Published in final edited form as:

Bioconjug Chem. 2011 June 15; 22(6): 1056–1065. doi:10.1021/bc100542r.

Acid-Degradable Cationic Dextran Particles for the Delivery of siRNA Therapeutics

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Abstract

We report a new acid-sensitive, biocompatible and biodegradable microparticulate delivery system, spermine modified acetalated-dextran (Spermine-Ac-DEX), which can be used to efficiently encapsulate siRNA. These particles demonstrated efficient gene knockdown in HeLa-*luc* cells with minimal toxicity. This knockdown was comparable to that obtained using Lipofectamine, a commercially available transfection reagent generally limited to *in vitro* use due to its high toxicity.

Introduction

RNA interference (RNAi) has drawn much attention in the field of medicine due to its potential for treating chronic diseases and genetic disorders by harnessing the endogenous RNAi pathway.¹⁻³ RNAi is a biological mechanism wherein double-stranded RNAs can be used to reduce expression of target proteins.^{4,5} Once the RNA is present in the cytoplasm of the cell, it is shortened and processed by the RNase III enzyme, Dicer,⁶ and incorporated into a protein complex called the RNA-induced silencing complex (RISC).⁷ One of the two strands of the short, double-stranded RNA is cleaved, and the activated RISC (which contains the guide strand of the RNA) binds to a complementary sequence of mRNA and results in its degradation.⁸ The activated RISC is capable of multiple rounds of mRNA cleavage, which propagates gene silencing.⁹ Due to its potential to silence genes in a sequence-specific manner, RNAi holds promise for treating many diseases that may not otherwise be accessed with current therapeutic technology.¹

Various approaches have been developed that allow for exploitation of the RNAi process, principally through the use of exogenous synthetic small interfering RNA (siRNA), double-stranded RNAs that are typically 19-23 base pairs in length. Synthetic siRNA can be designed to target nearly any gene in the body, and is therefore attractive for a variety of medical applications. Previous reports have demonstrated that synthetic siRNAs are capable of knocking down targets in several diseases *in vivo*, including hepatitis B virus, human papillomavirus, and ovarian cancer.¹⁰ Despite great therapeutic potential, the clinical application of siRNA is limited by delivery problems. siRNA does not cross cellular membranes efficiently due to its relatively large size, negative charge, and hydrophilicity. In addition, siRNA is unstable under *in vivo* conditions due to rapid degradation by serum nucleases.¹¹ Thus, the widespread use of RNAi therapeutics for disease prevention and treatment requires the development of clinically suitable, safe, and effective delivery

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vehicles.¹⁰ In order to induce effective RNAi, these vehicles must overcome a variety of extracellular and intracellular obstacles; i.e. they should provide protection against nuclease activity and facilitate internalization and intracellular trafficking of the siRNA.¹² Even though significant advances have been made in the field, the development of vehicles that can efficiently deliver RNAi therapeutics both *in vitro* and *in vivo* remains a major challenge.

Both viral and non-viral carriers have been developed for the delivery of siRNA.¹²⁻¹⁶ Although viral vectors are very efficient, they can cause immunogenic and inflammatory responses,^{17, 18} which raise concerns about their safety as delivery vectors. Non-viral vectors provide opportunities for improved safety, greater flexibility and more facile manufacturing, however, most of the existing carriers suffer from low delivery efficiencies. The most common non-viral vectors involve complexes formed between cationic lipids^{19, 20} or polymers^{21, 22} and siRNA through electrostatic interactions between the negative phosphates along the nucleic acid backbone and the positive charges displayed on the vector. In addition to low transfection efficiencies, these systems also suffer from high toxicity due to their polycationic nature and limited stability *in vivo* due to non-specific interactions with serum proteins. The limitations associated with current delivery vehicles motivate the development of novel systems for siRNA delivery that may be able to overcome these obstacles.

Among many alternatives to cationic polymers and lipids commonly used to form polyplexes/lipoplexes with genetic material,^{10, 23, 24} particles made from biodegradable and non-toxic materials such as slow-hydrolyzing poly(lactic-co-glycolic acid) (PLGA),^{25, 26} fast-degrading polyesters (such as DEAPA-PVA-PLGA),²⁷ and acid-sensitive poly(orthoesters) (POEs)²⁸ have been explored as *in vivo* gene delivery vectors. Another system based on acid-sensitive polyketals has also recently shown promise for delivery of siRNA both *in vitro* and *in vivo*.²⁹ These prior examples of particulate systems for siRNA and DNA delivery typically employ small quantities of cationic polymers^{25, 30} (i.e. poly(β -amino ester), PBAE, or polyethyleneimine, PEI), lipids such as DOTAP,²⁹ or small molecules such as spermine²⁶ blended with the carrier polymer to enhance loading of DNA/RNA and delivery efficiency. Formed by standard emulsion techniques, these particles combine physical entrapment of their payload with electrostatic complexation of genetic material while retaining biocompatible degradation mechanisms. However, some limitations of these systems relate to the synthetic flexibility, biocompatibility of the degradation products, and the paucity of chemical methods for the modification of the particle surface. For example, despite the promising transfection results obtained from PLGA microspheres, they still suffer from slow release rates³¹ and the formation of DNA-damaging acidic by-products.^{32, 33}

