Published in final edited form as:

J Control Release. 2016 November 28; 242: 80–88. doi:10.1016/j.jconrel.2016.09.003.

Therapeutic RNAi robed with ionic liquid moieties as a simple, scalable prodrug platform for treating skin disease

Michael Zakrewsky and Samir Mitragotri¹

Center for Bioengineering and Department of Chemical Engineering, University of California, Santa Barbara, California 93106, USA

Abstract

The high prevalence of skin diseases and their visible symptoms result in major physical, emotional, and economic burden for which few solutions exist. To address this unmet medical need, topical delivery of RNAi such as siRNA holds many advantages including direct access to the diseased site, potent knockdown of disease symptoms, and limited off-target effects. Unfortunately, delivering drugs into skin is extremely difficult. To address these concerns, we present RNAi robed with ionic liquid moieties. Specifically, we show that robed-siRNAs can be synthesized by a simple two-step process from bulk materials. Robing affords tuneability of properties necessary for dermal drug delivery including octanol-water partitioning, skin transport, and cell internalization. The efficacy and safety of robed-siRNA for treating skin disease was confirmed by its ability to limit breakdown of elastin, a major cause of premature aging, following UVB exposure to human reconstructed skin tissue. Together, the data strongly support that therapeutic RNAi robed with ionic liquid moieties are a simple, scalable prodrug platform for treating skin disease.

Graphical Abstract

RNAi robed with ionic liquid moieties for enhanced transdermal delivery and cell internalization is a simple, scalable gene-silencing therapy for treating skin disease

¹To whom correspondence should be addressed: Professor Samir Mitragotri, Department of Chemical Engineering, University of California, Santa Barbara, CA 93106, Phone: 805-893-7532, samir@engineering.ucsb.edu.

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

Keywords

Topical delivery; RNAi; Ionic liquids; Robed-siRNA; Skin disease

INTRODUCTION

Skin disease is one of the most common human illnesses affecting upwards of 70% of the population globally [1]. Symptoms of skin disease range from purely cosmetic (*e.g.* cellulite, wrinkling, and brown spots) to debilitating and even deadly (*e.g.* severe pain, skin barrier disruption, dehydration, and systemic infection) [2]. High prevalence of skin disease and outward presentation of symptoms combined with high rates of morbidity and mortality associated with severe skin disease results in significant physical, emotional, and economic burden [1, 3]. In fact, skin disease is estimated to be the 4th leading cause of non-fatal disease burden globally, more burdensome than chronic obstructive pulmonary disease, diabetes mellitus, osteoarthritis, and drug abuse [1]. Yet, effective treatment of skin disease remains poorly addressed [1, 3, 4].

For effective treatment of skin disease, topical delivery of RNAi such as siRNA holds many advantages. Advantages of topical siRNA delivery include direct access to the diseased site, easy observation of treatment progress, easy observation and excision of sites of adverse reaction, sustained and controlled drug delivery, and potent and specific knockdown of causative proteins [2]. Further, topical application avoids drug degradation in the GI tract and limits systemic toxicity and drug clearance from the diseased tissue [5, 6]. However, delivering siRNA into the skin remains challenging. The challenge originates from the outermost layer of the skin – the stratum corneum (SC) – which presents a formidable barrier to the external environment and prevents accumulation of therapeutically relevant quantities of hydrophilic macromolecules such as siRNA in the skin.

Over the years, extensive efforts have been expended to achieve more effective delivery of topical siRNA. Strategies to overcome the SC barrier include physical methods such as microneedle patches [7] and laser ablation [8], active methods such as sonophoresis [9] and iontophoresis [10], and passive methods such as peptides [11–14] and spherical nucleic acids [15, 16]. Skin disease symptoms, however, typically manifest over large surface areas and often limit the use of device-based methods. Passive delivery methods, on the other hand, are suitable for application on large surface areas; however, current passive methods have certain limitations including complex synthesis and the necessity to use siRNA conjugation chemistries [11, 17–19].

As an alternative, we propose RNAi robed with ionic liquid moieties (robed-siRNA). Here, we demonstrate that robed-siRNA can be synthesized using a simple, scalable two-step process that affords tuneability of physicochemical properties, skin transport, cell internalization, and biocompatibility. Using this platform, we identify one robed-siRNA in particular, benzyl dimethyl octyl ammonium (BDOA)-siRNA, which possessed excellent skin transport and the ability to enhance cell internalization compared to naked siRNA. Biocompatibility and gene-silencing of BDOA-siRNA was confirmed *in vitro* on human adult epidermal keratinocytes. Clinical potential of BDOA-siRNA was confirmed against a

skin aging model in human reconstructed skin tissue. Together, the data strongly suggest that therapeutic RNAi robed with ionic liquid moieties provides a simple, scalable produg platform for treating skin diseases.

MATERIALS AND METHODS

Synthesis and characterization of robed-siRNAs

siRNA robed with ionic liquid (IL) moieties were synthesized by acid-base neutralization as described previously [20]. Benzyl dimethyl alkyl ammonium chloride salts were purchased from Sigma Aldrich (St. Louis, MO). FAM-GAPDH siRNA (siRNA1, 5'-FAM-GAC GUA AAC GGC CAC AAG UUC-3'), FAM-GAPDH siRNA (siRNA2, 5'-FAM-GUG UGA ACC ACG AGA AAU AUU-3'), FAM-Elastase siRNA (siRNA3, 5'-FAM-UCA CUU ACA GGA UCU AUA AUU-3'), and FAM-Control siRNA (siRNA4, 5'-FAM-UAA GGC UAU GAA GAG AUA CUU-3') were purchased from Dharmacon, Thermo Fisher Scientific (Waltham, MA).

