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Research Paper

Factors influencing polycation/siRNA colloidal stability toward aerosol lung delivery

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ABSTRACT

Hexanediol diacrylate cross-linked oligoethylenimine (OEI-HD) is a non-viral polymeric vector designed to deliver siRNA. To achieve safe and effective in vivo siRNA delivery using this vector, the polyplex must have sufficient colloidal stability if administered intravenously or nebulized for delivery by the pulmonary route. In this study, polyplexes from OEI-HD and siRNA were formulated for aerosol-based lung delivery, regarding their colloidal stability, optimal particle size, and in vitro biological activity. Herein, we describe how these properties are dependent upon the polymer-to siRNA weight ratios, buffer composition they were complexed in, PEG-grafting, and the addition of commercial lung surfactants and/or non-ionic surfactants to the formulation. Lastly, the effects of nebulization of the formulation into aerosol droplets, on the polyplex particle size and transfection efficiency, were evaluated.

Polyplex size was monitored for up to 2 h after polyplex formation to determine the extent of aggregation and final particle sizes when stability was achieved. Our results suggest that PEG-grafting and polyethylenimine-PEG mixing were effective in achieving colloidal stability in isotonic saline buffers. In addition, colloidal stability was achieved in isotonic glucose buffers using commercially available non-ionic surfactant Pluronic™ P68 or the lung-derived surfactant Alveofact™. The smallest particle size, 140 nm, was obtained with Pluronic™ F68. For transfection efficiency, both Alveofact™ and Pluronic™ F68 achieved equal or better transfection when added to the OEI-HD/siRNA polyplexes. For long term storage of OEI-HD/siRNA formulations, we propose a lyophilization method that created in situ polyplexes upon addition of water. Preparation of OEI-HD/siRNA polyplexes by this method allowed dry storage at room temperature for up to the 3 months. In conclusion, we have identified approaches to achieve formulation and colloidal stability of OEI-HD/siRNA complexes, a step toward successful application of polyplexes for in vivo siRNA delivery.

1. Introduction

RNA interference (iRNA) represents a promising strategy to treat pathogenic and heritable diseases by regulating RNA expression (see review [1]). Through this pathway, the suppression of specific mRNA sequences can be achieved by using short, sequence specific, synthetic, small interfering RNA (siRNA), micro-RNA (miRNA), or vector-based short hairpin RNA (shRNA). Overall, synthetic siRNAs have an advantage as they are amenable to large scale manufacturing, and their medicinal and pharmacologic properties can be improved through chemical modifications [2].

For pharmaceutical companies looking to bring siRNA therapeutics quickly to patients, diseases of the lung are prime candidates because of their relatively accessibility by delivery systems. Patients could inhale the nebulized formulations, ensuring deep lung delivery without systemic applications or side effects.

Before treatment can commence, siRNA must reach the cytosol site of action. However, siRNA molecules by themselves do not fare well as classical drugs due to their negatively charged phosphate backbone. These properties severely hinder siRNA's ability to cross cell membranes and enter into the cytosol, where the siRNA is therapeutically active. To counter the poor uptake, vectors for RNAi have been investigated to package, shuttle, and release the short oligonucleotides into the cell environment. These vectors have taken the form of engineered viruses [3], liposomes [4,5], nanoparticles [6,7], and cationic polymer polyplexes [8,9].

Polyethylenimine (PEI) is a cationic polymer well known for its oligonucleotide transfection properties with mixed results concerning siRNA transfection [8,10]. To improve on PEI, several biodegradable variants have been synthesized to reduce the

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cytotoxicity while keeping similar or improved oligonucleotide transfection efficiency. The strategy uses relatively non-toxic small MW oligoethylenimine (OEI) that is cross-linked with biodegradable linkages, which include disulfides [11], ketals [12], esters [13], and amide bonds [14]. The polymerized OEI generally has better transfection efficiency due to the higher MW, while the low MW degraded fragments are non-toxic.

Of particular interest to the present work was 1,6-hexanediol diacrylate crosslinked oligoethylenimine (OEI-HD). OEI-HD was synthesized by crosslinking 800 molecular weight (Mw) oligoethylenimine with 1,6-hexanediol diacrylate with subsequent aminolysis [14,15]. Reproducible transfection was demonstrated both in vitro (Neuro2a, B16F10, HUH7, and H1299 cell lines) and in vivo [13,16]. Of particular interest is its improved cytotoxicity over PEI of similar Mw and its non-toxic degraded fragments [17]. Concomitant blockage of toxic amino groups was believed to be one factor of better performance over PEI, not only the polymer degradation. Degradation of the cationic polymer is slow, as 77% of the original MW was retained after 10 days of hydrolysis in a pH 7 environment [14]. These properties offer the possibility of repeated treatments without associated accumulation effects.

Preparation of the cationic vectors for oligonucleotide transfection is usually straightforward; at physiological pH, the amines are positively charged and the phosphates from the oligonucleotide backbone are negatively charged. The electrostatic interactions draw the two macromolecules together to form heterozygous aggregates that range from 100 nm to over 1000 nm. The 100 nm size is ideal for the coated pits in endocytosis [18]. These cationic polymer/oligonucleotide complexes are typically called polyplexes, and their diameter depends most on salt concentration. However, polymer molecular weight and charge density can also influence size and stability [19].

The tendency of the polyplexes (or other small particles) to reach a stable size over a given time is known as colloidal stability. Should the polyplexes be colloidally unstable, then the polyplexes will continue to grow in size, known as aggregation or flocculation. Under the right conditions, this flocculation can be useful, as in the case for purifying DNA binding proteins with PEI [20]. However for oligonucleotide transfection, this effect is undesirable. Alternatively, the polyplex can dissociate into the original macromolecules, a process known to occur in high salt conditions (>1 M NaCl) [21].

Colloidal stability of polyplexes is known to fall quite short of expected norms for typical formulation stability, especially when compared to liposomes or PLGA based nanoparticles. In low salt conditions (<10 mM), polyplexes prepared with an excess of polycationic polymer tend to show higher colloidal stability. The excess cationic charge leads to positive ζ-potentials and the polyplexes electrostatically repel one another. As salt ions are added into the solution, the electrostatic repulsion is decreased, leading to aggregation. This is especially relevant at isotonic salt concentrations [19].

Methods to overcome the aggregation and induce colloidal stability involve the use of hydrophilic groups grafted onto the polycationic polymers. This includes the use of poly(ethylene glycol) (PEG) [22–24], and dextrans [25]. Grafting hydrophilic polymers on polycationic polymers provides a number of benefits, especially with the use of PEG. These advantages include reduced cytotoxicity, increased water solubility, diminished interactions with blood components, and a spacer for additional ligands [26]. However, mixed results have also been seen in the integrity of the polyplexes themselves. The steric interactions of the PEG side chains can cause the polyplex to dissociate faster than non-pegylated polymers in in vivo circulation. [27] Thus, other preparation methods are needed for improved colloidal stability.

The addition of anionic and cationic surfactants to polyplexes has yielded successful results in in vitro [28] and in vivo [29] transfection. Polyplex formulations are first prepared, and then, the surfactants are added in a subsequent step. The resulting surfactant coating gives the particle a more virus-like composition, with the internally condensed oligonucleotide and a lipid shell. This affects both the size and ζ-potential of the tertiary surfactant incorporated polyplex [21]. However, the overall effect on the colloidal stability in isotonic solutions has not been elucidated.