Dextran, a homopolysaccharide of glucose, appears to be well poised for use as a polymeric carrier due to its biodegradability, wide availability, and ease of modification.³⁴ In addition, dextran already has a history of human use in clinical applications for plasma volume expansion and plasma substitution. The potential application of dextran for siRNA delivery has recently been demonstrated.³⁵⁻³⁷ Previously, we described the development of a modular and tunable particle system based on acetal-modified dextran (Ac-DEX).^{38, 39} We have shown that Ac-DEX particles prepared by standard emulsion techniques - either water in oil (w/o) or water in oil in water (w/o/w) - have high encapsulation efficiencies for both hydrophobic small molecules⁴⁰ and high molecular weight hydrophilic cargoes,^{38, 39} such as proteins, and that the release rate of the encapsulated cargo was tunable.³⁹ These acid-degradable Ac-DEX particles were capable of delivering protein antigens to macrophages and dendritic cells. Besides their use as a successful vaccine carrier, microparticles prepared from Ac-DEX blended with a cationic polymer proved effective at delivering plasmid DNA

to both phagocytic and non-phagocytic cells.⁴¹ Although siRNA and plasmid can be applied to achieve similar functional outcomes, successful plasmid delivery carriers cannot necessarily achieve efficient siRNA delivery due to the major intrinsic structural differences and different location of action for siRNA and plasmid.^{42, 43, 44} For example, reports from other groups have shown the difficulty of encapsulating the highly charged, hydrophilic and rigid siRNA in particles by w/o/w emulsion, presumably due to leakage into the outer water phase.⁴⁵

We now describe the preparation and preliminary evaluation of a new polymeric platform – spermine-modified Ac-DEX (spermine-Ac-DEX) – for the delivery of siRNA. The new system combines facile synthesis and biocompatibility with the additional benefit of controlled payload release sensitive to physiologically relevant acidic conditions. Acid-sensitive systems have particularly desirable characteristics, as cargo release can be triggered in response to endosomal acidification upon cellular uptake. Acid-catalyzed hydrolysis of spermine-Ac-DEX generates spermine-modified dextran, which may be able to be further metabolized by enzymes *in vivo*.⁴⁶⁻⁵²

The ability of spermine-Ac-DEX particles to overcome a variety of cellular obstacles and function as an efficient delivery vehicle for siRNA can be rationalized by its tailor-made design (Figure 1). We hypothesize particulate formulation should provide protection of the encapsulated siRNA against chemical and enzymatic degradation. The cationic characteristics of spermine-Ac-DEX can facilitate the encapsulation of siRNA inside particles, and it may also favorably contribute to cellular uptake by enhancing interaction of the particles with negatively-charged cell membranes. Once inside cells, hydrolysis of the polymer in the acidic endolysosomal compartment can allow the siRNA to be released from the particles. Endosomal escape may be achieved via “proton sponge” effect of the amine moieties, as well as increased endosomal osmotic pressure by degradation of the spermine-Ac-DEX material. Overall, we speculate that it is the combination of protection and endolysosomal release that would be the most likely contributors to the successful delivery of siRNA using a spermine-Ac-DEX carrier.

Experimental Procedures

General Materials and Methods

All chemicals were from Sigma-Aldrich (St. Louis, MO) unless otherwise noted. The anti-luciferase siRNA (sense strand: 5'-CUU ACG CUG AGU ACU UCG A dTdT-3') was obtained from Dharmacon (Lafayette, CO) and Silencer Negative Control #1 siRNA was purchased from Ambion (Austin, TX). Phosphate buffered saline (PBS, pH 7.4) was from Invitrogen (Carlsbad, CA). Reactions requiring anhydrous conditions were performed in flame-dried vessels and under a positive pressure of dry nitrogen. Water (dd-H₂O) for buffers and particle washing steps was purified to a resistance of 18 MΩ using a NANOpure purification system (Barnstead, USA). When used in the presence of acetal containing materials, dd-H₂O was rendered basic (pH 8) by the addition of triethylamine (TEA) (approximately 0.01%). ¹H NMR spectra were recorded at 400 or 500 MHz on a Bruker spectrometer. Elemental analyses were performed at the UC Berkeley Mass Spectrometry Facility. Fluorescence measurements were obtained using a Spectra Max Gemini XS plate-reading fluorimeter (Molecular Devices, USA), usage courtesy of Prof. Carolyn Bertozzi. Absorbance measurements were obtained using a Spectra Max 190 microplate reader (Molecular Devices, USA), usage courtesy of Prof. Carolyn Bertozzi. Luminescence measurements were obtained using a GloMax 96 microplate luminometer (Promega, USA), usage courtesy of Prof. Eva Harris.

