL-PEG(5K) in murine breast microvascular endothelial cells (Ambardekar et al., 2011) and murine breast cancer epithelial cells (Ambardekar et al., 2013). To determine whether modifying DsiRNA with 3'-cholesterol and/or PLL block length also affects the potency of DsiRNA polyplexes of PLL-PEG(5K) in murine breast cancer epithelial cells *in vitro*, the suppression of luciferase activity in 4T1 cells that stably express firefly luciferase (4T1-Luc) by DsiLuc or Chol-DsiLuc polyplexes of PLL(30)-PEG(5K) or

PLL(50)-PEG(5K) at minimum N/P ratios (Table 1) was compared by luminescence imaging after 24 hrs (Fig.3).

DsiLuc (Fig.3A, dark grey bars) and Chol-DsiLuc (Fig.3A, black bars) polyplexes decreased luciferase activity between 49% to 64% below untreated 4T1-Luc, whereas comparable polyplexes of inactive DsiCtrl (Fig.3B, light grey bars) or Chol-DsiCtrl (Fig.3B, white bars) or uncomplexed DsiLuc or Chol-DsiLuc (Fig.3B, Alone) had no effect. Furthermore, trypan blue exclusion and total number of 4T1-Luc in all treatment groups were statistically similar to untreated 4T1-Luc (~96 to 100%; not shown), indicating that the inhibition of luciferase activity was due to the suppression of luciferase mRNA and not cytotoxic or cytostatic effects of the polyplexes.

Modifying DsiLuc with 3'-cholesterol increased the suppression of luciferase activity ~12% over DsiLuc polyplexes at each PLL block length (Fig.3, dark grey bars vs. black bars). In contrast, increasing PLL block length from 30 to 50 lysines did not affect the extent that DsiLuc (Fig.3A, dark grey bars) [$P = 0.6230$] or Chol-DsiLuc (Fig.3A, black bars) [$P = 0.9988$] suppressed luciferase activity in 4T1-Luc. Thus, modifying DsiRNA with 3'-cholesterol increases the potency of DsiRNA polyplexes of PLL-PEG(5K) in murine breast cancer epithelial cells *in vitro*, whereas increasing PLL block length from 30 to 50 poly-L-lysines has no effect on polyplexes of DsiRNA or Chol-DsiRNA with the current N/P ratios. Furthermore, there was no difference between the activities of Chol-DsiLuc (N/P 1) and Chol-siLuc (N/P 3) polyplexes of PLL(30)-PEG(5K) in 4T1-Luc under the same transfection conditions over 72 hours (not shown).

3.6 Effect of complexation with PLL(30)-PEG(5K) on the activity of DsiRNA and Chol-DsiRNA in primary murine syngeneic breast tumors after i.v. administration

To determine if complexation with PLL-PEG(5K) increases the potency of mRNA suppression by DsiRNA or Chol-DsiRNA in primary murine syngeneic breast tumors after i.v. administration, we intravenously administered vehicle alone, DsiLuc alone, active (DsiLuc or Chol-DsiLuc) or inactive (DsiCtrl or Chol-DsiCtrl) polyplexes of PLL(30)-PEG(5K) (N/P 1/1), then compared luciferase activity from primary murine 4T1-Luc breast tumors over time relative to the first day of treatment by IVIS (Fig.4). We focused on Chol-DsiRNA polyplexes of PLL(30)-PEG(5K) and not PLL(50)-PEG(5K) because they formed neutral complexes at the same low N/P ratio (Table 1), had smaller hydrodynamic diameters with relatively low polydispersity (Table 2), completely protected Chol-DsiRNA in 90% (v/v) murine serum at 37°C for 24 hrs (Fig.1), and suppressed stably expressed luciferase in murine 4T1 breast cancer epithelial cells to the same extent *in vitro* (Fig.3). Furthermore, PLL(30)-PEG(5K) is potentially less toxic than PLL(50)-PEG(5K) as increasing the MW of PLL alone from 24 kDa to 124 kDa increases toxicity after i.v. administration (~11.3 mg/kg) (Moreau et al., 2002) although endotoxin levels of the respective PLL MWs were not reported.