The difficulty in controlling the complex formulations after in situ generation has direct consequences on animal testing and future therapies. Unlike stable, small molecule drugs, polycationic polymer/siRNA complexes are dynamic in respect to size and colloidal stability. This currently limits them to small (sometimes unknown) time frames after in situ generation to maintain reproducible results.

This represents a significant challenge when working with interdisciplinary collaborations, as inappropriate handling of the complex formulation can introduce unknown errors into the final results. Before expensive animal experiments were carried out, we were determined to provide robust methods to control colloidal stability for subsequent reproducible siRNA transfection. Since greater stability of complexes is an important parameter for superior transfection [30], methods to control aggregation and flocculation are required to advance the field of gene therapy.

Herein, it is hypothesized that addition of surfactants should stabilize polyplexes in both salted and non-salted isotonic solutions. Three types of surfactants were used: Alveofact™, Survanta™, and Pluronic™ F68. The former two consist of lung extracts from bovine tissue, and the latter is a non-ionic surfactant. The second condition tested was the type of isotonic aqueous environment, using non-ionic glucose or sodium chloride to achieve isotonicity. An ionic environment is more relevant to simulate in vivo conditions and happens to be the more difficult medium to achieve colloidal stability in using polyplexes. These formulations were also subjected to ultrasonic nebulization, to note any changes in size or colloidal stability, an important consideration if used toward lung delivery.

The second method for inducing colloid stability onto the polycationic polymer was PEG grafting. Two types of PEG with different MWs were grafted and tested for their effects on size, ζ-potential, and amount of aggregation. To further test the effectiveness of the PEG grafting, our polycationic polymer was mixed with a known colloidally stable PEI–PEG formulations. A mixture of these PEI-grafted and non-grafted polycationic polymers was hypothesized to achieve stability, at an unknown mixing ratio.

A third method to avoid aggregation for long term storage is a simple technique taking advantage of lyophilization and in situ formation of the polyplexes. This method has allowed greater reproducibility in polyplex size, as mixing of polycationic polymer and oligonucleotide can vary from batch, individuals, and laboratories. This method eliminates this variability and can be used for the long term storage of polyplexes at room temperature with or without the previously mentioned stabilization techniques.

2. Materials and methods

The polymer OEI was synthesized as previously described by Tarcha et al. [15]. The anti-luciferase siRNA: 5′-GAUUAUGUCC GGUUAUGUAdTdT-3′ (cat. no. D-002050-01-20), siCONTROL Non-Targeting siRNA #3 (cat. no. D-001210-03-20), and anti-rgRSV 5#-CGAUAAUAUAACAGCdTdT-3 was purchased from Dharmacon (Lafayette, CO, USA). Survanta™ was provided by Abbott Laboratories, North Chicago, IL, USA). Pluoronic™ F68 was purchased from Sigma–Aldrich (cat. no. P1300, Munich, Germany). Alveofact™ was purchased from Boehringer Ingelheim (Ingelheim, Germany). From RAPP Polymere (Tübingen, Germany), 2000 and 5000 MW

polyethylene glycol α-methoxy-ώ-NHS esters were purchased. Aerogen Lab Micropump Nebulizer was purchased from Aerogen Ltd. (Galway, Ireland).

2.1. Polyplex formation

OEI-HD, PEG–PEI, or both were dissolved in 10 mM HEPES buffered glucose (HBG, pH 7.4) at 1 mg/mL. siRNA was dissolved at 1 mg/mL with HBG. Typically, 10 μL of siRNA solution was diluted to 100 μL with HBG and added to 20 μg OEI-HD, PEG–PEI, or a mixture of both that had been diluted to 100 μL HBG to form 30 μg of 2/1 polymer/siRNA in 200 μL buffer. Spontaneous complexation occurred immediately. The solution was used within 20–30 min after preparation for transfection studies. A 2/1 weight ratio (w/w) corresponds to a 10/1 nitrogen/phosphate ratio, present from the OEI-HD and siRNA, respectively. When lung or synthetic surfactants effects were studied, 10× surfactant stocks dissolved in HBG were added separately to 10 μg siRNA in 100 μL HBG and 20–30 μg OEI-HD in 100 μL HBG buffer, and then immediately mixed.

2.2. One-pot lyophilization of siRNA and OEI-HD without polyplex formation

In a 1.5 mL E-tube, 50 μL of OEI-HD (10 μg) in HBG buffer was flash frozen in liquid nitrogen. After 1 min, 50 μL of siRNA (5 μg) in HBG buffer was immediately added to the frozen pellet in the liquid nitrogen bath. The 100 μL frozen pellet was immediately lyophilized to form a dried, powder pellet. Dried powder pellets were prepared in bulk and stored in a desiccant atmosphere at RT and reconstituted with 100 μL dH_2O when polyplex formation was desired.

2.3. Measurement of particle size, zeta-potential and colloidal stability

Hydrodynamic diameter of polyplex suspensions was measured by photon correlation spectroscopy (PCS) using the Zetasizer, Nano ZS, Malvern Instruments (Herrenberg, Germany). Polyplexes were prepared in a total volume of 200 μL HBG buffer at different w/w ratios containing 10 μg siRNA. For determination of the zeta-potential, the polyplexes were further diluted in PBS solution to 700 μL. Colloidal stability was assessed by plotting particle sizes over time.

2.4. OEI-HD synthesis [15]

The polymer OEI was synthesized as previously described by Tarcha et al. [15]. Briefly, 800 MW polyethylenimine (5 g, 6.3 μmoles) was dissolved in 7.5 mL DMSO and added to a 50 mL round bottom flask with magnetic stir bar containing 1.4 g (6.3 μmoles) of 1,6-hexanedioldiacrylate dissolved in 3.3 mL of DMSO. The flask was loosely stoppered and mixed for 4 days at 60 °C. The solution was added dropwise to 200 mL rapidly stirred dioxane, causing a viscous material to collect on the sides of the reaction flask. The solvent was decanted and subsequently washed with additional dioxane. The viscous material was dissolved in water and dialyzed against 12 L of distilled dH_2O using a 3500 MW cut-off dialysis membrane for a 4 days with a change in dH_2O every 24 h. The OEI-HD was freeze dried providing a 30% final yield with 23 K MW. Before use, samples were diluted in dH_2O, and pH was adjusted to 7.1–7.5.

2.5. Synthesis of PEG-g-OEI-HD polymer

OEI-HD was dissolved at 20 mg/mL in 0.1 M Na_3PO_4, pH 7.5 solution and cooled to 4 °C. Polyethylene glycol α-methoxy-ώ-NHS esters, 2000 and 5000 MW, were preweighed in 3 mL glass vials to give a final w/w ratio of 25% and 50% PEG/OEI-HD. Magnetic stir bars were inserted into the glass vials, precooled to 4 °C, and 1.0 mL of OEI-HD solution was added and allowed to react overnight. Polymers were purified using serially connected Novema 30 Å and 300 Å GPC columns (PSS, Mainz, Germany) in 1% formic acid. PEG-g-OEI-HD fractions were pooled, lyophilized, and redissolved at 1 mg/mL in HBG buffer. Polymers were characterized on a DAWN EOS multiangle-laser light scattering instrument (Wyatt, CA, USA).