Synthesis of Spermine-Ac-DEX

Partial Oxidation of Dextran—Dextran (5.0 g, 30.9 mmol, M_w 9–11,000 g/mol, from *Leuconostoc mesenteroides*) was dissolved in 20 mL water. After adding sodium periodate (1.1 g, 51.4 mmol), the solution was stirred for 5 h at rt. The product was purified by dialysis of the solution against distilled water using a regenerated cellulose membrane with a MWCO of 3,500 g/mol. The water was changed 5 times and the sample was lyophilized to obtain a white powder (4.2 g, 8.4 mol aldehyde functions/100 mol anhydroglucose unit, AGU). The degree of oxidation was determined colorimetrically (UV absorption at 562 nm) using a microplate reductometric bicinchoninic acid assay (Micro BCA Protein Assay Kit, Pierce, USA) according to the manufacturer's protocol and glucose monohydrate for calibration.

Synthesis of Partially-oxidized Acetalated Dextran—Acetalation of partially oxidized dextran was performed in a similar manner as described previously.³⁸ Briefly, 3.0 g partially oxidized dextran (18.5 mmol, 8.4 mol aldehyde functions/100 mol AGU) was modified with 2-methoxypropene (10.6 mL, 111 mmol) yielding partially-oxidized acetalated dextran (4.3 g) containing 100 mol acyclic and 72.5 mol cyclic acetals/100 mol AGU. The degree of functionalization was determined by ^1H NMR spectroscopy in $\text{DCl}/\text{D}_2\text{O}$ according to the method described by Broaders *et al.*³⁹ ^1H NMR (400 MHz, CDCl_3): δ 1.40 (s, br, acetal), 3.25 (br, acetal), 3.45, 3.50–4.10, 4.90, 5.10 (br, dextran).

Synthesis of Spermine-Ac-DEX—Partially oxidized Ac-DEX (2.0 g, 12.3 mmol) was stirred with spermine (4.0 g, 19.8 mmol) in 10 mL DMSO at 50°C for 22 h. The reduction was performed for 18 h at room temperature by adding NaBH_4 (2.0 g, 52.9 mmol) to the DMSO solution. The spermine modified dextran was precipitated in dd- H_2O (40 mL). The product was isolated by centrifugation at $4000 \times g$ for 5 min, and the resulting pellet was washed thoroughly with dd- H_2O (5×40 mL, pH 8) by resuspension followed by centrifugation and removal of the supernatant. Residual water was removed by lyophilization, yielding spermine functionalized acetalated dextran, spermine-Ac-DEX, (1.6 g) as a white powder containing 6.6 mol spermine/100 mol AGU. The degree of functionalization was determined by elemental analysis using the nitrogen content. Anal. (spermine-Ac-DEX) C: found, 55.79; H: found, 8.29; N: found, 1.24. ^1H NMR (400 MHz, CDCl_3): δ 1.40 (s, br, acetal), 1.60, 1.80, 2.60, 2.65, 2.75 (br, spermine), 3.25 (br, acetal), 3.45, 3.50–4.10, 4.90, 5.10 (br, dextran).

Synthesis of PBAE

PBAE, a white solid, was synthesized by the Michael-type addition of 4,4'-trimethylenedipiperidine to 1,4-butanediol diacrylate in THF as the solvent according to the method described by Lynn *et al.* (GPC: $M_n = 42.6$ kDa, PDI = 2.78).⁵³

Preparation of Particles

Particles Encapsulating siRNA—Ac-DEX particles containing siRNA were prepared using a double emulsion water/oil/water (w/o/w) evaporation method similar to that described previously.^{38, 39} Stock siRNA solutions (2.66, 5.32, or 10.64 mg/mL depending on the desired initial siRNA feed) were prepared in nuclease-free distilled water. Spermine-Ac-DEX (25 mg) was dissolved in ice-cold CH_2Cl_2 (0.5 mL). The stock siRNA solution (25 μL) was added and the mixture was sonicated for 30 s on ice using a probe sonicator (Branson Sonifier 450) with a $\frac{1}{2}$ " flat tip, an output setting of 5, and a duty cycle of 80%. This primary emulsion was then added to an aqueous solution of poly(vinyl alcohol) (PVA, $M_w = 13000 - 23000$ g/mol, 87–89% hydrolyzed) (1 mL, 3% w/w in PBS) and sonicated for an additional 30 s on ice using the same settings. The resulting double emulsion was

immediately poured into a second PVA solution (5 mL, 0.3% w/w in PBS) and stirred for 3 h at rt allowing the organic solvent to evaporate. The particles were isolated by centrifugation (10,250 rpm, 30 min) and washed with PBS (25 mL) and dd-H₂O (2 × 25 mL, pH 8) by vortexing and sonication followed by centrifugation and removal of the supernatant. The washed particles were resuspended in dd-H₂O (2 mL, pH 8) and lyophilized to yield a white fluffy solid. Yields were typically between 50-85% per batch based on starting polymer and siRNA mass (12.5 – 21 mg of particles).

Ac-DEX/PBAE Particles—Particles consisting of Ac-DEX and PBAE were made in the same manner as described above, except that unmodified Ac-DEX and 10 wt% PBAE were used instead of spermine-Ac-DEX.