Chol-DsiLuc polyplexes of PLL(30)-PEG(5K) (Fig.4B, black squares) decreased luciferase activity in 4T1-Luc tumors ~78% by the last day of treatment [Day 0 vs. Day 2, $P = 0.0019$] and maintained ~73% to 78% suppression somewhere between 48 to 72 hours after the last day of treatment [Day 0 vs. Day 3 ($P = 0.0010$); Day 0 vs. Day 4, $P = 0.0203$]. DsiLuc

polyplexes of PLL(30)-PEG(5K) (Fig.4B, black squares) decreased luciferase activity in primary 4T1-Luc tumors by ~60% but up to 24 hours after the last day of treatment [Day 0 vs. Day 3, $P=0.0002$] and only maintained suppression between 24 to 48 hours [Day 0 vs. Day 4, $P=0.0036$]. In contrast, Vehicle alone (Fig.4A, white triangles), Chol-DsiLuc alone (Fig.4A, black triangles), or inactive polyplexes of DsiCtrl (Fig.4B, white circles) or Chol-DsiCtrl (Fig.4C, white squares) had no effect on the increase in luciferase activity with increasing 4T1-Luc tumor volume over time. Furthermore, none of the treatments affected the growth of 4T1-Luc tumors (Fig.5A) or body weights of the mice (Fig.5B) over the course of the study, indicating that changes in luciferase activity from 4T1 tumors were not due to changes in the rate of tumor growth or acute toxicity (Lim et al., 2009). Thus, complexation with PLL(30)-PEG(5K) increases the potency of mRNA suppression by DsiRNA in primary murine syngeneic breast tumors after i.v. administration and modifying DsiRNA with 3'-cholesterol increases the rate, potency, and duration of mRNA suppression by polyplexes of PLL(30)-PEG(5K) with the current N/P ratio and dosage regimen.

3.7 Comparison of Chol-DsiRNA and Chol-siRNA PLL(30)-PEG(5K) polyplex activities in primary murine syngeneic breast tumors after i.v. administration

To determine if Chol-DsiRNA polyplexes of PLL(30)-PEG(5K) increase the potency and/or duration of mRNA suppression over Chol-siRNA polyplexes of PLL(30)-PEG(5K) in primary murine breast tumors after i.v. administration, we compared relative luciferase activities from primary murine breast tumors of 4T1-Luc over time after i.v. administration of polyplexes of Chol-DsiLuc (taken from Fig.4C) or nuclease-resistant Chol-*siLuc (taken from (Ambardekar et al., 2013)) and PLL(30)-PEG(5K) under the same dosage regimen (Fig.6). Chol-DsiLuc polyplexes (Fig.6, black squares, N/P 1) decreased luciferase activity in 4T1-Luc tumors at the same rate and extent (~78% by Day 2) as Chol-*siLuc polyplexes over the course of treatment (Fig.6, black inverted triangles, N/P 3). Chol-DsiLuc polyplexes, however, maintained suppression of luciferase activity (~73 to 78%) at least 48 hours longer (~72 hrs vs. ~24 hrs) than Chol-*siRNA polyplexes after the final day of treatment (Day 3, $P=0.0357$; Day 4, $P=0.0347$) despite administering ~19% fewer Chol-DsiLuc molecules due to a higher MW (17,258 g Chol-DsiLuc/mol vs 13,998 g Chol-siLuc/mol). Thus, Chol-DsiRNA polyplexes of PLL(30)-PEG(5K) have a similar potency to Chol-siRNA polyplexes of PLL(30)-PEG(5K) in primary murine syngeneic breast tumors after i.v. administration but maintain mRNA suppression for a much longer duration with the current N/P ratios and dosage regimen.

4. Discussion

This study provides evidence that complexation of PLL(30)-PEG(5K) with Chol-DsiRNA in place of Chol-siRNA increases polyplex loading, nuclease protection in high concentrations of serum, and the duration of mRNA suppression in primary murine syngeneic breast tumors after i.v. administration. We found that (i.) Chol-DsiRNA required a lower minimum N/P ratio for complexation with PLL(30)-PEG(5K) than Chol-siRNA (N/P 3 vs. N/P 1) (Table 1), (ii.) Complexation with PLL(30)-PEG(5K) fully protected Chol-DsiRNA from nuclease degradation in murine serum (90% v/v) at 37°C for at least 24 hr but only ~60% of Chol-siRNA under the same conditions (Fig.1), and (iii.) Chol-DsiLuc polyplexes of PLL(30)-