Chloride salts were converted to their corresponding hydroxide salts using Amberlite IRA-402 hydroxide form anion exchange resin (Santa Cruz Biotechnology, Dallas, TX). Benzyl dimethyl alkyl ammonium chloride salts were dissolved in ultrapure ddH₂O (Life Technologies, Grand Island, NY) at a concentration of 1.0% wt and mixed with excess resin for 1 hour under constant agitation. The slurry was then centrifuged to pellet the resin and collect supernatant. Complete anion exchange was verified by the lack of precipitate following dropwise addition of silver nitrate (2 g/mL in ddH₂O, Sigma Aldrich, St. Louis, MO). The final solution of benzyl dimethyl alkyl ammonium hydroxide was freeze-dried to remove ddH₂O. Similarly, siRNA sodium salts were converted to acidic form using Amberlite IR-120 hydrogen cation exchange resin (Santa Cruz Biotechnology, Dallas, TX). Complete cation exchange was verified by titration. The final solution of hydrogen form of siRNA was freeze-dried to remove ddH₂O. Acidic groups on siRNA were neutralized by addition of equivalent benzyl dimethyl alkyl ammonium hydroxide in methanol. Neutralization was allowed to proceed overnight at room temperature with constant agitation. Methanol was then removed by rotary evaporation and subsequent freeze-drying, and robed-siRNA was stored at -20°C until further use.

Composition of robed-siRNA was confirmed by elemental analysis. CHN elemental analysis was performed with a CE-440 Rapid Analysis Elemental Analyzer (Exeter Analytical, North Chelmsford, MA). Sample size was ~1 mg weighed in small aluminum capsules. Capsules were placed in protective nickel sleeves, loaded into the autosampler wheel, and introduced into the combustion furnace by means of a mechanically operated quartz ladle. Percent weights of carbon, nitrogen, and hydrogen were determined by high-temperature combustion at 1000°C in an oxygen-enriched helium atmosphere.

Robed-siRNA were characterized by measuring partitioning into octanol and water (Log P_{o/w}). 5 mL ddH₂O was mixed overnight at room temperature with 5 mL octanol. FAM-siRNA or robed-FAM-siRNA was then added to the mixture and again allowed to mix overnight at room temperature in the dark. After overnight incubation, the solution was centrifuged to separate the octanol layer and water layer. The concentration of FAM-siRNA

present in each layer was quantified by fluorescence spectroscopy using a SAFIRE, XFLUOR4, V4.50 microplate reader (Tecan Group Ltd, Morrisville, NY). Fluorescence detection was performed at an excitation of 485 nm and an emission of 520 nm, and the method was validated for linearity, accuracy, and precision. The linear range during the measurements was from 0.25 pmol/mL to 25 pmol/mL ($r^2 = 0.9999$). Log $P_{o/w}$ was calculated as the logarithm of the ratio of fluorescence in the octanol layer compared to the water layer.

Aggregation propensity was determined by photon correlation spectroscopy (Zetasizer Nano series, Malvern Instruments Ltd., Worcestershire, UK). Robed-FAM-siRNA were dissolved in ddH₂O or octanol and sonicated for 30 minutes. Measurements were made at 25 °C with a fixed angle of 173°. Measurements were performed using a red laser to avoid interference from the FAM-labeled siRNA. Sizes quoted here are the number means for the robed-siRNA hydrodynamic diameter.

Measurement of skin transport

Full thickness pig skin (Lampire Biological Laboratories, Pipersville, PA) was used in this study. All skin samples were stored at -80°C. Skin was defrosted and hair trimmed immediately prior to use. Skin pieces were cleaned with PBS (pH 7.4). Skin conductivity was measured to ensure that the samples were intact. 100 mV RMS at 100 Hz was applied using an Agilent 33120 waveform generator (Palo Alto, CA). Conductivity was measured using nickel plated leads and a Fluke 189 multimeter (Everett, WA). Skin pieces with conductivity < 0.033 mS/cm² were deemed intact. Skin penetration was assessed in Franz diffusion cells (FDCs, 1.77 cm² penetration area, 12.0 mL receptor volume) as described previously [17, 21]. Briefly, the receptor compartment was filled with PBS at pH 7.4. Each test formulation was assessed in triplicate. Skin was mounted with the SC facing up and the donor compartment left dry and open to atmosphere for 30 minutes before applying the test formulation. Caution was taken to remove all air bubbles between the underside of the skin (dermis) and the receptor solution. 150 µL of 50 µM FAM-siRNA or robed-FAM-siRNA was applied to skin surface. FDCs were incubated for 24 hours at 37°C under occlusive condition with moderate stirring. After 24 hours the formulations were removed from the skin by washing five times with PBS (pH 7.4). All washes were collected in a glass vial for further analysis. The SC was isolated from the epidermis by stripping with an adhesive tape (Scotch® Transparent Tape, 3M Corporate, St. Paul, MN). Ten tape strips were performed consecutively. The stripped tapes were collected in glass vials as follows: SC 1 = 1st strip and SC 2–10 = 2nd–10th strips. 10 strips were assumed to remove the majority of the SC [22]. After tape-stripping, the epidermis was isolated from the dermis with a surgical sterile scalpel. The epidermis and dermis were cut into small pieces and transferred into separate glass vials. Finally, 3 mL volume from the acceptor solution was transferred to glass vial. For extraction of fluorescent siRNA, 3 mL of methanol and PBS pH 7.4 (1:1, v/v) mixture was added to each vial and shaken overnight at room temperature. Afterwards the solutions were centrifuged for 10 minutes to pellet skin tissue. Supernatants were withdrawn, diluted if necessary, and concentrations of fluorescent probes were determined by fluorescence spectroscopy as described above.