Empty Particles—Particles that did not contain siRNA were made in the same manner as described above, except that the aqueous buffer in the primary emulsion consisted of dd-H₂O (25 µL) and no siRNA.

Quantification of Encapsulated siRNA

Particles containing siRNA were suspended at a concentration of 10 mg/mL in a 0.3 M acetate buffer (pH 5) and incubated at 37°C under gentle agitation for 3 d using a Thermomixer R heating block (Eppendorf). After the particles had been fully degraded, aliquots were taken and analyzed for siRNA content using the Quant-iT Picogreen dsDNA assay (Molecular Probes) according to the manufacturer's instructions. Empty Ac-DEX particles were degraded in a similar fashion and used to determine background fluorescence. For this experiment, all solutions included heparin at 10 mg/mL to disrupt electrostatic interactions between blend polymers and siRNA to enable quantification. The results were compared to a standard curve and the mass of siRNA encapsulated was calculated. Fluorescence was measured using a SpectraMax Gemini XS microplate reader (Molecular Devices, Sunnyvale, CA, ex. 480 nm, em. 520 nm).

Zeta-Potential Analysis

Zeta-potentials of particles were measured using a Nano ZS ZetaSizer (Malvern Instruments, UK) at 25°C after suspending particles in HEPES buffer (5 mM, pH 7.4) at 0.1 mg/mL. Data shown represent the average zeta-potential ± standard deviation of distributions of five sequential measurements.

Characterization of Particle Size by Scanning Electron Microscopy (SEM)

Particles were suspended in dd-H₂O (pH 8) at a concentration of 0.3 mg/mL and the resulting dispersions were dripped onto silicon wafers. After 15 min, the water was wicked away using tissue paper and the samples were further dried under a stream of N₂ gas. The particles were then sputter coated with a 2 nm layer of a palladium/gold alloy and imaged using a scanning electron microscope (S5000, Hitachi). The particle diameter and size distribution of the particles were determined by measuring 100 particles and analyzing the data using Excel. These micrographs were also used to assess particle morphology.

pH-Dependent Degradation of Ac-DEX Particles

Empty Ac-DEX particles were suspended in triplicate at a concentration of 1 mg/mL in either a 0.3 M acetate buffer (pH 5.0) or PBS (pH 7.4) and incubated at 37°C under gentle agitation using a Thermomixer R heating block (Eppendorf). At various time points, 50 µL aliquots were removed, centrifuged at 14,000 × g for 4 min to pellet out insoluble materials, and the supernatant was stored at -20°C. The collected supernatant samples were analyzed for the presence of reducing polysaccharides using a microplate reductometric bicinchoninic

acid based assay (UV absorption at 562 nm) according to the manufacturer's protocol (Micro BCA Protein Assay Kit, Pierce, USA). The curve was made by applying a Boltzmann fit.

pH-Dependent Release of siRNA from Ac-DEX Particles

This experiment was performed essentially in the same manner as above except siRNA-loaded particles were used instead of empty particles. The quantity of siRNA in the supernatant samples was determined by using the Quant-iT Picogreen dsDNA assay (Molecular Probes) according to the manufacturer's instructions. The amount of siRNA in each sample was calculated by fitting the emission to a calibration curve using the Quant-iT Picogreen dsDNA assay (Molecular Probes). For this experiment, all solutions included heparin at 12.5 mg/mL to disrupt electrostatic interactions between blend polymers and siRNA to enable quantification. The curve was made by applying a Boltzmann fit.

Cell Lines and Culture

HeLa cell line stably expressing firefly luciferase (HeLa-*luc*) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 1% GlutaMAX, and 500 µg/mL Zeocin (all from Invitrogen except the serum, which was from Hyclone (Logan, UT)). Cell incubations were performed in a water-jacketed 37°C/5% CO₂ incubator.

In Vitro siRNA Transfection Assay

HeLa-*luc* cells were seeded (15,000 cells/well) into each well of a 96-well clear tissue culture plate (Costar, Corning, NY) and allowed to attach overnight in growth medium. Growth medium was composed of DMEM (with phenol red), 10% FBS, and 1% GlutaMAX. Particle samples (encapsulating either luciferase siRNA or control siRNA) were prepared in culture medium (without antibiotics) by alternately vortexing and sonicating in a Branson 2510 water bath for 20 s to generate homogeneous suspensions. The samples were then serially diluted in medium to give the indicated particle concentrations or equivalent siRNA doses. Existing medium was replaced with 100 µL of each particle dilution (2.19 – 35 pmol siRNA) in quadruple wells. The cells were allowed to grow for an additional 48 h before being analyzed for gene expression. Lipofectamine 2000 (Invitrogen, Carlsbad, CA) was used as a positive control for siRNA delivery and was prepared according to the manufacturer's instructions. Complexes containing equivalent doses of siRNA to particles were prepared by mixing Lipofectamine 2000 and siRNA (1:50 ratio). As negative controls, both equivalent doses of free siRNA in medium and medium alone were used.