PEG(5K) decreased luciferase activity in 4T1-Luc tumors at the same rate and extent as Chol-*siLuc polyplexes of PLL(30)-PEG(5K) (Ambardekar et al., 2013) over the course of treatment but maintained suppression at least 48 hours longer after the final day of treatment (~72 hours vs. ~24 hours) (Fig.6) despite administering ~19% fewer Chol-DsiLuc molecules than Chol-siLuc molecules. Although no differences in the potencies of Chol-siRNA and Chol-DsiRNA polyplexes of PLL(30)-PEG(5K) were observed (Fig.6), it remains possible that both polyplexes saturate primary 4T1 breast tumors under the current dosage regimen.

In contrast to our study in primary murine breast tumors, there were no differences in the potencies or duration of mRNA suppression in the livers of C57BL/6 mice after i.v. administration of PEGylated lipid nanoparticles (LPN) containing DsiRNA or siRNA against *PTEN* or *Factor VII* (Foster et al., 2012). This may be because the physicochemical properties and subsequent activities of DsiRNA and siRNA LPN are similar *in vivo*, especially in the liver, whereas the physicochemical properties of Chol-DsiRNA and Chol-siRNA polyplexes are different enough to affect local distribution and activities in primary 4T1 breast tumors after i.v. administration. It's also possible that nanocarrier distribution and activity in the liver is less affected by differences in physicochemical properties than in solid tumors.

4.1 Possible reasons that Chol-DsiRNA polyplexes suppress mRNA for a longer duration than Chol-siRNA polyplexes in primary murine syngeneic breast tumors after i.v. administration

There are several possible reasons that may singly or collectively explain why Chol-DsiRNA polyplexes of PLL(30)-PEG(5K) increase the duration of mRNA suppression in primary murine tumors over Chol-siRNA polyplexes of PLL(30)-PEG(5K). The first possibility, assuming that Chol-DsiRNA and Chol-siRNA polyplexes distribute to primary murine syngeneic breast tumors at similar levels, is that DsiRNA alone or Chol-DsiRNA polyplexes suppress mRNA for a longer duration in 4T1-Luc cells within the primary tumor than siRNA alone or Chol-siRNA polyplexes, respectively. This is unlikely, however, because electroporation with siLuc or DsiLuc suppressed luciferase activity to the same extent over 72 hours (Fig.2) and transfection of 4T1-Luc with Chol-DsiLuc or Chol-siLuc polyplexes suppressed luciferase activity to the same extent after 24 hours (Fig.3) (Ambardekar et al., 2013) up to 72 hours (not shown), indicating that the potencies and kinetics of mRNA suppression by DsiLuc and siLuc are similar in 4T1 cells either alone or after complexation of their cholesterol-modified forms with PLL(30)-PEG(5K).

The second and most likely possibility is that a higher proportion of Chol-DsiRNA polyplexes localize to primary 4T1 tumors than Chol-siRNA polyplexes after i.v. administration and have higher levels of loading (Table 1) that deliver more RNAi molecules per localized polyplex. A higher proportion of the Chol-DsiRNA delivered to the primary tumor may also be active considering that PLL(30)-PEG(5K) fully protects Chol-DsiRNA from nuclease degradation for 24 hrs in 90% (v/v) murine serum (Fig.1) but requires modifications that increase the nuclease resistance of siRNA before completely protecting Chol-siRNA under the same conditions (Ambardekar et al., 2013). Furthermore, considering that 3' nucleotide overhangs on the antisense (guide) strand of siRNA are critical for RNAi

activity (Caplen et al., 2001; Elbashir et al., 2001), Chol-DsiRNA polyplexes might be less susceptible to exonuclease removal of 3' overhangs than Chol-siRNA polyplexes while localizing to the primary tumor and deliver a larger proportion of fully active Chol-DsiRNA. Although our data shows that PLL(30)-PEG(5K) completely protected Chol-DsiRNA (Fig. 1) and nuclease-resistant Chol-siRNA (Ambardekar et al., 2013), it remains possible that differences in protection of 3' overhangs of Chol-DsiRNA vs. Chol-siRNA cannot be determined by polyacrylamide gel electrophoresis because the loss of ssRNA overhangs does not decrease fluorescent dye binding enough to affect the overall intensities of the fluorescent bands.