2.6. In vitro luciferase gene knockdown experiments

H1299 luc cells, stably expressing luciferase, were cultured in RPMI medium and supplemented with 10% heat inactivated fetal bovine serum. Twenty-four hours before transfection, cells were seeded in 96-well plates at a density of 0.6×10^4 per well. Polyplexes were prepared in w/w ratios ranging from 2/1 to 4/1, and 10–20 μL (10–20 pmol siRNA) of the suspension was added to each well containing 100 μL fresh RPMI. After 4 h of incubation, the medium was replaced and the cells were allowed to grow for 44 h. In addition to the anti-luc siRNA, a non-silencing siRNA sequence was used to ensure that the decrease in luciferase expression is due to the anti-luciferase siRNA and not to cytotoxicity effects or the vector. Lipofectamine 2000 (Invitrogen, Carlsbad, USA) was used as a positive control for siRNA delivery and was prepared according to the manufacturer's protocol. Luciferase gene silencing activity was measured according to the protocol provided by Promega (Madison, WI, USA). Briefly, cells were lysed in 100 μL cell culture lysis buffer for 15 min. Luciferase activity was quantified by injection of 50 μL luciferase assay buffer, containing 10 mM luciferin, to 20 μL of the cell lysate. The relative light units (RLU) were measured with a plate luminometer (LumiSTAR Optima, BMG Labtech GMBH, Offenburg, Germany). All experiments were performed in quadruplicates, and data were expressed as percentage of control (untreated cells).

2.7. Nebulization of polyplex suspension and droplet size characterization

The prepared polyplexes (200 μL) were pipetted directly onto the membrane of a sterilized Aeroneb Laboratory Nebulizer. The aerosol was collected and condensed using a sterile 1.5 mL Eppendorf tube (cap removed) pressed to the bottom of the nebulizer. Condensate was briefly centrifuged and analyzed or transfected immediately. Typical recoveries of the aerosol were >80% by volume.

Polyplex solutions were diluted 1:5 with 0.9% NaCl or HBG solution. The diluted solution (1000 μL) was placed into the Aeroneb Laboratory Nebulizer. A stream of nitrogen (10 L/min) was used to propel the aerosol through the laser beam of the Frauenhofer Laser Diffractor, and six measurements were taken of each formulation. Saline served as control. Droplet sizes are expressed as mass median aerodynamic diameter (MMAD).

2.8. Statistics

Significance between the mean values was calculated using One-way ANOVA with Benferroni means comparison on OriginPro 8.0 software. Probability values $p < 0.05$ were marked with *, and $p < 0.01$ with **.

3. Results and discussion

3.1. Colloidal stability vs. salt- and glucose-based isotonic buffers

One of the many parameters affecting successful delivery of oligonucleotides was the particle size of the cationic polymer/oligonucleotide complexes, also known in the gene therapy field as polyplexes. Particle size refers to the hydrodynamic radius of the complex when in solution. Many different factors affect the size of the polyplexes: from salt concentration, polymer/oligonucleotide weight ratio, cationic charge density, and cationic substituent (i.e., 1° vs. 2° amines) to name a few. These factors will determine whether they reach equilibrium (colloidal stability) or aggregate until precipitation. In Fig. 1, two parameters were varied for cationic OEI-HD polymer and siRNA: salt concentration and polymer/oligonucleotide weight ratios. Salt concentration had the most striking effect on the colloidal stability—at 150 mM isotonic PBS buffer, 2/1 and 3/1 w/w ratios of OEI-HD polymer and siRNA had flocculated to >700 nm in less than 60 min. At 20 min, the zeta-potentials (surface charge) were recorded as 2.4 ± 0.5 and 5.6 ± 1.1 mV, respectively. The HBG buffer, containing only 10 mM salt concentrations, stabilized the 2/1 and 3/1 w/w ratios within 20 min, but small increases continued over time, giving the polyplexes a 'shelf-life' of a few hours after preparation. The zeta-potentials were smaller with values of 2.0 ± 0.4 and 3.0 ± 0.6 mV, respectively. Raising the w/w ratio from 2/1 to 3/1 reduced the polyplex size to roughly half of the 2/1 ratio. In general, ratios from 2/1 to 3/1 showed optimal transfection efficiency with minimal non-specific knockdown. Smaller w/w ratios were non-toxic but had little transfection efficiency due to almost immediate aggregation. Higher w/w ratios had higher transfection efficiency but had more non-specific siRNA knockdown, suggesting cytotoxicity, disruption of protein metabolism, or both (data not shown).

This was in accordance to the combined Derjaguin, Verwey, Landau, and Overbeek (DVLO) theory. The DVLO theory describes the balance of surface forces will either attract particles to aggregate or repel based on van der Waals attraction or electrostatic repulsion, respectively. The electrostatic repulsion exists from the formation of a double layer of counter ions upon solvation [31,32]. For the OEI-HD polymer and siRNA polyplexes, the double layer was composed from ions present from the positively charged amines on the OEI-HD polymer and negatively charged ions present in the buffer. The first charge layer exists from the surface charge present on the polyplex surface (assuming homogenous distribution). Due to the excess amines within OEI-HD over phosphates present in siRNA, the surface will be positively charged. The next layer (outer layer) consists of the oppositely charged ions in PBS or HEPES solutions: (bi)phosphate, chloride, and hydroxide ions. For glucose isotonic solutions, the neutrally charged sugars have little effect on the electric double layers. The outer layer can extend many times that of the core (microns or more) in low salt conditions (such as the HEPES/glucose isotonic buffer). This allowed polyplex stability due to the large hydrated shell present in these low salt buffer conditions. Formation of a double layer of sufficient thickness to prevent aggregation is termed peptization [32]. The addition of ions from the PBS/saline buffer decreases the thickness of the outer shell. By decreasing the hydration shell thickness, less particle to particle kinetic energy is needed to overcome the electrostatic repulsion. The van der Waal attraction forces will fuse the polyplex cores as Brownian motion continually impacts the particles against one another, tipping the equilibrium in favor of the aggregation, as seen in Fig. 1.

For the colloidal stability studies, 2 h of monitoring was sufficient to determine whether aggregation could be arrested or not. In our experience with polyplexes, if a solution was unstable, it was apparent within this time. We conjecture that this would be the minimum time needed for polyplex stability in a clinical setting, from pharmacist formulation to patient application. Most formulations displaying little particle size deviation over 2 h were seen to be stable for 24 h (data not shown).

3.2. Nebulization of siRNA polyplexes and their effects on aerosol droplet sizes

A variety of nebulizers are available for producing droplets in the 1–5 μm range needed for lung delivery based on compressor, ultrasonic, or mesh-based technologies. The Aerogen Lab Micropump Nebulizer was found to be ideal for preclinical studies, where 100–200 μL sample volumes could be aerosolized with >80% recovery—droplet condensation—into a standard 1.5 mL E-tube. Aerogen nebulization droplets containing the siRNA polyplexes were measured using laser diffraction. A key parameter was forming the droplets in the 1–5 μm range. Droplets less than 1 μm are not deposited and larger particles than 5 μm impact the bronchial airways before reaching the alveolar sacs. Saline droplets had a mean average of 3.8 ± 0.2 μm. Those containing the siRNA polyplexes had a mean average of 3.9 ± 0.2 μm (Fig. 2). The inclusion of the siRNA polyplexes into the droplets did not significantly alter the droplet sizes. Using isotonic glucose buffer, there was a significant difference ($p < 0.05$) between the droplet sizes: 3.2 ± 0.2 μm for buffer alone and 3.8 ± 0.3 for buffer with the siRNA polyplexes, displayed in Fig. 2. The increase in size is

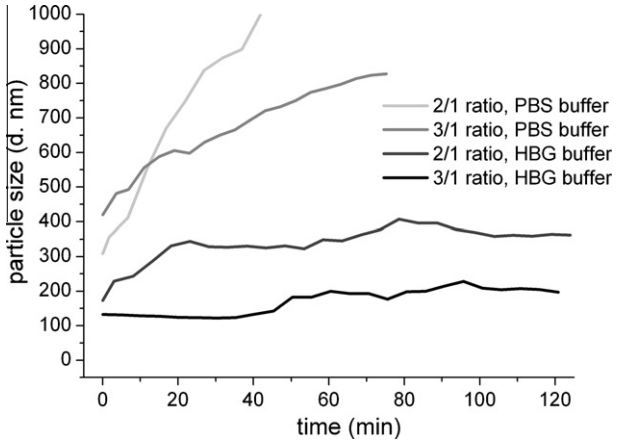

Fig. 1. Colloidal stability of 2/1 and 3/1 w/w ratios of OEI-HD and siRNA (siCONTROL) polyplexes formed in phosphate buffered saline and HEPES (10 mM) buffered isotonic glucose buffers.