After 48 h, the cells were washed with PBS (containing Mg²⁺ and Ca²⁺, 3 × 100 µL), Glo Lysis Buffer (120 µL, Promega, Madison, WI) was added to each well, and the plate was vortexed at rt for 20 min. Samples from each well (100 µL) were transferred to the wells of a white 96-well tissue culture plate (Corning, Lowell, MA). Steady-Glo luciferase assay reagent (Promega) was reconstituted according to the manufacturer's instructions and injected into each well in series (100 µL/well) using a GloMax 96 microplate luminometer (Promega). After a 10 s post-injection delay, each well was read with a 2 s integration time.

Total Protein Assay

Cells treated identically and in parallel with transfection assays were tested on a second 96-well plate. After washing, the cells were lysed with M-PER Mammalian Protein Extraction Reagent (50 µL/well, Pierce, Rockford, IL) by incubating for 10 min at rt. PBS (50 µL/well) was then added, and the plate was briefly vortexed. Samples from each well (50 µL) were transferred to a black 96-well plate (Corning) already containing PBS (100 µL/well). A solution of 3 mg/mL fluorescamine in acetone (50 µL) was added to each well and mixed

well using a multi-channel pipette. After 5 min, fluorescence was measured using a SpectraMax Gemini XS reader (ex. 400 nm, em. 460 nm). Protein concentrations were determined using bovine serum albumin as a standard. Relative light units (RLU) from the luminometer were normalized to the total mass of cellular protein. The resulting data (RLU/mg of protein) are given as a mean $\pm$ standard deviation of four independent measurements. Percentage knockdown was calculated by comparison of treated cells to untreated cells. The data was compared to the knockdown of cells treated with particles loaded with control siRNA or control siRNA/Lipofectamine complexes.

Viability Assay

Cells treated identically and in parallel with transfection assays were tested on a third 96-well plate. A 3.0 mg/mL solution of MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) in medium (40 μ L) was added directly to each well, and the plate was incubated for an additional 30 min. The medium was then replaced with DMSO (200 μ L/well), 100 μ L of which was transferred to another clear-bottom 96-well assay plate (Pro-Bind, Falcon) containing 100 μ L DMSO and 25 μ L of glycine buffer (0.1 M glycine, 0.1 M NaCl, pH 10.5) per well. The absorbances at 570 nm were measured using a SpectraMax 190 reader (Molecular Devices). Cell viability was normalized to the absorbance measured from untreated cells. Data are represented as a mean $\pm$ standard deviation of four measurements.

Results and Discussion

Synthesis of Spermine-modified Ac-DEX

Ac-DEX has several characteristics that make it well suited for the delivery of bioactive cargoes. Although this system has been used to successfully deliver protein antigens, adjuvants and small molecule agents, the neutrally charged polymer can not efficiently encapsulate siRNA. Taking advantage of the blend approach described above, we have recently reported the preparation of particles encapsulating plasmid DNA by formulating Ac-DEX with small amounts of the cationic polymer PBAE.⁴¹ These particles were able to efficiently transfect both phagocytic and non-phagocytic cells *in vitro*. Inspired by this work, we tried to prepare Ac-DEX particles encapsulating siRNA by blending with either small polyamines or cationic polymers. While high loading and efficient delivery of plasmid DNA was achieved by blending Ac-DEX with PBAE, attempts to prepare siRNA-loaded particles by the same method only afforded particles with low loading and low encapsulation efficiency.

Domb and coworkers have reported the synthesis of various oligoamine polysaccharide conjugates for use in gene delivery.⁵⁴ They found that, of 300 different polycations prepared, only a few were active in transfecting cells. Dextran-spermine displayed especially high transfection efficiency, which they attributed to unique complexation properties between DNA and the grafted spermine moieties.⁵⁴ Dextran-spermine, and derivatives thereof, have shown high transfection of plasmid DNA both *in vitro* and *in vivo*.^{55, 56} Combining this pioneering work with the unique characteristics of our Ac-DEX, we have modified Ac-DEX with spermine for use in siRNA delivery.

Spermine-Ac-DEX was prepared by using reductive amination chemistry for the conjugation of spermine to Ac-DEX (Scheme 1). To increase the number of available aldehyde functionalization sites beyond the reducing chain ends of the polysaccharide, dextran was first lightly oxidized with sodium periodate thus increasing significantly its aldehyde content⁵⁷ as evaluated by a reductometric bicinchoninic acid assay (BCA assay). Using this method, we settled on a loading of 8.4 aldehyde functions per 100

anhydroglucose units (AGU). Reaction of the remaining hydroxyl groups with 2-methoxypropene afforded partially-oxidized Ac-DEX, an acid-sensitive, hydrophobic polymer that can be easily processed into microparticles. Finally, amine modification was performed between spermine and aldehyde-containing-Ac-DEX using sodium borohydride as the reducing agent. The amount of spermine conjugated to the dextran, calculated from the nitrogen content (%N) as determined by elemental analysis, averaged 6.6 spermine units per 100 AGU.