Visualization of skin transport

To visualize transport of FAM-siRNA and robed-FAM-siRNA into skin, formulations were applied to skin in FDCs as described above. After incubation for 24 hours, thin sections (20 μm) corresponding to the application area were prepared using a Leica Cryostat CM1850 (Leica, Buffalo Grove, IL). Sections were prepared with the microtome edge perpendicular to the skin surface to minimize potential redistribution of FAM-siRNA along the z-axis (depth) of the skin. Sections were imaged using an Olympus Fluoview 1000 Spectral confocal microscope (Olympus, Center Valley, PA). All instrument settings were kept constant between samples for comparison between experimental conditions.

Cell culture

All cell culture materials were acquired from Life Technologies (Grand Island, NY). Human adult epidermal keratinocytes were cultured in EpiLife Medium supplemented with Human Keratinocyte Growth Supplement, 25 U/mL penicillin, 25 $\mu\text{g/mL}$ streptomycin, and 50 $\mu\text{g/mL}$ neomycin. Cultures were grown at 37°C with 5% CO_2 .

Evaluation of biocompatibility in cell culture

Cells were seeded in a 96-well microplate (Corning Inc., Corning, NY) and were allowed to attach and proliferate. Once cells reached ~80% confluency, the media was removed and FAM-siRNA, robed-FAM-siRNA, or benzyl dimethyl alkyl ammonium chloride salts in media was added. Media alone was used as a control. Cells were incubated with test solution for 4 hours at 37°C and 5% CO_2 . After incubation, test solutions were removed, cells were washed with HBSS, and fresh media was added to each well. Cells were allowed to proliferate overnight before being assessed for viability using the MTT Cell Proliferation Assay (ATCC, Manassas, VA). Viability was determined according to the manufacturer's recommended protocol using a SAFIRE, XFLUOR4, V4.50 microplate reader (Tecan Group Ltd, Morrisville, NY).

Visualization of cell internalization

Cells were seeded on poly-D-lysine-coated glass bottom culture dishes (MatTek Corporation, Ashland, MA) and were allowed to attach and proliferate. Once cells reached ~80% confluency, the media was removed and FAM-siRNA or robed-FAM-siRNA in media was added. Cells were incubated with test formulations for 4 hours under standard culture conditions. After incubation with test solutions, cells were stained with 5 $\mu\text{g/mL}$ Hoechst 33342 (Life Technologies, Grand Island, NY) for 5 min at room temperature and then washed 3 times for 5 min each with Hank's Balanced Salt Solution (HBSS, Lonza Group Ltd., Basel, Switzerland). Cells were imaged using an Olympus Fluoview 1000 Spectral confocal microscope (Olympus, Center Valley, PA). All instrument settings were kept constant for any comparisons between experimental conditions and a 30X silicon immersion objective was used to capture the entire thickness of the cell.

Evaluation of gene silencing in cell culture

Cells were seeded in a 96-well microplate (Corning Inc., Corning, NY) and were allowed to attach and proliferate. Once cells reached ~80% confluency, media was removed and FAM-

siRNA or robed-FAM-siRNA in media was added. Media alone was used as a control. Cells were incubated with test solution for 72 hours at 37°C and 5% CO₂. After incubation, total protein and GAPDH expression were quantified. Total protein was assessed with the Bicinchoninic Acid Protein Assay Kit (Thermo Scientific, Rockford, IL) and GAPDH levels were measured using the KDalert GAPDH Assay Kit (Life Technologies, Grand Island, NY) according to the manufacturer's recommended protocol and using a SAFIRE, XFLUOR4, V4.50 microplate reader (Tecan Group Ltd, Morrisville, NY).

Evaluation of disease treatment and biocompatibility in MatTek Epiderm™ tissue

MatTek Epiderm™ human skin equivalent tissues were used as described previously [23] to evaluate treatment of premature wrinkle formation using robed-siRNA. Briefly, Epiderm™ tissues were allowed to acclimate at 37°C and 5% CO₂ for 24 hours with 2.5 mL media in 6-well culture plates (Corning Inc., Corning, NY). After 24 hours incubation, media was replaced and 100 µL of ddH₂O (control) or 50 µM native elastase siRNA (siRNA3), 50 µM BDOA-control non-silencing siRNA (siRNA4), 25 µM BDOA-siRNA3, or 50 µM BDOA-siRNA3 was applied to the apical side of the skin tissues. After 24 hours incubation, test formulations were removed and the skin was exposed to 200 mJ/cm² UVB irradiation using a 6-watt UV lamp with 302nm wavelength and white light tubes (Cole Parmer, Vernon, IL). Irradiation was measured with a Solarmeter model 6.2 UV meter (Solar Light Company Inc., Glenside, PA). Following exposure freshly prepared formulations were reapplied to the skin tissues and incubated for an additional 96 hours. At the conclusion of the experiment, 2 mL of media was collected and analyzed for elastin and elastase content using a Human Elastin ELISA kit and Human MMP-12 ELISA kit (Biomatik USA LLC., Wilmington, DE), respectively, following the manufacturer's recommended protocol. Biocompatibility of the test formulations was confirmed using the MTT assay following MatTek's recommended protocol.

Statistical analysis

Data reported are mean±SD except where otherwise noted. Where appropriate, statistical significance was confirmed by one way-ANOVA and post-hoc test or the two-tailed, unpaired Student's t-test in Microsoft Excel. The level of significance was set at $p < 0.05$.

RESULTS

Synthesis and characterization of robed-siRNAs

siRNAs robed with ionic liquid (IL) moieties were synthesized using a simple, scalable two-step process consisting of cation/anion exchange followed by acid-base neutralization (Fig. 1).

Benzyl dimethyl alkyl ammoniums were used as the IL moieties as described previously [20]. Three different alkyl chain lengths were used: octyl (BDOA), tetradecyl (BDTA), and stearyl (BDSA) (Fig. S1). Further, several different siRNA sequences were used including two different glyceraldehyde-3-phosphate dehydrogenase (GAPDH) knockdown sequences (abbreviated as siRNA1 and siRNA2) as well as a matrix metalloproteinase-12 (MMP-12) knockdown sequence (abbreviated as siRNA3) and a non-silencing control siRNA sequence

(abbreviated as siRNA4) (Fig. S1). Complexes were then lyophilized to remove all residual water. 1:1 ion pairing and final compositions were confirmed using elemental analysis (Table S1). Specifically, percent weights of carbon, nitrogen, and hydrogen were not significantly different than expected values.