The third possibility is that low levels of Dicer substrate activity in primary tumors such as 4T1 (Grelrier et al., 2009) decrease the rate that localized Chol-DsiRNA is converted into active siRNA. This would potentially maintain relatively constant cytosolic levels of active siRNA in 4T1 cells within the tumor for a longer duration than Chol-siRNA because the entire dose of Chol-DsiRNA would not be immediately available for incorporation into the RNAi pathway. Although transfection of 4T1-Luc with Chol-DsiLuc or Chol-siLuc polyplexes suppressed luciferase activity to the same extent up to 72 hours as discussed above, it remains possible that Dicer activity in 4T1 cells within primary tumors is much lower than in 4T1 cells *in vitro*.

5. Conclusions

In summary, our results indicate that complexation of Chol-DsiRNA in place of Chol-siRNA increases the duration of mRNA suppression by polyplexes of PLL(30)-PEG(5K) in primary murine syngeneic breast tumors after i.v. administration at least 48 hrs longer than comparable Chol-siRNA polyplexes and suggest that this is due to differences between the activities of Chol-DsiRNA and Chol-siRNA polyplexes *in vivo*. Thus, replacing Chol-siRNA with Chol-DsiRNA may be a simple approach to significantly increase the duration of mRNA suppression by polyplexes of PLL(30)-PEG(5K) and possibly other PEGylated polycationic polymers in primary solid tumors and metastases after i.v. administration.

Acknowledgments

This work was supported by NIH/NIGMS RR021937 (Nebraska Center for Nanomedicine) (JAV), NIH/NCATS 1R41TR001902-01A1 (JAV), Fred & Pamela Buffett Cancer Center Support Grant (P30CA036727) and Pediatric Cancer Research Group Support, State of Nebraska, LB905 (DC, TM, ZY), Susan G. Komen for the Cure Grant KG090860 (RKS), and the University of Nebraska Presidential Graduate Fellowship (VVA). The IVIS instrument was purchased through the Nebraska Tobacco Settlement Biomedical Research Development Fund (NTSBRDF).

References

- Aigner A. Cellular delivery in vivo of siRNA-based therapeutics. *Curr Pharm Des.* 2008; 14:3603–3619. [PubMed: 19075737]
- Ambardekar VV, Han HY, Varney ML, Vinogradov SV, Singh RK, Vetro JA. The modification of siRNA with 3' cholesterol to increase nuclease protection and suppression of native mRNA by select siRNA polyplexes. *Biomaterials.* 2011; 32:1404–1411. [PubMed: 21047680]
- Ambardekar VV, Wakaskar RR, Sharma B, Bowman J, Vayaboury W, Singh RK, Vetro JA. The efficacy of nuclease-resistant Chol-siRNA in primary breast tumors following complexation with PLL-PEG(5K). *Biomaterials.* 2013; 34:4839–4848. [PubMed: 23557861]