Preparation and Characterization of Spermine-Ac-DEX Particles

Spermine-Ac-DEX particles encapsulating siRNA were prepared via a standard double-emulsion technique (Figure 2). Particles prepared from unmodified Ac-DEX and Ac-DEX blended with 10 wt% PBAE, a formulation previously used for plasmid DNA, were also made for comparison purposes. The particles were visualized by scanning electron microscopy (SEM) to determine the average particle size and morphology. All particles were found to be spherical in shape with diameters in the range of 180 to 230 nm in the dry state, irrespective of particle formulation. The surface charge of the particles was determined by zeta-potential measurements. Unmodified Ac-DEX particles have a slightly negative zeta-potential due to the encapsulated siRNA. Blending the polymer with PBAE made the surface charge less negative owing to the cationic nature of the polymer, while the surface charge of spermine-Ac-DEX particles was positive. As expected, the surface charge decreased with increasing loadings of siRNA but remained positive for all particle formulations. Various particle characteristics are known to influence particle behavior *in vivo*, including particle size, shape, and surface charge.⁵⁸ When administered intravenously, positively-charged particles are susceptible to opsonization by negatively-charged serum proteins and clearance by cells of the reticuloendothelial system (RES). While a positive surface charge might be disadvantageous for delivery into the bloodstream, pulmonary delivery represents an attractive alternative delivery strategy for which there is precedent of success utilizing positively charged particles.^{59, 60} Indeed, we envision utilizing aerosolization for the delivery of our particles to the lungs.

The siRNA loading and loading efficiencies were determined by degrading a sample of particles under acidic conditions and analyzing the siRNA concentration using a Picogreen dsDNA assay (Table 1). The siRNA loading efficiency for unmodified Ac-DEX particles was low (5%), as expected based on previous reports with other particle systems, such as PLGA.²⁶ Blend particles formulated from Ac-DEX and a cationic polymer poly(β -amino ester) (PBAE) also showed low encapsulation efficiency for siRNA (7%), much lower than the plasmid encapsulation achieved with a similar particle formulation. This lower encapsulation efficiency for siRNA as compared to plasmid DNA may be due to the fact that siRNA has a lower molar mass than plasmid DNA (approximately 250 $\times$ less) and, thus, may be more able to diffuse through the condensing polymer during the emulsion process, resulting in reduced loading efficiency.⁶¹ Improved loading efficiencies (75-98%) were obtained for particles prepared from spermine-Ac-DEX. This loading compares favorably with other particle formulations, such as PLGA blended with spermidine for which 56% of the siRNA was encapsulated.²⁶ In contrast to previously reported particulate delivery systems, however, particles prepared using spermine-Ac-DEX did not require blending of a cationic material with the polymer due to the ability of the polymer to electrostatically interact with siRNA.

A significant limitation of other commonly used particle delivery systems is their lack of ability to tune the release rate of the encapsulated cargo. Ac-DEX particles are designed to be relatively stable under physiological conditions (pH 7.4) but degrade under the mildly acidic conditions typically found in the lysosome (pH 5.0-5.5), thus allowing for the selective release of the therapeutic agent once inside the cell. We have shown that the rate of

microparticle degradation can be easily varied by controlling the type of acetals (i.e. cyclic vs. acyclic) formed on the dextran.³⁹ To determine the degradation rate of spermine-Ac-DEX particles, empty particles were incubated at 37°C at either pH 5.0 or pH 7.4 and monitored for the release of soluble dextran (Figure 3a). No soluble dextran was detected after 48 h for the particles incubated at pH 7.4. In contrast, particles incubated in pH 5.0 buffer showed continuous release of dextran in the first 24 h at 37°C, with a degradation half-life of 8 h. We also monitored the release of siRNA from the particles at pH 5.0 and pH 7.4 to determine if the release occurred in a pH-controlled manner (Figure 3b). Little or no release was observed at pH 7.4 after 48 h, while continuous release of siRNA was observed over 24 h at pH 5.0.

To study siRNA loading, three differently-loaded batches of spermine-Ac-DEX particles encapsulating luciferase siRNA were prepared. The feed values for these particles were 2.5, 5.0, and 10 µg of siRNA per mg of polymer, respectively. Following preparation, the siRNA loading of the particles was quantified as described above (Figure 4a). Loadings of up to 8.0 µg of siRNA per mg of particle were achieved and the loading efficiency was above 75% for all particle batches. Efficient loading of siRNA into spermine-Ac-DEX particles is important because of the high cost of siRNA.

***In Vitro* Analysis of siRNA Delivery to HeLa Cells**

To test the transfection efficiency of spermine-Ac-DEX particles *in vitro*, we chose to knockdown a model reporter protein, firefly luciferase. HeLa cells that stably express firefly luciferase (HeLa-*luc* cells) were used as a model cell line. A more effective delivery system will lead to more cytosolic siRNA and luciferase mRNA cleavage, and thus a lower expression of luciferase protein. Firefly luciferase catalyzes the mono-oxygenation of luciferin and during this process a photon of light is produced. Thus, reduced expression of luciferase will result in the generation of fewer photons when the cells are incubated with the enzyme substrate.