Robed-siRNAs were characterized by octanol/water partitioning ($P_{o/w}$) (Table 1). As expected all robed-siRNAs partition well into octanol. While naked siRNA1 was highly hydrophilic ($\text{Log } P_{o/w} = -3.76 \pm 0.13$), robed-siRNA1s were hydrophobic with $\text{Log } P_{o/w}$ ranging from 1.43 ± 0.04 for BDOA-siRNA1 to 3.79 ± 0.38 for BDTA-siRNA1. Similar results were observed regardless of the siRNA sequence used (Table 1). In addition, all robed-siRNAs were more hydrophobic than their corresponding benzyl dimethyl alkyl ammonium salt. For example, BDOA chloride is only slightly hydrophobic with $\text{Log } P_{o/w} = 0.85$ (predicted value). In contrast, BDOA-siRNA1 was ~4-fold more hydrophobic.

The size and aggregation propensity of robed-siRNAs in octanol and water were determined using dynamic light scattering (DLS). In contrast to polyplexes which do not readily penetrate skin, our goal was to produce siRNA robed with IL moieties at the molecular scale. Since siRNA sequence does not appear to affect partitioning, only siRNA1 was studied here. Importantly, DLS measurement suggests robed-siRNA1 form individual complexes and do not aggregate in octanol (representative of the SC) (Table 2). Hydrophilic naked siRNA1 was not soluble in octanol and thus DLS was not performed. In water, however, the hydrodynamic diameter of naked siRNA1 was 1.06 ± 0.32 nm. All robed-siRNAs were soluble in octanol. Hydrodynamic diameters of robed-siRNA1 in octanol were larger than for naked siRNA1 in water and increased with increasing alkyl chain length of the IL moieties. Aggregation was observed in water for BDTA- and BDSA-siRNA1. Specifically, BDTA- and BDSA-siRNA1 possessed hydrodynamic diameters in water of 182.0 ± 92.4 and 846.4 ± 176.1 nm, respectively. In contrast, BDOA-siRNA1 was not observed to aggregate in water (hydrodynamic diameter = 1.63 ± 0.16 nm). As a control, the size of a siRNA polyplex (Lipofectamine® RNAiMax, Life Technologies, Grand Island, NY) was also determined. Similar to naked siRNA1, RNAiMax-siRNA1 was insoluble in octanol thus DLS was not performed. In water, the hydrodynamic radius was 88.3 ± 1.2 nm. Taken together the data indicate that robed-siRNAs do not aggregate in octanol and therefore may move through the hydrophobic SC as single complexes as opposed to polyplexes.

Skin transport of robed-siRNAs

Skin penetration was assessed using Franz diffusion cells (FDCs) as described previously [17, 21]. Importantly, skin transport of siRNA1 was significantly enhanced by robing with IL moieties (Fig. 2 and Fig. S2). % applied dose delivered into the viable epidermis was 9.85 ± 2.64 , 9.60 ± 2.85 , and 7.33 ± 0.24 for BDOA-, BDTA-, and BDSA-siRNA1 compared to only 2.06 ± 0.15 for naked siRNA1. Similarly, % applied dose delivered into the deeper skin tissue layer of the dermis was 12.94 ± 2.56 and 8.89 ± 0.77 for BDOA- and BDSA-siRNA1 compared to only 2.26 ± 1.16 for naked siRNA1. Interestingly, % applied dose delivered into the dermis was actually retarded (0.49 ± 0.18) when siRNA1 was robed with BDTA. Similar results were observed regardless of siRNA sequence used (Fig. S3).

Cell internalization and biocompatibility of robed-siRNAs

The ability of robed-siRNA to cross cell membranes was assessed *in vitro* by confocal laser scanning microscopy (CLSM). Human adult epidermal keratinocytes (HEKa cells) are the primary cell type in the viable epidermis and thus were used for all *in vitro* studies. HEKa cells were incubated with 100 nM robed-siRNA1 for 4 hours. Cells were then washed, nuclei stained with Hoechst 33342, and imaged to visualize siRNA1 internalization. Internalization of robed-siRNA1 was compared to internalization of naked siRNA1 (Fig. 3). Importantly, compared to naked siRNA1, cell internalization was enhanced by robing with IL moieties. On the other hand, the extent of cell internalization does appear to depend on the IL moiety used. For example, BDTA showed the most internalization enhancement while BDOA showed the least enhancement (Fig. 3).

Biocompatibility of robed-siRNAs was assessed *in vitro* against HEKa cells using the MTT assay (ATCC, Manassas, VA). Importantly, robed-siRNA1 shows negligible cytotoxicity to HEKa cells up to 100 nM (Fig. 4a). Above 100 nM, however, cytotoxicity was observed for BDTA-siRNA1 and BDSA-siRNA1. Specifically, following incubation with 1000 nM BDTA- or BDSA-siRNA1 % cell viability was 18.48 ± 2.66 and 67.60 ± 9.92 compared to HEKa cells incubated with culture media alone. In contrast, no significant cytotoxicity was observed following incubation with 1000 nM BDOA-siRNA1. Naked siRNA1 was non-toxic for all concentrations studied (Fig. 4). In addition, BDOA, BDTA, and BDSA appear to be more toxic as free salts than when delivered as robed with siRNAs (Fig. 4b). This observation was most apparent at higher concentrations; however, the differences were not statistically significant ($p > 0.05$). Again, similar results were observed regardless of the siRNA sequence used (Fig. S4).