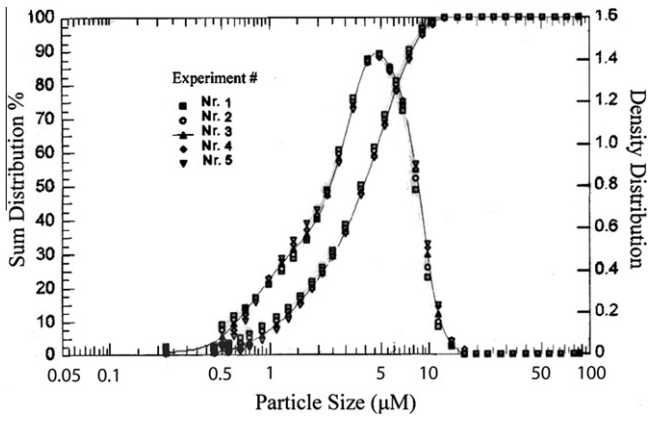

Fig. 2. Laser diffraction studies of the nebulized OEI-HD/siRNA polyplexes in isotonic glucose buffer, in pentuplicate. Mean average diameter was 3.8 ± 0.3 μm.

likely due to the increase in ions and the subsequent change of surface tension. The data support that siRNA polyplexes in isotonic saline or isotonic glucose buffer was within the parameters needed for full lung distribution.

3.3. SiRNA Polyplex sizes before and after nebulization

The polyplex size measurements were measured before and after nebulization for the OEI-HD/siRNA polymers at the indicated w/w ratios. This was investigated to observe any changes in the size of the complexes due to the sudden high intensity energy of the ultrasound nebulization. Airjet nebulizers aerosolize by a different mechanism, although this mechanism is considered milder in its potential for particle disruption. The effects of the nebulization for two batches of OEI-HD polymer were studied. OEI-HD/siRNA complexes were formed at the 2/1 and 3/1 weight ratios, and half the solution was nebulized, condensed, and measured for particle size in comparison to the non-nebulized control solution. The particle sizes were 390 ± 40 and 260 ± 30 nm for the 2/1 and 3/1 ratios control solutions, respectively—after nebulization, 380 ± 40 and 330 ± 30, respectively. The 3/1 ratio was significantly different after nebulization, and it appeared the aggregation had been slightly advanced. No significant difference was seen for 2/1 w/w ratios before or after nebulization. This does not mean that the polyplexes were unaffected, as they could have de-complexed under nebulization and reformed similar size polyplexes. During this possible decomplexation, the siRNA could have been compromised. Overall, the additional energy provided by the Aerogen nebulizer seemed to advance the equilibrium conditions. To check for any damaging effects to the siRNA, the biological activity of the siRNA was confirmed in a luciferase knockdown assay.

3.4. Knockdown of H1299 cells before and after nebulization

Knockdown efficiency of the siRNA/polymers was assessed for any damage that could have happened during the nebulization. This could include splitting of the siRNA duplex, siRNA nicking, or a reorganization of the polymer/siRNA complex. To be as precise as possible, non-nebulized and nebulized comparisons were always from the same formulation; every lot prepared was divided into two, with one lot used for nebulization and the other lot used as control to the nebulization. Results for the OEI-HD polymer at two w/w ratios are shown in Fig. 3. Neither the anti-luciferase siRNA (measuring knockdown efficiency) nor the non-specific siRNA (measuring cytotoxicity of the polymer) was significantly different when comparing non-nebulized vs. nebulized polymers. OEI-HD/siRNA polyplexes were still able to reduce the luciferase protein by 60% or more after nebulization, compared to non-specific control. A commercially available transfection vector, Lipofectamine 2000, was also seen to be unaffected by the nebulization. Taken together, these results demonstrate that nebulization for these formulations had no negative effects on the knockdown efficiency.

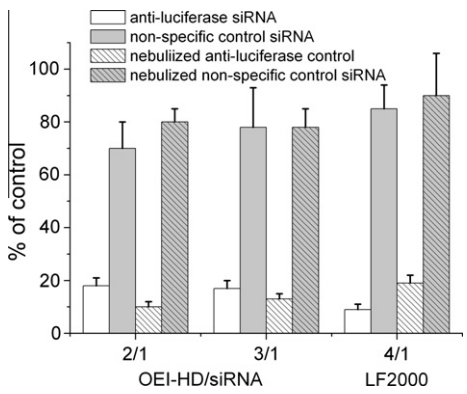

Fig. 3. Knockdown efficiency of nebulized OEI-HD/siRNA polyplexes (40 pmol of anti-luciferase or non-specific siRNA). LF2000: Lipofectamine 2000 positive control.

3.5. Flash freezing, lyophilization, and then in situ formation of siRNA polyplexes

Colloidal stability of the polymer siRNA complexes was difficult to achieve, and most cationic polymer preparations have to be made immediately before transfection. This was often inconvenient for collaborations, as methods of preparation can vary from laboratory to laboratory. Freezing or lyophilization of the polymer/siRNA complexes leads to large aggregation sizes that are unsuitable for transfection. Hobel et al. found some specific Mw fractions of polyethylenimine where siRNA polyplexes could be lyophilized with no detrimental effects on complex size or biological activity [10]. However, this seems to be one of the few exceptions, rather than the rule for polycation transfection agents. Miyata et al. was able to achieve a stable freeze-dried polyplex formulation by thiol cross-linking of newly formed polyplexes. The procedure was laborious however, as it require 13% or more of the primary amines be converted to thiols, following a 3-day crosslinking procedure that requires the initial polymer/oligonucleotide mixtures to have colloidal stability for 24 h [33].