We were interested in determining if particle loading or concentration influences the delivery efficiency of siRNA-loaded particles. Differentially loaded particles were prepared and HeLa-*luc* cells were incubated with particle doses equivalent to 17.2 pmol of siRNA per well. All of the particles could reduce luciferase expression of the cells (approximately 55-60% compared to untreated cells) with low toxicity (greater than 85% viability) (Figure 4b). The luciferase expression was comparable for all three batches of particles indicating that the particle loading and concentration within the tested range have a minimal impact on the overall performance, and the knockdown resulted from successful delivery of siRNA.

To determine if the knockdown was dose-dependent, HeLa-*luc* cells were incubated with varying siRNA doses for 48 h and analyzed for the expression of luciferase. As shown in Figure 5, spermine-Ac-DEX particles containing luciferase-specific siRNA efficiently knockdown luciferase expression at all siRNA concentrations tested. Transfection with luciferase siRNA loaded spermine-Ac-DEX particles resulted in significant gene silencing (up to 60% knockdown compared with untreated cells). In comparison, treatment of cells with free siRNA did not affect the luciferase expression, indicating the importance of encapsulation of siRNA in particles. The extent of gene silencing depends on the amount of siRNA incubated with the cells (Figure 5). Treating cells with lower particle concentrations, and thus less siRNA, resulted in reduced knockdown. The optimal knockdown was obtained with the three highest siRNA dosages tested (8.75, 17.5, and 35 pmol of siRNA). This silencing effect was sequence specific as spermine-Ac-DEX particles loaded with a non-specific siRNA sequence (Silencer Negative control #1 siRNA) did not result in obvious reduction of luciferase expression. Importantly, there was no significant cytotoxicity associated with intracellular accumulation of spermine-Ac-DEX particles. MTT assay of

cells incubated with particles showed greater than 80% cell viability for all particle concentrations tested (Figure 6).

Lipofectamine 2000, a commercially available cationic lipid-based reagent, was used as a positive control in the transfection experiment. This reagent is commonly used for *in vitro* transfection⁶², but it faces several obstacles for clinical translation.⁶³ Lipofectamine complexes with the luciferase-targeting siRNA as well as with the control siRNA were prepared so that the dose of siRNA would match that used with two of the particle concentrations tested (35 pmol siRNA per well and 8.75 pmol siRNA per well). We observed some non-specific reduction of luciferase expression using Lipofectamine 2000, most likely due to slight toxicity of the complexes. Thus, the knockdown was normalized to the data from the cells treated with the control siRNA complexes. The knockdown obtained with the spermine-Ac-DEX particles was comparable to that obtained with Lipofectamine 2000 at both siRNA doses tested (Figure 7). Due to their ability to provide efficient siRNA delivery with reduced cytotoxicity (97% cell viability for spermine-Ac-DEX particles compared to 75% for Lipofectamine), spermine-Ac-DEX particles represent an attractive delivery system that may offer several advantages over previously reported materials for siRNA delivery.

Conclusion

In summary, spermine-Ac-DEX particles represent a novel delivery vehicle which can efficiently deliver siRNA to cancer cells with minimal toxicity. Spermine-Ac-DEX combines the attractive properties of cationic polymers with those of polymeric particles affording a material that is safe and effective for gene delivery. Spermine modification of Ac-DEX facilitated the preparation of particles capable of encapsulating siRNA with high loading efficiency. *In vitro* evaluation demonstrated that the particles are capable of knocking down luciferase expression in HeLa-*luc* cells in a dose-dependent manner with low cytotoxicity. In comparison to previously reported gene delivery materials, spermine-Ac-DEX shows attractive new transfection and biodegradability features, and in addition can be easily modified⁶⁴ (for example with peptide targeting ligands). Due to its acid-sensitivity, spermine-Ac-DEX allows for the selective release of siRNA after cellular internalization, with potential tunability of cargo release rate. Thus, spermine-Ac-DEX expands the potential application of Ac-DEX based particles and represents a promising vehicle for siRNA delivery. Future studies will explore the feasibility of using these particles to regulate protein expression *in vivo*.

Acknowledgments

This project has been funded in part with Federal funds from the National Heart, Lung, and Blood Institute, National Institutes of Health, Department of Health and Human Services, under Contract No. HHSN268201000043C, and in part through the Fréchet “various gifts” fund for the support of research in new materials. We thank Ann Fischer and Michelle Yasukawa for help with cell culture, Dr. Peter Friebe and Dr. Eva Harris for assistance with and the use of their luminescence plate reader, and Dr. Chris Contag for kindly providing the HeLa-*luc* cell line. S. Schubert gratefully acknowledges the Deutsche Forschungsgemeinschaft (DFG) and P. R. Wich thanks the Alexander von Humboldt Foundation (AvH) for funding.

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Figure 1.