Taken together, BDOA-siRNA emerged as the best candidate for topical RNAi therapy due to its excellent transport into deep viable skin layers (epidermis and dermis), its ability to improve cell internalization compared to naked siRNA, and its superior biocompatibility compared to BDTA- and BDSA-siRNA. Since siRNA in cell culture typically requires extended incubation times to elicit a response, BDOA-siRNA cell internalization and biocompatibility was also confirmed following 72 hour incubation. As expected, cell internalization in HEKa cells was significantly enhanced following 72 hour exposure to BDOA-siRNA1 compared to naked siRNA1 (Fig. 5a). Further, negligible cytotoxicity was observed up to 300 nM BDOA-siRNA relative to cells incubated with media alone (Fig. 5b).

As an additional control, cell internalization of BDOA-siRNA1 was compared to Lipofectamine RNAiMax-siRNA1. In contrast to RNAiMax-siRNA1, BDOA-siRNA1 shows significantly less cell internalization (Fig. S5a–b). Interestingly, however, the internalization pattern of BDOA-siRNA1 was strikingly different (Fig. S5c–d). Specifically, BDOA-siRNA1 demonstrated diffuse fluorescence indicative of cytoplasmic localization. In contrast, RNAiMax-siRNA1 demonstrated punctate fluorescence indicative of endosomal sequestration.

In vitro gene silencing

The ability of BDOA-siRNA to elicit a therapeutic response was first confirmed *in vitro*. GAPDH was used as a model knockdown target. BDOA-siRNA1 was incubated with HEKa cells at various concentrations and GAPDH knockdown was assessed as described previously [17]. As controls, naked siRNA1 and BDOA-siRNA4 (non-silencing control siRNA) were also studied. As an additional control RNAiMax-siRNA1 and RNAiMax-siRNA4 were also used at the manufacturer's recommended concentration (10 nM). As expected, naked siRNA1 as well as non-silencing siRNA4 formulations did not reduce GAPDH expression in HEKa cells after 72 hour incubation (Fig. 6). In addition, 100 nM and 200 nM BDOA-siRNA1 showed no significant difference in GAPDH expression compared to the untreated control. Incubation with 300 nM BDOA-siRNA1, however, did result in significant GAPDH knockdown (58.7 ± 2.0 % GAPDH expression compared to the untreated control) (Fig. 6). Similarly, incubation with 10 nM RNAiMax-siRNA1 resulted in significant GAPDH knockdown (36.9 ± 3.2 % GAPDH expression compared to the untreated control).

Clinical potential

The clinical potential of BDOA-siRNA was assessed against a skin aging model in human skin equivalent tissues [22]. Elastase upregulation has been proposed as an important factor for ultraviolet irradiation induced wrinkle formation [24]. Therefore, knockdown of elastase with topically applied RNAi is a proposed option for the treatment and prevention of skin wrinkling. Following UVB irradiation and application of BDOA-siRNAs there was no observed decrease in tissue viability (Fig. 7). This result confirms 1) irradiation of a suberythral dose and 2) biocompatibility of BDOA-siRNA. Moreover, UVB irradiation resulted in significant upregulation of elastase (Fig. 8a) and significant reduction in elastin (Fig. 8b) compared to tissues not exposed to UVB irradiation confirming the validity of the disease model. Topical application of naked siRNA3 or BDOA-siRNA4 was unable to protect against elastase upregulation and consequent elastin degradation. In contrast, application of BDOA-siRNA3 at both 50 μ M and 25 μ M were able to maintain the non-altered state of the skin following UVB irradiation thus confirming the clinical potential of BDOA-siRNAs (Fig. 8).

DISCUSSION

Skin wrinkling is a cosmetic condition that affects nearly everyone as they age. Unfortunately, few effective therapies exist for the reduction of wrinkles, and thus, patients seeking treatment must resort to costly, invasive procedures such as intradermal injections of botulinum toxin A. Alternatively, topical RNAi therapies may be beneficial as both a treatment and prevention strategy. For example, elastase upregulation has been proposed as an important factor for ultraviolet irradiation induced wrinkle formation. Inhibition of elastase with N-phenethylphosphonyl-L-leucyl-L-tryptophane resulted in reduced formation of wrinkles in mice exposed to daily doses of ultraviolet irradiation [24]. Therefore, knockdown of elastase with topically applied RNAi may be a viable option for the treatment and prevention of skin wrinkling, as well as provide patients with a safe alternative to current options.

Topically applied RNAi must transport through the skin and into cells to elicit a therapeutic response. In addition, the therapy must be biocompatible. BDOA-siRNA exhibited the most enhancement into the deep viable tissue layers of the skin (epidermis and dermis) (Fig. 2 and Fig. S2–S3). In addition, although cell internalization was less efficient compared to BDTA- and BDSA-siRNA, BDOA-siRNA shows negligible cytotoxicity to skin cells at significantly higher concentrations (Fig. 4 and Fig. S4). Biocompatibility becomes particularly important when applying robed-siRNA on the skin where we would reasonably expect $> 1 \mu\text{M}$ concentrations in the epidermis and dermis (Fig. 2 and Fig. S2–S3, and assuming thickness of human skin on average 26, 66, and 3338 μm for the SC, epidermis, and dermis, respectively [25]). BDOA was the only counter species studied in this work that did not exhibit significant cytotoxicity at concentrations up to $1 \mu\text{M}$ (Fig. 4 and Fig. S4). Thus, BDOA-siRNA emerged as the best candidate robed-siRNA for topical RNAi therapy.