We attempted a simpler approach by physically separating the OEI-HD and siRNA into two frozen pellets present within the same tube, called one-pot lyophilization. While frozen and separated, both pellets were then lyophilized. In this method, the OEI-HD and siRNA were not allowed to mix or complex as the ice and glucose (when dried) will force a physical separation. Upon reconstitution, they will rapidly dissolve, mix, and complex (in situ complexation). Dynamic light scattering confirmed that the in situ lyophilized complexes had the same hydrodynamic diameters as that of the fresh preparations with particle sizes of 210 ± 30 and 200 ± 20 (2/1 w/w ratio, 10 min after preparation) but size will still aggregate similarly to Fig. 1, respectively. In general, no differences were distinguished between the polyplex sizes of fresh preparations or in situ lyophilized complexes. The lyophilized complexes (OEI-HD/siRNA polyplexes allowed to complex for 20 min, and then flash-frozen and lyophilized) resulted in large complexes (590 ± 60 nm) that may reduce polyplex uptake, endosomal release, or cytosomal release, ultimately reducing the efficiency. Fig. 4 displays the results of the transfection efficiency of freshly prepared polyplexes, in situ lyophilized polyplexes, and lyophilized polyplexes. In situ lyophilized complexes displayed similar transfection behavior to that of fresh preparations. The 'tt' formulations and the '**' formulations marked in Fig. 4 were attempted to compare the most obvious differences. As a test marker for polycation cytotoxicity, complexes were transfected with non-specific siRNA, whose sequence does not target any known mammalian proteins. If the luciferase enzyme activity drops with non-specific siRNA, it is an indicator of cell cytotoxicity, disruption of protein metabolism, or both. As one can see in Fig. 4, the lyophilized polyplexes had poor transfection and the non-specific siRNA was also seen to diminish luciferase activity. This was likely caused by the aggregation of the lyophilized complexes. Generally, the larger the difference between the specific and non-specific siRNA knockdown for a particular formulation, the better the therapeutic effect (more specificity, less cytotoxicity). This was observed by noting the diminished difference between luciferase of specific siLUC siRNA (gray bars, Fig. 4) and the amount of non-specific siNC siRNA (black bars) for the individual formulations when lyophilized in situ complexes was compared with lyophilized complexes. Also,

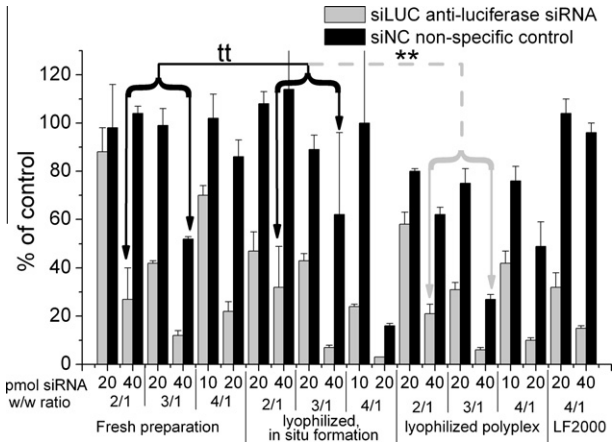

Fig. 4. Lyophilized formulations of OEI-HD/siRNA polyplexes in HBG buffer. Lyophilized, in situ formation: OEI-HD and siRNA were flash frozen separately in HBG buffer, within the same tube, and then lyophilized. OEI-HD/siRNA polyplexes were then formed when distilled water was added. Lyophilized polyplex: OEI-HD/siRNA polyplexes were frozen, lyophilized, and reconstituted with distilled water. tt: No significant differences were seen between values, or if significant ($p < 0.05$), differences were less than 10%. **Significant differences were seen between individual values or (siNC – siLUC per cent) differences ($p < 0.05$).

as the w/w ratios increase, toxicity and transfection generally increase, so optimal conditions must always be defined.

Lyophilized powder pellets were stored up to 3 months at RT with no appreciable loss in activity, as long as the hydrophilic powders were kept desiccated. This opens the possibility of powder mixtures of siRNA and cationic polymers that can be prepared in bulk, developed for dry powder nebulization, or simply prepared in standard pill weights for lab-to-lab reproducibility. By encapsulating the lyophilized powder in surface eroding polymer matrices, localized, long term release of in situ-forming polyplexes can also be envisioned.

3.6. Natural ionic lung surfactants on OEI-HD/siRNA polyplex—effects on colloidal stability and polyplex size

Positively charged phospholipid/oligonucleotide complexes (also known as lipoplexes) have proved to be reliable transfection agents for in vitro transfection. Their transfection properties have displayed reproducibility and ease of use while forming oligonucleotide-lipoplexes that have been shown to be stable, biologically and colloidally, for hours to days. This has spawned several commercial transfection reagents, including the one used for positive control throughout our experiments: Lipofectamine™ 2000®.

One drawback to gene transfer by lipoplexes is their incompatibility with pulmonary surfactants. In vitro transfection with lipoplexes displayed marked decrease in vector efficiency when pulmonary surfactants are present, making them an unlikely candidate for lung delivery. In vitro use of cationic polymers for transfection with airway epithelial cells was possible, however [34]. To capitalize on the advantages of both type of carriers (positively charged phospholipids and cationic polymers), hybrids of both have been utilized. Hybrid alkyl-PEI polymers attempting to combine both cationic polymer and lipofection mechanisms of action did not improve on transfection efficiency enough to justify the complex syntheses [35]. A simpler approach of encapsulating polyplexes with liposomes has been a more successful strategy. Such tertiary complexes have seen increased systemic colloidal stability, with only a slight impairment of transfection efficiency [36,37].

With this in mind, lung-derived phospholipid surfactants were added to enhance the colloidal stability of the OEI-HD/siRNA complexes. The commercially prepared surfactants were Alveofact™ and Survanta™—they are typically used for surfactant replacement therapy for the treatment of respiratory distress in premature infants. Both consist of phospholipids whose primary component is dipalmitoylphosphatidylcholine (DPPC), a phospholipid consisting of two 16-carbon saturated chains with a positively charged head group. They differ in their methods of extraction, which can change their overall proportion of fatty acids, phosphatidylcholine subtypes (alkyl chain lengths and hydrocarbon saturation), and surfactant proteins. Alveofact™ was lipid extracted from bovine lung lavage, which produces a lower concentration of hydrophilic surfactant proteins and fatty acids. Survanta™ was extracted from minced bovine lung and supplemented with additional fatty acids and DPPC. The final amount of Survanta™ DPPC was twice the molar percentage as that of Alveofact™ [38,39].

Fig. 5 displays the colloidal stability of the polyplexes in HBG buffer with the lung surfactants. The lung surfactant concentrations were at 10 μg/mL or 2 μg for 30 μg of OEI-HD/siRNA complexes measured. The hydrodynamic diameters in Fig. 5 were derived from dynamic light scattering measurements. To verify the light scattering was from the polyplex/surfactants mixing and not surfactant/siRNA or surfactant/OEI-HD interactions, the derived count rate was measured for each of the aforementioned solutions. Polyplex/surfactant mixtures had ~50 times the amount of light scattering compared to surfactant/siRNA or surfactant/OEI-HD solutions.

A lower concentration (2.5 μg/mL) gave similar results as the polyplex with no surfactant. Higher concentrations (50 μg/mL) gave erratic light scattering and particle sizes (data not shown). Erratic light scattering was likely due to the large amounts of DPPC micelle or laminar structures formed (DPPC critical micelle concentration was ~1 μg/mL in pure water at 25 °C [40]).