The particles are expected to efficiently transfect HeLa-*luc* cells due to their ability to overcome several obstacles to gene delivery. Ac-DEX particles should protect siRNA from degradation. (1) The particles are endocytosed by HeLa cells, and the vesicle is acidified upon fusion of the endosome with the lysosome. (2) Spermine Ac-DEX particles degrade in the acidic environment of the endolysosome and (3) the siRNA is released into the cytoplasm.

Figure 2.

Preparation of spermine-modified Ac-DEX particles encapsulating siRNA showing a representative scanning electron micrograph of particles (scale bar = 500 nm) (center) and a cartoon illustration of siRNA-loaded particles (right).

Figure 3.

(a) Degradation of particles when incubated at pH 7.4 (black circles) and pH 5 (white circles), as determined by analysis of released soluble dextran. (b) Release of siRNA from spermine Ac-DEX particles when incubated at pH 7.4 (black circles) or pH 5 (white circles). Data represent the mean $\pm$ standard deviation of triplicate measurements.

Figure 4.

Optimized siRNA loading in particles and its effect on luciferase knockdown. (a) siRNA loading in particles compared with the feed amount of siRNA used in particle synthesis. Dashed diagonal line represents 100% loading efficiency. (b) Knockdown of luciferase activity with varying siRNA loadings. HeLa-*luc* cells were treated with particle doses equivalent to 17.2 pmol siRNA/well. Results were compared to untreated cells and the percentage knockdown of luciferase expression was calculated. Results (columns) are combined with results from a concurrently performed cytotoxicity assay (closed circles and line). Knockdown correlates with siRNA concentration, not particle loading.

Figure 5.

Spermine Ac-DEX particles can efficiently deliver siRNA to HeLa-*luc* cells. *In vitro* transfection of HeLa-*luc* cells with siRNA-loaded spermine Ac-DEX particles. HeLa-*luc* cells were treated with particles encapsulating either luciferase siRNA or control siRNA at various concentrations. Relative light units (RLU) from the luminometer were normalized to the total mass of cellular protein determined from a fluorescamine assay. Data represent the mean $\pm$ standard deviation of quadruplicate measurements. Statistical difference was performed with Student's *t*-test between untreated cells and each treatment group, $p < 0.05$ were marked with *, $p < 0.01$ with **, and $p < 0.001$ with ***.

Figure 6.

Spermine Ac-DEX particles are non-toxic at concentrations up to 1 mg/mL. MTT assay was used to measure cell viability compared with untreated cells. Data represent the mean $\pm$ standard deviation of quadruplicate measurements.

Figure 7. Spermine Ac-DEX particles show comparable activity compared with Lipofectamine 2000. Knockdown was normalized to the data from the cells treated with the control siRNA particles or complexes. Data represent the mean $\pm$ standard deviation.

Scheme 1.

Schematic illustration of the synthesis of spermine-modified Ac-DEX.

Table 1

Characterization of particle formulations.

Particle Formulation (siRNA, feed) ^a	Diameter ^{b,c} [nm]	Zeta-potential ^b [mV]	siRNA Loading ^{b,d} [$\mu\text{g mg}^{-1}$]	Loading Efficiency ^b (%)
Ac-DEX (Luc, 5)	230 $\pm$ 80	-5.5 $\pm$ 0.42	0.26	5
Ac-DEX/10% PBAE (Luc, 5)	N.D.	-3.4 $\pm$ 0.44	0.34	7
Spermine Ac-DEX (Luc, 10)	178 $\pm$ 76	9.2 $\pm$ 0.69	7.95	76 ^e
Spermine Ac-DEX (Luc, 5)	229 $\pm$ 59	16.2 $\pm$ 0.58	4.70	91
Spermine Ac-DEX (Luc, 2.5)	185 $\pm$ 65	19.7 $\pm$ 0.63	2.28	95 ^f
Spermine Ac-DEX (Control, 5)	195 $\pm$ 45	16.1 $\pm$ 0.71	5.11	98

^a Luc – anti-luciferase siRNA, Control – Silencer Negative Control #1 siRNA, Feed – amount of siRNA used in particle preparation, μg of siRNA per mg of polymer.

^b Characterized as described in the main text.

^c Some particle aggregation was observed upon resuspension following lyophilization. The mean particle diameter determined by light scattering for Spermine Ac-DEX (Luc, 5) and Spermine Ac-DEX (Control, 5) was $2.71 \pm 1.54 \mu\text{m}$ and $6.45 \pm 2.47 \mu\text{m}$, respectively (Horiba Partica LA-950, Horiba Scientific).

^d siRNA loading in μg of siRNA per mg of particles (100% efficiency $\approx 5.0 \mu\text{g}$ of siRNA per mg of particles). A loading of $5 \mu\text{g}$ of siRNA per mg of particles corresponds to an N/P ratio of approximately 100.

^e 100% efficiency $\approx 10 \mu\text{g}$ of siRNA per mg of particles.

^f 100% efficiency $\approx 2.5 \mu\text{g}$ of siRNA per mg of particles.

N.D. = not determined