The ability of BDOA-siRNA to prevent wrinkle formation was assessed against a skin aging model in human skin equivalent tissues as described previously [23]. BDOA robed-anti-elastase siRNA was applied on the apical surface of human skin equivalent tissues. As controls, saline, naked anti-elastase siRNA, and BDOA robed-non-silencing control siRNA were also tested. UVB irradiation induced significant elastase upregulation and elastin degradation in control tissues (Fig. 8). In contrast, tissues treated with $25 \mu\text{M}$ or $50 \mu\text{M}$ BDOA robed-anti-elastase siRNA showed identical elastase and elastin content as unexposed, healthy tissues (*i.e.* no UVB treatment).

Efficacy and safety of BDOA-siRNA is related to its physicochemical properties. The extent of transport correlates well with hydrophobicity of robed-siRNAs. Naked siRNA is hydrophilic and transport through the SC is minimal (Fig. 2 and Fig. S2–S3). In contrast, the hydrophobic robed-siRNAs were able to penetrate the SC in significant quantities. BDTA was the most hydrophobic and transport into the SC was highest. However, due to high fusogenicity between BDTA and SC lipids, BDTA-siRNA was retained in superficial layers of the skin and did not penetrate into deep tissue layers. Thus, a balance must be sought to allow enhanced partitioning into the SC from the donor solution as well as out of the SC into viable tissue layers of the skin. Porcine skin is commonly recognized as representative of human skin for drug transport studies. For example thickness of porcine skin layers, follicular structure and density, and chemical composition of pig and human skin are similar [26]. There may, however, be some differences in the penetration into porcine and human skin. Porcine skin has been reported to be slightly more permeable to hydrophobic alkanes [26]. Also note that the transport studies performed here were conducted with intact skin. Transport kinetics in diseased skin would likely differ compared to intact skin.

Cell internalization of robed-siRNAs also correlates well with hydrophobicity. Log $P_{o/w}$ of BDTA-siRNA was highest among robed-siRNAs, and BDTA-siRNA1 possessed the highest degree of cell internalization. In contrast, Log $P_{o/w}$ of BDOA-siRNA was lowest among robed-siRNAs, and BDOA-siRNA1 possessed the lowest degree of cell internalization albeit still significantly higher than was observed for naked siRNA1 (Fig. 3, 5a and Fig. S5).

The relationships between hydrophobicity and transport properties of robed-siRNAs presents an opportunity for optimizing their efficacy through facile manipulation of the IL

moiety counter species. Hydrophobicity appears tunable to an extent by varying the alkyl chain length of the counter species. Robing with BDOA resulted in the lowest Log $P_{o/w}$ while BDTA- and BDSA-siRNAs were significantly more hydrophobic. However, the relation between hydrophobicity and chain length was not linear. Specifically, BDTA (alkyl chain = 14 carbons) was significantly more hydrophobic than BDSA (alkyl chain = 18 carbons). This is in contrast to the Log $P_{o/w}$ of individual benzyl dimethyl alkyl ammonium species which increase linearly with chain length (Fig. S6, $r^2 = 0.9497$) according to predicted and experimentally determined values. Aggregation propensity and the size of aggregates also appear to depend on alkyl chain length (Table 2). Ideally, robed-siRNAs would transport as single, hydrophobic molecules as opposed to polyplexes to maximize transdermal and transcellular transport. Aggregation into polyplexes is expected to significantly hinder the kinetics of skin transport as well as the depth of delivery thus aggregation should be avoided through optimization of the IL moiety alkyl chain length. Studies exploring the efficacy of additional IL moieties other than benzyl dimethyl alkyl ammoniums for RNAi delivery is a major focus of ongoing work that will be communicated at a later date.

Lipofectamine RNAiMax is a commercially available siRNA polyplex platform that has been optimized over many years to afford consistent, fast, and efficient delivery of siRNA into cells. The significant difference in cell fluorescence following incubation with BDOA-siRNA1 compared to unrobed siRNA1 (Fig. 5), coupled with the significant gene-silencing that was observed for BDOA-siRNA1 compared to the appropriate controls (Fig. 6) supports that BDOA-siRNA is internalized into the cytoplasm of keratinocytes. However, RNAiMax-siRNA outperformed BDOA-siRNA at 30-fold lower concentrations (Fig. 6). Therefore, the data do not support the use of BDOA-siRNA for gene-silencing applications in cell culture *in vitro*. Optimizing cell internalization of robed-siRNA will be necessary to realize its full potential both in the laboratory and in the clinic. The difference could be related to the stability of robed-siRNAs in aqueous solution. RNAiMax-siRNA polyplexes are stable in high salt and buffered solutions while robed-siRNAs are held together simply by 1:1 ion pairing and thus are expected to dissolve in dilute aqueous conditions. Maximizing ion association between siRNA phosphate groups and the IL moiety may be one potential option to mitigate dissociation and subsequently maximize cell internalization and gene silencing at lower concentrations in solution. Ionic liquids are known to have varying degrees of ion association both *neat* as well as in solution depending on the choice of ion pairing [27]. Again the relationship between ion association and transport properties of robed-siRNAs presents an opportunity for optimizing their efficacy through facile manipulation of the IL moiety counter species. This should be explored in future studies.

High concentrations of benzyl dimethyl alkyl ammonium robed-siRNA needed to minimize dissociation were also met with significant observed cytotoxicity. As with transport properties, cytotoxicity correlates well with hydrophobicity. This is not surprising given hydrophobicity is commonly used as an indicator for IL moiety cytotoxicity [28, 29], but may impose some limitations of robed-siRNA use as a balance may need to be struck between transport enhancement into skin and cells and biocompatibility. Interestingly, however, robed-siRNAs do appear to be less toxic than equal concentrations of the IL moiety chloride salts (Fig. 4). This suggests ion pairing may reduce cytotoxicity of the

individual components somewhat, which is in agreement with previous studies of ionic liquid toxicity [30, 31]. Thus, the relationship between ion association and cytotoxicity properties of robed-siRNAs presents an opportunity for optimizing their safety through facile manipulation of the IL moiety counter species.