The colloidal stability of the OEI-HD/siRNA complexes was dissimilar when mixed with Alveofact or Survanta. The Alveofact-mixed polyplexes had smaller hydrodynamic diameters of ~250 nm. The polyplexes appeared to have more colloidal stability, with minimized aggregation over 2 h. The behavior of the Survanta lung surfactant was more complicated. At 25 mg/mL, the Survanta stock solution appeared opaque to the naked eye, and this opacity was maintained upon dilution, typical of a stable suspension. The suspension was treated in an ultrasonic bath (for dissociation) and filtered through a 0.22 μm membrane to remove any large particles that would produce light scattering interferences with polyplex size determinations. Despite these extra measures, the Survanta lung surfactant did not provide a decrease in particle size or beneficial colloidal stability. For isotonic saline buffers (i.e., phosphate buffered saline), no colloidal stability was displayed,

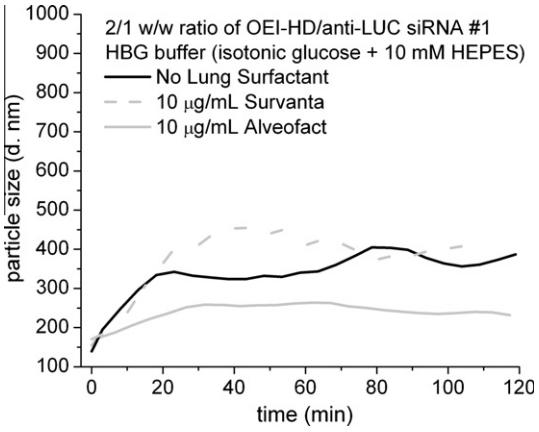

Fig. 5. Effect of lung surfactants on the colloidal stability of OEI-HD/siRNA polyplexes.

and particle aggregation tended to be faster with the lung surfactants, making it a poor choice as a preparation buffer for lung administration. If systemic administration was sought in saline buffers, serum proteins need to be present to avoid aggregation, as serum albumin tends to absorb onto the nanoparticles, stabilizing them [36]. Particle sizing utilizing dynamic light scattering in such a complex environment (i.e., 6–8% protein in serum) yields high background noise (large amounts of light scattering), making accurate interpretation of the data unfeasible (data not shown).

3.7. Non-ionic surfactants on OEI-HD/siRNA polyplexes-enhancement of colloidal stability and polyplex size

The third type of surfactant tested was a non-ionic surfactant generically known as poloxamer. The specific type used was Pluronic F68 and consisted of polyethylene glycol/polypropylene glycol block polymers in a ratio of $E_{76}P_{30}E_{76}$ with a Mw of 8400 Da. This non-ionic surfactant has been considered safe when working with mammalian cell lines—it enhanced cell growth and decreases cell lysis during production of recombinant proteins [41]. The relatively non-toxic Pluronic F68 has been pre-approved for medicinal formulations by the FDA.

Only one concentration of the Pluronic F68 was tested (1 mg/mL or 0.1% w/v), but the results indicate that it was particularly promising for colloidal stability and achieving small particle sizes. At this concentration, only a minimal amount of Pluronic F68 micelle formation was detected. The polyplexes incubated with 0.1% Pluronic F68 were stable at ~140 nm; there was significantly less standard deviation compared to the lung ionic surfactants of Survanta and Alveofact, as seen in Fig. 6.

Further testing with different siRNA nucleotide compositions was performed to ensure the effects were not sequence dependent. Although the sizes were slightly dependent on the siRNA sequence, the Pluronic F68 was able to achieve colloidal stability for all tested in isotonic HBG buffer. The same tests were repeated for isotonic saline solutions. No arrest of aggregation was seen, and in most cases, it increased the rate of aggregation compared to that of no surfactant, similar to the lung ionic surfactants. After 20 min, all polyplexes were >600 nm and continued to aggregate (data not shown).

Similarly to the lung surfactants, the polyplex/Pluronic F68, siRNA/Pluronic F68, OEI-HD/Pluronic F68, and 0.1% Pluronic F68 (by itself in HBG buffer) were measured for the derived light scattering count rate for each of the solutions. The critical micelle concentration (CMC) for Pluronic F68 was reported at 1.2 mg/mL at 30 °C, with CMC inversely related to temperature. The light scattering of the 0.1% Pluronic F68 with siRNA, OEI-HD, or neither was 2–3 times that of HBG buffer light scattering counts, suggesting a small amount of micelle structures present from the non-ionic surfactant at conditions of 0.1% conc. in 37 °C HBG buffer. Particle sizes were under 30 nm. Polyplex/Pluronic F68 complexes were still 30–40 times that of HBG buffer in light scattering counts, as was expected from the 140 nm complexes.

The exact method by which the pluronic F68 induces colloid stability in the particles was not determined, but likely follows typical surfactant mechanisms for colloid stability. The more hydrophobic polypropylene glycol segment was speculated to concentrate itself near the polyplex core, with the hydrophilic polyethylene glycol arms extending out from the surface, creating a more viscous water layer on the polyplex surface. This method is in agreement to more sophisticated, covalently-bound pluronic polyplexes [42].

3.8. Tertiary complexes of Alveofact or Pluronic F68 surfactants enhance OEI-HD/siRNA polyplex transfection efficiency

Polyplex formulations incorporating the colloid stabilizing surfactants of Alveofact or Pluronic F68 were transfected to see the effects of cell cytotoxicity and siRNA delivery. Fig. 7 displays the results of 20 pmol siRNA transfected at 2/1 and 3/1 ratios of OEI-HD/siRNA with no surfactant, Alveofact, and Pluronic F68.

Addition of both surfactants improved the transfection efficiency without affecting the cytotoxicity, when compared with the control. The addition of Pluronic F68 had the most significant effect ($p < 0.01$). The expression of luciferase decreased by 3–4× for the 2/1 and 3/1 ratio formulations. For the non-specific siRNA transfection, expression of luciferase was not significantly altered. This suggests the cell viability remained unaffected upon addition of the Pluronic F68.

After mixing the OEI-HD and siRNA, the polyplexes are left to incubate for 20–30 min before addition into the serum free RPMI medium. In this time range, the 2/1 w/w ratios would have particle sizes of 330, 250, and 140 nm for no surfactant, Alveofact, and Pluronic F68, respectively. This correlates well with the empirical findings that colloid stability, and (smaller) particle sizes have a beneficial effect on transfection of oligonucleotides [43,44]. It is interesting to note that even though none of the formulations displayed colloidal stability in saline buffers (i.e., RPMI medium), transfection was still influenced by the *initial* particle size. It was conjectured that the polyplexes are only potent in the first few

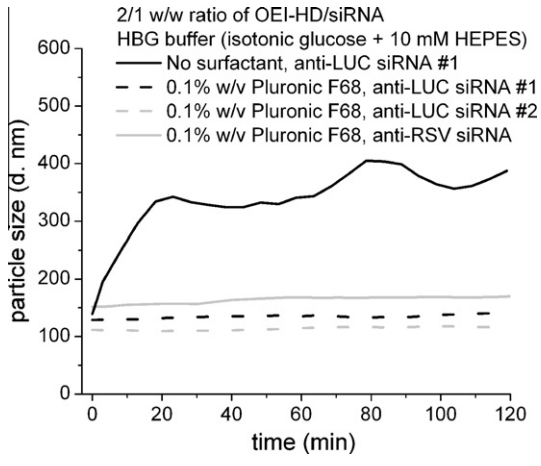

Fig. 6. Effect of non-ionic surfactant, Pluronic F68, on the colloidal stability of OEI-HD/siRNA polyplexes.

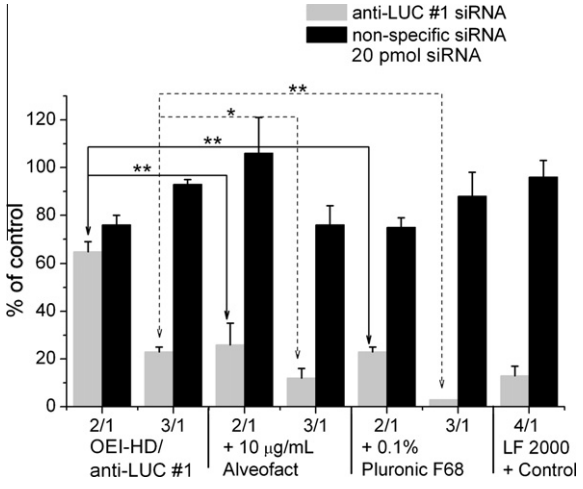

Fig. 7. Transfection of OEI-HD/siRNA polyplexes with Alveofact and Pluronic F68 surfactants. $^{*}p < 0.05$. $^{**}p < 0.01$.

minutes of transfection, before aggregation precipitates the polyplexes. As the RPMI transfection medium dilutes the polyplexes, aggregation should be slower than that seen in Fig. 1 for the PBS buffer. For lung delivery, this is mute point, since the main challenge for polyplexes is penetrating the mucus layer to reach the alveolar cells.