- Caplen NJ, Parrish S, Imani F, Fire A, Morgan RA. Specific inhibition of gene expression by small double-stranded RNAs in invertebrate and vertebrate systems. *Proceedings of the National Academy of Sciences of the United States of America*. 2001; 98:9742–9747. [PubMed: 11481446]
- Elbashir SM, Lendeckel W, Tuschl T. RNA interference is mediated by 21- and 22-nucleotide RNAs. *Genes Dev*. 2001; 15:188–200. [PubMed: 11157775]
- Foster DJ, Barros S, Duncan R, Shaikh S, Cantley W, Dell A, Bulgakova E, O'Shea J, Taneja N, Kuchimanchi S, Sherrill CB, Akinc A, Hinkle G, Seila White AC, Pang B, Charisse K, Meyers R, Manoharan M, Elbashir SM. Comprehensive evaluation of canonical versus Dicer-substrate siRNA in vitro and in vivo. *Rna*. 2012; 18:557–568. [PubMed: 22294662]
- Grelier G, Voirin N, Ay AS, Cox DG, Chabaud S, Treilleux I, Leon-Goddard S, Rimokh R, Mikaelian I, Venoux C, Puisieux A, Lasset C, Moyret-Lalle C. Prognostic value of Dicer expression in human breast cancers and association with the mesenchymal phenotype. *Br J Cancer*. 2009; 101:673–683. [PubMed: 19672267]
- Howard KA. Delivery of RNA interference therapeutics using polycation-based nanoparticles. *Advanced drug delivery reviews*. 2009; 61:710–720. [PubMed: 19356738]
- Ignowski JM, Schaffer DV. Kinetic analysis and modeling of firefly luciferase as a quantitative reporter gene in live mammalian cells. *Biotechnol Bioeng*. 2004; 86:827–834. [PubMed: 15162459]
- Kim DH, Behlke MA, Rose SD, Chang MS, Choi S, Rossi JJ. Synthetic dsRNA Dicer substrates enhance RNAi potency and efficacy. *Nat Biotechnol*. 2005; 23:222–226. [PubMed: 15619617]
- Kim DH, Rossi JJ. Strategies for silencing human disease using RNA interference. *Nature reviews. Genetics*. 2007; 8:173–184.
- Lim E, Modi KD, Kim J. In vivo bioluminescent imaging of mammary tumors using IVIS spectrum. *Journal of visualized experiments : JoVE*. 2009
- Moreau E, Domurado M, Chapon P, Vert M, Domurad D. Biocompatibility of polycations: in vitro agglutination and lysis of red blood cells and in vivo toxicity. *Journal of drug targeting*. 2002; 10:161–173. [PubMed: 12074543]
- Morrissey DV, Lockridge JA, Shaw L, Blanchard K, Jensen K, Breen W, Hartsough K, Machemer L, Radka S, Jadhav V, Vaish N, Zinnen S, Vargeese C, Bowman K, Shaffer CS, Jeffs LB, Judge A, MacLachlan I, Polisky B. Potent and persistent in vivo anti-HBV activity of chemically modified siRNAs. *Nature biotechnology*. 2005; 23:1002–1007.
- Oe Y, Christie RJ, Naito M, Low SA, Fukushima S, Toh K, Miura Y, Matsumoto Y, Nishiyama N, Miyata K, Kataoka K. Actively-targeted polyion complex micelles stabilized by cholesterol and disulfide cross-linking for systemic delivery of siRNA to solid tumors. *Biomaterials*. 2014; 35:7887–7895. [PubMed: 24930854]
- Sarett SM, Werfel TA, Chandra I, Jackson MA, Kavanaugh TE, Hattaway ME, Giorgio TD, Duvall CL. Hydrophobic interactions between polymeric carrier and palmitic acid-conjugated siRNA improve PEGylated polyplex stability and enhance in vivo pharmacokinetics and tumor gene silencing. *Biomaterials*. 2016; 97:122–132. [PubMed: 27163624]
- Soutschek J, Akinc A, Bramlage B, Charisse K, Constien R, Donoghue M, Elbashir S, Geick A, Hadwiger P, Harborth J, John M, Kesavan V, Lavine G, Pandey RK, Racie T, Rajeev KG, Rohl I, Toudjarska I, Wang G, Wuschko S, Bumcrot D, Kotliansky V, Limmer S, Manoharan M, Vornlocher HP. Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs. *Nature*. 2004; 432:173–178. [PubMed: 15538359]
- Thompson JF, Hayes LS, Lloyd DB. Modulation of firefly luciferase stability and impact on studies of gene regulation. *Gene*. 1991; 103:171–177. [PubMed: 1889744]
- Whitehead KA, Langer R, Anderson DG. Knocking down barriers: advances in siRNA delivery. *Nat Rev Drug Discov*. 2009; 8:129–138. [PubMed: 19180106]

Fig.1.

Effect of increasing PLL block length and modification with 3'-cholesterol on the protection of complexed DsiRNA and siRNA from nuclease degradation by PLL-PEG(5K) in high concentrations of murine serum. Polyplexes of DsiCtrl, Chol-DsiCtrl, siCtrl, or Chol-siCtrl and PLL-PEG(5K) were prepared as described (Table 1) at the indicated minimum N/P ratio required to form neutral polyplexes, then incubated in buffer or 90% (v/v) murine serum at 37°C for 24 h. Remaining full-length DsiRNA (white bars), Chol-DsiRNA (black bars), siRNA (not detected), or Chol-siRNA (grey bars) were released from the polyplexes by heparin in the presence of a broad-spectrum nuclease inhibitor. Chol-DsiCtrl and Chol-siCtrl were additionally separated from serum proteins with water-soluble cholesterol, then quantitated by agarose gel electrophoresis. Average percent protection $\pm$ SD (n=2) after 24 hours was determined by normalizing the signal density from the remaining single band of the indicated RNAi molecule from serum-treated polyplexes to the signal density from the single band of the indicated RNAi molecule from the respective buffer-treated polyplexes and compared by one-way ANOVA with Tukey's post-test where $*P < 0.05$ and $**P < 0.01$. DsiRNA, siRNA, Chol-DsiRNA, siRNA, and Chol-siRNA alone were undetectable under these conditions (not shown). Results are representative of at least three independent experiments. ^aData taken from Ambardekar et al., 2013.