On the other hand, no toxicity effects were observed in human skin equivalent tissues. Therefore, biocompatibility may only be an issue for extending this strategy for knockdown in cell culture or following systemic administration *in vivo*. Cell internalization enhancement relative to naked siRNA and avoidance or release from endosomal compartments (Fig. S5) in combination with excellent dermal delivery highly supports the use of BDOA-siRNA for gene-silencing applications in skin. Indeed, the skin presents a unique environment where robed-siRNAs can penetrate through the hydrophobic SC as single complexes and then interact immediately with diseased cells in the viable epidermis, thus limiting the extent of dissociation and enhancing cell internalization and gene silencing. This is consistent with our observations after application of BDOA-siRNA on human skin equivalent tissues (Fig. 7 and Fig. 8). In addition, robed-siRNA properties and behavior are independent of siRNA sequence. This opens up the possibilities of using robed-siRNAs for the treatment of myriad other skin diseases for which known protein knockdown targets exist such as psoriasis, atopic dermatitis, skin cancer, melasma, pachyonychia congenita, and many others [2]. Further, this opens up the possibility for more effective treatments through the use robed-siRNA cocktails that knockdown several protein targets simultaneously.

CONCLUSION

Topical delivery of RNAi such as siRNA holds potential to address the growing burden of skin disease. However, skin disease symptoms typically present over a large surface area which precludes the use of most delivery enhancement strategies identified to-date. To address these concerns, we present RNAi robed with ionic liquid moieties. We show robed-siRNAs can be synthesized by a simple two-step process from bulk materials. Moreover, robing affords tuneability of properties necessary for dermal drug delivery including skin transport, cell internalization, and biocompatibility irrespective of siRNA sequence. Through a comprehensive screening strategy, we identified BDOA-siRNA which was able to significantly knockdown proteins in cell culture and human skin equivalent tissues. BDOA-siRNA was also shown to protect against elastin degradation in skin following UVB exposure. Together, the data strongly support therapeutic RNAi robed with ionic liquid moieties as a simple, scalable prodrug platform for treating skin disease. Future work should focus on IL moiety optimization for improved cell internalization and biocompatibility, as well as applying this platform for the treatment of other skin diseases such as psoriasis, atopic dermatitis, and melasma.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We want to acknowledge the NRI-MCDB Microscopy Facility for access to the Leica Cryostat CM1850 and the Olympus Fluoview 1000 Spectral confocal microscope which was supported by NIH Grant 1 S10 OD010610-01A1. Authors acknowledge support from National Institutes of Health (5R21CA19133-02). We also thank the Biological Nanostructures Laboratory within the California NanoSystems Institute, supported by UCSB. M.Z. was supported by UCSB's Center for Bioengineering under the Mellichamp Fellowship for Graduate Students. SM acknowledges support from Duncan and Suzanne Mellichamp Chair.

References

1. Hay RJ, Johns NE, Williams HC, Bolliger IW, Dellavalle RP, Margolis DJ, Marks R, Naldi L, Weinstock MA, Wulf SK, Michaud C, Murray CJL, Naghavi M. The Global Burden of Skin Disease in 2010: An Analysis of the Prevalence and Impact of Skin Conditions. *Journal of Investigative Dermatology*. 2014; 134:1527–1534. [PubMed: 24166134]
2. Zakrewsky M, Kumar S, Mitragotri S. Nucleic acid delivery into skin for the treatment of skin disease: Proofs-of-concept, potential impact, and remaining challenges. *Journal of Controlled Release*. 2015; 219:445–456. [PubMed: 26385169]
3. Bickers DR, Lim HW, Margolis D, Weinstock MA, Goodman C, Faulkner E, Gould C, Gemmen E, Dall T. The burden of skin diseases: 2004: A joint project of the American Academy of Dermatology Association and the Society for Investigative Dermatology. *Journal of the American Academy of Dermatology*. 2006; 55:490–500. [PubMed: 16908356]
4. Freeman EE. A Seat at the Big Table: Expanding the Role of Dermatology at the World Health Organization and Beyond. *Journal of Investigative Dermatology*. 2014; 134:2663–2665. [PubMed: 25318423]
5. Prausnitz MR, Mitragotri S, Langer R. Current status and future potential of transdermal drug delivery. *Nature reviews Drug discovery*. 2004; 3:115–124. [PubMed: 15040576]
6. Brown MB, Martin GP, Jones SA, Akomeah FK. Dermal and transdermal drug delivery systems: current and future prospects. *Drug delivery*. 2006; 13:175–187. [PubMed: 16556569]
7. Chong RHE, Gonzalez-Gonzalez E, Lara MF, Speaker TJ, Contag CH, Kaspar RL, Coulman SA, Hargest R, Birchall JC. Gene silencing following siRNA delivery to skin via coated steel microneedles: In vitro and in vivo proof-of-concept. *Journal of Controlled Release*. 2013; 166:211–219. [PubMed: 23313112]
8. Lee WR, Shen SC, Zhuo RZ, Wang KC, Fang JY. Enhancement of topical small interfering RNA delivery and expression by low-fluence erbium:YAG laser pretreatment of skin. *Human gene therapy*. 2009; 20:580–588. [PubMed: 19239381]
9. Tran, MA., Gowda, R., Park, E-J., Adair, J., Smith, N., Kester, M., Robertson, GP. Noninvasive Drug Delivery Using Ultrasound: Targeting Melanoma Using siRNA Against Mutant (V600E) B-Raf. In: Ebbini, ES., editor. 8th International Symposium on Therapeutic Ultrasound; 2009. p. 423–427.
10. Kigasawa K, Kajimoto K, Hama S, Saito A, Kanamura K, Kogure K. Noninvasive delivery of siRNA into the epidermis by iontophoresis using an atopic dermatitis-like model rat. *International journal of pharmaceutics*. 2010; 383:157–160. [PubMed: 19732811]
11. Hsu T, Mitragotri S. Delivery of siRNA and other macromolecules into skin and cells using a peptide enhancer. *Proceedings of the National Academy of Sciences of the United States of America*. 2011; 108:15816–15821. [PubMed: 21903933]
12. Lin CM, Huang K, Zeng Y, Chen XC, Wang S, Li Y. A simple, noninvasive and efficient method for transdermal delivery of siRNA. *Archives of Dermatological Research*. 2012; 304:139–144. [PubMed: 22009459]
13. Uchida T, Kanazawa T, Kawai M, Takashima Y, Okada H. Therapeutic Effects on Atopic Dermatitis by Anti-RelA Short Interfering RNA Combined with Functional Peptides Tat and AT1002. *Journal of Pharmacology and Experimental Therapeutics*. 2011; 338:443–450. [PubMed: 21531792]