Although our work suggests that transfection was improved by Pluronics beneficial effect on the colloidal stability and particle size, Yang et al. results support different conclusions [45]. Their results displayed little difference in particle size upon addition of Pluronic P123. Differences between our conclusions and theirs are attributed to a number of experimental differences, i.e., particle sizing using complete media (serum present), different polycations (PEI), and characterization of DNA instead of siRNA. Nevertheless, the most notable finding was that Pluronics enhanced the cellular uptake regardless of size, and this may be the cause of beneficial effects found in our work.

Alternatively, Lee et al. demonstrated that covalently bound Pluronics had endosome disruption capabilities, which would free nanoparticles into the cell cytosol after entry via the endocytotic vesicle pathway [42]. Thus, Pluronic polymers may increase OEI-HD/siRNA transfection by providing colloidal stability, small particle size, cell uptake, endosome disruption, or combination thereof.

3.9. Grafting of 2 and 5 kDa PEG onto OEI-HD enhances colloidal stability with mixed effects on transfection

For isotonic salt solutions, aggregation of the polyplexes occurs rapidly. The aggregation was slower with higher w/w ratio of OEI-HD/siRNA, but after 40 min, both had hydrodynamic diameters above 600 nm, as seen in Fig. 1. Cytotoxicity effects prevent polymer-to-siRNA w/w ratios above 5, and surfactants previously mentioned (see Section 3.7–3.9) did not did slow or prevent aggregation. The next attempt to achieve colloidal stability in both saline and glucose buffers was to graft polyethylene glycol (PEG) onto the OEI-HD. Modified PEG–polycation complexes have been widely examined in terms of colloidal stability for in vivo blood delivery [46], siRNA lung delivery [47] and siRNA gastric delivery [48]. By grafting PEG onto polycations, oligonucleotide polyplexes are effectively shielded from the cationic charge—their surface charge becomes, or is close to zero. While this increases colloidal stability, effects on transfection can be detrimental [23]. Without the surface charge, the electrostatic forces that drove the polyplexes to the negatively charged cell surface are non-existent.

PEG grafting was introduced to the OEI-HD polymer via PEG-succinimide esters in Mw of 2 kDa and 5 kDa at 25% and 50% w/w ratios of PEG to OEIHD (or 1/3 and 1/1 w/w ratios of PEG/OEI-HD), respectively). These four OEI-HD-PEG formulations were then complexed to siRNA and measured for colloidal stability and transfection, similar to above. Table 1 lists the Mw properties of the OEI-HD and the OEI-HD-PEG grafts. The OEI-HD-PEG/siRNA complexes displayed colloidal stability in phosphate buffered saline, with particle sizes ranging from 225 to 325 nm, as seen in Fig. 8. The 2 kDa PEG grafts had the smallest particle size, with no difference in size at the two graft ratios.

Addition of PEG required a considerable effort in transfection optimization. Fig. 9 shows the parameters varied with respect to siRNA transfection efficiency: PEG Mw, PEG grafting ratio, pmol siRNA transfected, and w/w ratios of OEI-HD-PEG/siRNA. The therapeutic index—by the amount of drug that causes cytotoxicity divided by the amount needed for therapeutic benefits—of the OEI-HD-PEG was much smaller than OEI-HD alone. For example, the OEI-HD-PEG 5 K (25%) yielded no siRNA knockdown at 3/1 polymer/siRNA ratio at 20 pmol. After doubling the amount to 40 pmol, luciferase knockdown was 20% of control (at 3/1 ratio). However, when the 40 pmol siRNA dosage was changed from 3/1 to 4/1 polymer/siRNA ratio, the non-specific control was reduced to 20% of control—a sign of major cytotoxicity.

Table 1
Characterization of Mw and ratio of OEI-HD-PEG grafts.

Polymer	Molar ratio of PEG/OEI-HD	GPC[a] (M_w/M_n)	PDI	PEG[b] (%)
OEI-HD	0	21,000/16,000	1.31	0
OEI-HD-PEG 2 kDa (25%)	5	28,000/23,000	1.22	25
OEI-HD-PEG 5 kDa (25%)	2	30,000/26,000	1.15	30
OEI-HD-PEG 2 kDa (50%)	10	35,000/29,000	1.20	40
OEI-HD-PEG 5 kDa (50%)	5	34,000/29,000	1.17	38
PEI–PEG 2 kDa (50%)	10	36,000/33,000	1.09	44

[a] GPC standard deviation was typically ±10% of Mw. M_w/M_n refer to molecular average by weight/molecular average by number.
[b] PEG per cent was calculated by ^{1}H NMR.

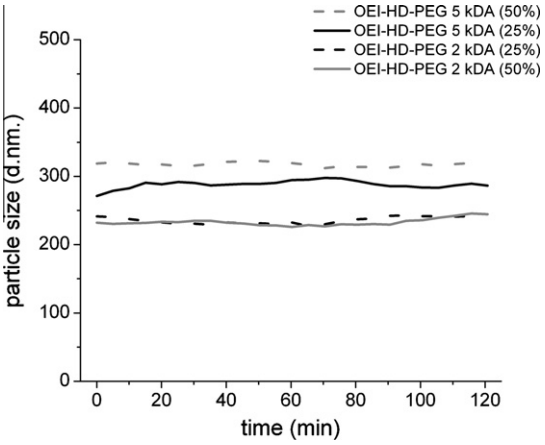

Fig. 8. Grafting of 2 kDa and 5 kDa polyethylene glycol (PEG) at two w/w ratios of 25% and 50% PEG/OEI-HD exhibited polyplex colloidal stability in phosphate buffered saline when the OEI-HD-PEG polymers were mixed with siRNA at 2/1 w/w ratios (OEI-HD-PEG/siRNA).

In general, only one of the four PEG-grafted formulations had substantial knockdown—the OEI-HD-PEG 2 kDa (50%) polymer, which also had one of the smallest particle sizes seen in the PEG series. Even though the OEI-HD-PEG 5 kDa grafts had stable particle sizes, this alone had little effect on transfection. As the PEG-grafts of various polycations have proven again and again, they impart colloidal stability in oligonucleotide complexes, but at cost of near zero surface charge (data not shown). With no surface charge, the electromotive force that normally drives the positively charged polyplexes to the negatively charged cell membranes was non-existent. To provide specificity and raise the binding potential, cell-receptor ligands have been engineered onto PEG–PEI grafts as well [49]. Recent investigations have revealed that PEG may not impart the inert safety designation once thought. PEI–PEG grafts—lower in cytotoxicity compared to PEI—still presented a elevated immune response in mice after intratracheal administration [50].