Fig. 2. Kinetics of siLuc and DsiLuc activity in murine 4T1 breast cancer epithelial cells over 72 hours after electroporation. A murine breast cancer epithelial cell line stably expressing firefly luciferase (4T1-Luc) was electroporated alone or with 300 nM anti-luciferase siRNA (siLuc, triangles) or DsiRNA (DsiLuc, circles) then grown at 37°C. Average percent luciferase activity $\pm$ SD ($n=2$ independent electroporation treatments) was calculated as the average radiance from siLuc- or DsiLuc-treated 4T1-Luc normalized to the average radiance from 4T1-Luc (electroporated alone) on the same plate and compared at the indicated time points by two-sided unpaired t-test. Results are representative of at least two independent experiments.

Fig.3.

Effect of increasing PLL block length and modifying DsiRNA with 3'-cholesterol on the activity of PLL-PEG(5K) polyplexes of DsiRNA in murine 4T1 breast cancer epithelial cells 24 hours after treatment. Murine 4T1 breast cancer cells stably expressing luciferase (4T1-Luc) were incubated for 4 h with serum-free complete DMEM containing 200 nM of DsiRNA or Chol-DsiRNA alone (*Alone*) or complexed with PLL30-PEG(5K) or PLL50-PEG(5K) at the indicated N/P ratio. An equal volume of complete DMEM containing 20% FBS was then added to each well and luciferase activity was measured 20 hrs later by bioluminescent imaging. Average percent luciferase activity $\pm$ SD (n=2 independent treatment wells) was calculated as the average radiance from treated 4T1-Luc normalized to the average radiance from untreated 4T1-Luc on the same plate and compared by one-way ANOVA with Tukey's post-test. Results are representative of two independent experiments. Trypan blue exclusion and number of 4T1-Luc in all treatment groups was between 96%–100% of untreated 4T1-Luc by cell counting with trypan blue exclusion (not shown). Results are representative of at least three independent experiments.

Fig. 4.

Effect of complexation with PLL(30)-PEG(5K) and modification with 3'-cholesterol on the potency and duration of DsiRNA activity in primary murine 4T1 breast tumors after i.v. administration. Primary syngeneic breast tumors were established by injecting 4T1-Luc cells (10^6) SQ into the mammary fat pad of female BALB/c mice, growing tumors to between 50 and 100 mm³, then determining a baseline luciferase signal on the first day of treatment (Day 0). (A) Vehicle alone (HEPES/saline, white triangles), Chol-DsiLuc alone (black triangles) or polyplexes of PLL(30)-PEG(5K) and (B) inactive DsiCtrl (white circles), DsiLuc (black circles), (C) inactive Chol-DsiCtrl, (white squares), or Chol-DsiLuc (black

squares) at the indicated N/P ratios were then injected i.v. at 2.5 mg RNAi molecule/kg on Days 0, 1, and 2 (black arrows). Average radiances from 4T1 tumors within the same treatment group at each timepoint were normalized to average radiances on the first day of treatment (Day 0) and expressed as percent luminescence $\pm$ SEM (n=4 to 5 mice). (Right inset) Representative images of luciferase activity in primary 4T1-Luc tumors on the first day of treatment (D0) or 24 hrs after the final day of treatment (D3). * P <0.05 or ** P <0.01 vs. average radiance on Day 0 within the same treatment group by repeated measures nonparametric one-way ANOVA with Dunn's post-test. Results are representative of at least two independent studies. (Color in pdf, black and white in print).