3.10. Ratios of (PEI–PEG 2 kDA (50%)/OEI-HD)/siRNA polyplexes had enhanced colloidal stability

Star shaped PEI–PEG 2 kDa grafts have been previously noted for their stable and compact particle sizes. PEI with a high degree

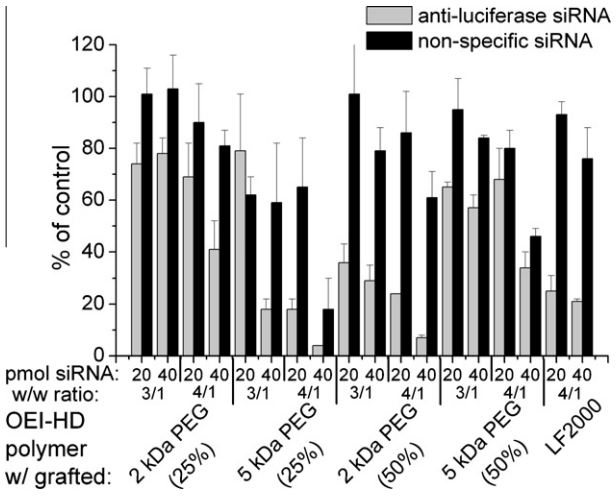

Fig. 9. Transfection of OEI-HD-PEG/siRNA polyplexes varying several parameters: PEG Mw, PEG grafting ratio, pmol siRNA transfected, and w/w ratios of OEI-HD-PEG/siRNA.

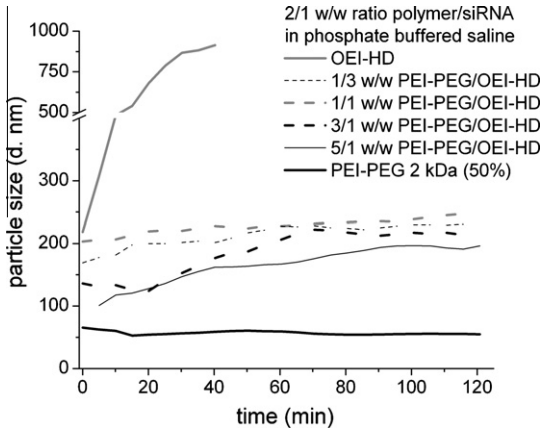

Fig. 10. Mixing of PEI–PEG 2 kDa with OEI-HD to form 4 polymer w/w ratios. Polymer ratios were then mixed with siRNA to form mixed polyplexes at a 2/1 polymer/siRNA w/w ratio.

of low Mw PEG substitution yield polyplexes in the range of 100 nm or less with siRNA and plasmid DNA. The superior complexation abilities of PEI–PEG 2 kDa polymers were theorized to be beneficial toward colloidal stability of OEI-HD/siRNA polyplexes as well (and other non-PEG grafted polycations). PEG grafting every polycation molecule (on average) creates a probable excess in terms of only the bare minimum needed for colloidal stability. Fig. 10 displays the results of this tested hypothesis by mixing different ratios of PEI–PEG 2 kDa and OEI-HD together before addition of siRNA. By itself, PEI–PEG 2 kDa condensed siRNA to 50 nm. With a ratio as low as 1/3 w/w (PEI–PEG 2 kDa/OEI-HD), colloidal stability was maintained even in phosphate buffered saline in an overall 2/1 mixed polymer/siRNA w/w ratio. As the ratio of PEI–PEG 2 kDa increased in the mixture, the particle sizes were generally smaller at first, but leveled off after 2 h around 200 nm. It accordance with the mechanism of PEG-induced colloidal stability; it was conjectured to that a PEI–PEG corona was formed on the PEI–PEG/OEI-HD/siRNA polyplexes. The hydrophilic PEI–PEG would be preferentially concentrated at the surface and OEI-HD/siRNA more concentrated toward the core. Further investigations will attempt to optimize this phenomenon to achieve smaller PEI–PEG/OEI-HD ratios while characterizing the physical properties of the siRNA polyplexes and their transfection efficiency.

4. Conclusions

SiRNA delivery to the lung alveolar epithelial cells offers an appealing new method to treat a variety of lung diseases that is not possible with traditional small-molecule drugs. Routes of administration include intranasal, intratracheal, and nebulized delivery. These routes avoid having to design vectors that can withstand the difficulties involved in systemic administration, i.e., interaction with blood components, uptake by scavenger cells, accumulation in the liver, etc. In this investigation, OEI-HD/siRNA polyplexes in small w/w ratios were examined for colloidal stability toward their use in pulmonary gene delivery. The polyplexes were found to have sufficient colloidal stability in isotonic glucose solution to enable formulation and application within a couple of hours. High loading efficiency of siRNA was possible using w/w ratios of 2–3/1, unlike chitosan systems that need an order of magnitude or above to maintain both the colloidal stability and transfection efficiency [51]. When using the Aeroneb ultrasonic nebulizer, the droplet sizes containing OEI-HD/siRNA were unaffected and were within the 1–5 μm size range needed for deep lung deposition.

The effects of nebulization have been previously studied on PEI/DNA polyplexes [52], but to our knowledge, no investigations with non-viral vectors and siRNA have been reported, except for that of siRNA encapsulated nanoparticles [7]. The data presented here suggest that the siRNA was not damaged during the nebulization, as transfection and luciferase knockdown activity were equal to that of non-nebulized formulations. The DLS characterization confirmed that the particle sizes were unaffected in size after nebulization and condensation of the aqueous droplets.

A simple method for extended storage of OEI-HD/siRNA formulations was also presented to complement the colloidal stability studies. The simple lyophilization method presented allows batch preparations that are useful for reproducibility and collaborations.

Two surfactants were seen promising for extending colloidal stability in isotonic glucose buffers, reducing polyplex sizes, and increasing transfection efficiency. The non-ionic surfactant Pluronic F68 had better physical properties than that seen in Alveofact, but the lung surfactant may prove more beneficial for in vivo studies—since it was derived from lung lavage fluid.

The only method that proved to have colloidal stability in phosphate buffered saline was covalent PEG-grafting onto the OEI-HD polymer. The PEG graft conferred very stable complexes, due to the non-existent surface charge. However, by removing this surface charge completely, transfection was greatly reduced, and more extended optimization was needed to restore modest amounts of transfection. Preliminary experiments in mixing PEI–PEG with OEI-HD to limit the amount of PEG within the polyplex were found to confer colloidal stability even at low PEI–PEG/OEI-HD ratios. Future work will continue optimizing this mixed system.

The methods presented here for OEI-HD/siRNA polyplex colloidal stability should be applicable to other polycation/oligonucleotide complexes as well. The method to achieve enhanced colloidal stability will be highly dependent on the gene delivery system and pathology. Our data suggests colloidal stability of siRNA complexes was improved the most by inclusion of Pluronic F68, which led to an enhancement of transfection results as well. PEG grafting offers the most promising colloidal stability but at the cost of decreased transfection. However, optimization of PEG grafting may be needed, as PEG has recently been shown to affect the shape of the polycation nanoparticles [53].

The non-viral gene therapist will have learned the following rules empirically, but for those getting familiar to the field, the following guidelines should prove useful for providing colloidal stability in polyplex systems: (1) Avoid saline based isotonic buffers if

possible—formulations using isotonic glucose solutions were easier to maintain stable, long term complexes; (2) Use the highest ratio of polycation to oligonucleotide allowed before cytotoxicity is apparent. High ratios tend to have high surface charges and decrease aggregation. (3) A careful choice of FDA approved surfactants can improve the particle stability and may improve biological efficacy, decrease cytotoxicity, or both; (4) Consider PEG grafting as a last resort for the most demanding colloidal stability requirements. Optimization of the PEG-grafted polycations will be more demanding, and a method to drive cell binding and uptake may need to be engineered simultaneously.

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