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 5. Effect of DsiRNA and Chol-DsiRNA polyplexes of PLL(30)-PEG(5K) on 4T1-Luc breast tumor growth and mouse body weight after i.v. administration. Mice were treated as described in Fig. 4 and tumor volumes and body weights were measured daily beginning the first day of treatment (Day 0). (A) Average tumor volumes or (B) body weights $\pm$ SEM (n=4 to 5 mice) at each timepoint within the same treatment group were compared to initial average tumor volumes or body weights on Day 0 by one-way ANOVA with Dunnett post-test. Results are representative of at least two independent studies.

Fig. 6. Activities of Chol-DsiRNA and nuclease-resistant Chol-siRNA polyplexes of PLL(30)-PEG(5K) in primary murine 4T1 breast tumors after i.v. administration. Changes in luminescence from primary 4T1-Luc breast tumors in female BALB/c mice after i.v. administration of polyplexes of PLL(30)-PEG(5K) and nuclease-resistant Chol-*siLuc (black inverted triangles, N/P 3) or Chol-DsiLuc (black squares, N/P 1, taken from Fig. 4C) at 2.5 mg RNAi molecule/kg (described in Fig. 4) were plotted together to facilitate comparison. Average percent changes in luminescence from 4T1-Luc tumors treated with Chol-DsiLuc polyplexes or Chol-*siLuc polyplexes at Days 1 through 4 were compared by two-tailed Mann-Whitney nonparametric unpaired t test (* $P < 0.05$). ^aData taken from Ambardekar et al., 2013.

Table 1

Effect of PLL block length and modifying the sense strand with 3'-cholesterol on DsiRNA and siRNA loading. DsiCtrl, Chol-DsiCtrl (DsiCtrl modified with 3'-cholesterol on the sense strand), siCtrl, or Chol-siCtrl was added to a solution of the indicated PLL-PEG(5K) in 0.1 M HEPES [pH 7.4] at various N/P ratios, briefly vortexed, incubated at room temperature for 30 min, then separated on a TBE agarose/SYBR Green II gel. The minimum N/P ratio required for complexation (N/P ratio) was defined as the first N/P ratio where polyplexes were completely retained in the agarose gel well. The lowest experimental N/P ratio was 1/1. All N/P ratios are representative of two independent experiments.

Polymer	DsiRNA		Chol-DsiRNA		siRNA ^a		Chol-siRNA ^a	
	N/P ratio	Loading (~wt%)	N/P ratio	Loading (~wt%)	N/P ratio	Loading (~wt%)	N/P ratio	Loading (~wt%)
PLL(30)-PEG(5K)	3	24	1	50	6	14	3	25
PLL(50)-PEG(5K)	3	29	1	56	5	20	2	39

^aData taken from Ambardekar et al. 2013. Results are representative of at least three independent experiments.

Table 2

Effect of PLL block length on the hydrodynamic diameter and polydispersity indices of DsiRNA, Chol-DsiRNA, and Chol-siRNA polyplexes of PLL-PEG(5K). Polyplexes of PLL-PEG(5K) were prepared as described at the indicated [minimum N/P ratio required to form neutral complexes] (Table 1). Average hydrodynamic diameters (Diam.) and polydispersity indices (PDI) $\pm$ SD (n=3 independent measurements) of PLL(30)-PEG(5K) and PLL(50)-PEG(5K) polyplexes were determined by DLS (z-average) and compared by two-sided unpaired t-test where

Polyplex	DsiRNA		Chol-DsiRNA		Chol-siRNA ^b	
	Diam. (nm $\pm$ SD)	PDI ($\pm$ SD)	Diam. (nm $\pm$ SD)	PDI ($\pm$ SD)	Diam. (nm $\pm$ SD)	PDI ($\pm$ SD)
PLL30-PEG(5K)	[N/P 3] n.d.	0.7 (0.1)	[N/P 1] 46 (1) ^a	0.26 (0.03) ^a	[N/P 3] 35 (1)	0.33 (0.02)
PLL50-PEG(5K)	[N/P 3] n.d.	0.6 (0.2)	[N/P 1] 80 (2) ^a	0.13 (0.01) ^a	[N/P 2] 35.0 (0.4)	0.36 (0.01)

^a $p < 0.001$.

^b Data taken from Ambardekar et al, 2013. n.d. – diameter not determined due to the high PDI of the polyplexes.