14. Yi X, Zhao G, Zhang H, Guan D, Meng R, Zhang Y, Yang Q, Jia H, Dou K, Liu C, Que F, Yin JQ. MITF-siRNA Formulation Is a Safe and Effective Therapy for Human Melasma. *Molecular Therapy*. 2011; 19:362–371. [PubMed: 21119619]
15. Randeria PS, Seeger MA, Wang XQ, Wilson H, Shipp D, Mirkin CA, Paller AS. siRNA-based spherical nucleic acids reverse impaired wound healing in diabetic mice by ganglioside GM3 synthase knockdown. *Proceedings of the National Academy of Sciences of the United States of America*. 2015; 112:5573–5578. [PubMed: 25902507]
16. Zheng D, Giljohann DA, Chen DL, Massich MD, Wang XQ, Iordanov H, Mirkin CA, Paller AS. Topical delivery of siRNA-based spherical nucleic acid nanoparticle conjugates for gene regulation. *Proceedings of the National Academy of Sciences of the United States of America*. 2012; 109:11975–11980. [PubMed: 22773805]
17. Chen M, Zakrewsky M, Gupta V, Anselmo AC, Slee DH, Muraski JA, Mitragotri S. Topical delivery of siRNA into skin using SPACE-peptide carriers. *Journal of Controlled Release*. 2014; 179:33–41. [PubMed: 24434423]
18. Meade BR, Gogoi K, Hamil AS, Palm-Apergi C, Berg Avd, Hagopian JC, Springer AD, Eguchi A, Kacsinta AD, Dowdy CF, Presente A, Lonn P, Kaulich M, Yoshioka N, Gros E, Cui X-S, Dowdy SF. Efficient delivery of RNAi prodrugs containing reversible charge-neutralizing phosphotriester backbone modifications. *Nat Biotech*. 2014; 32:1256–1261.
19. Cutler JJ, Auyeung E, Mirkin CA. Spherical Nucleic Acids. *Journal of the American Chemical Society*. 2012; 134:1376–1391. [PubMed: 22229439]
20. Nishimura N, Nomura Y, Nakamura N, Ohno H. DNA strands robed with ionic liquid moiety. *Biomaterials*. 2005; 26:5558–5563. [PubMed: 15860212]
21. Karande P, Jain A, Mitragotri S. Discovery of transdermal penetration enhancers by high-throughput screening. *Nature biotechnology*. 2004; 22:192–197.
22. Lindemann U, Weigmann H-J, Schaefer H, Sterry W, Lademann J. Evaluation of the Pseudo-Absorption Method to Quantify Human Stratum corneum Removed by Tape Stripping Using Protein Absorption. *Skin Pharmacol Physiol*. 2003; 16:228–236.
23. Afaq F, Zaid MA, Khan N, Dreher M, Mukhtar H. Protective effect of pomegranate-derived products on UVB-mediated damage in human reconstituted skin. *Exp Dermatol*. 2009; 18:553–561. [PubMed: 19320737]
24. Tsuji N, Moriwaki S, Suzuki Y, Takema Y, Imokawa G. The Role of Elastases Secreted by Fibroblasts in Wrinkle Formation: Implication Through Selective Inhibition of Elastase Activity¶. *Photochemistry and Photobiology*. 2001; 74:283–290. [PubMed: 11547567]
25. Bronaugh RL, Stewart RF, Congdon ER. Methods for in vitro percutaneous absorption studies II. Animal models for human skin. *Toxicology and applied pharmacology*. 1982; 62:481–8. [PubMed: 7071863]
26. Godin, Biana, Touitou, Elka. Transdermal skin delivery: Predictions for humans from in vivo, ex vivo and animal models. *Advanced Drug Delivery Reviews*. Sep 30; 2007 59(11):1152–1161. [PubMed: 17889400]
27. Zhang S, Sun N, He X, Lu X, Zhang X. Physical properties of ionic liquids: Database and evaluation. *Journal of Physical and Chemical Reference Data*. 2006; 35:1475–1517.
28. Ranke J, Mölter K, Stock F, Bottin-Weber U, Poczebott J, Hoffmann J, Ondruschka B, Filser J, Jastorff B. Biological effects of imidazolium ionic liquids with varying chain lengths in acute *Vibrio fischeri* and WST-1 cell viability assays. *Ecotoxicology and environmental safety*. 2004; 58:396–404. [PubMed: 15223265]
29. Ranke J, Müller A, Bottin-Weber U, Stock F, Stolte S, Arning J, Störmann R, Jastorff B. Lipophilicity parameters for ionic liquid cations and their correlation to in vitro cytotoxicity. *Ecotoxicology and environmental safety*. 2007; 67:430–438. [PubMed: 17034854]
30. Aoyagi K, Zakrewsky M, Mitragotri S. Formulating propranolol as an amorphous melt affords reduced skin irritation potential for transdermal drug delivery. *TECHNOLOGY*. 2015; 03:214–238.
31. Zakrewsky M, Lovejoy KS, Kern TL, Miller TE, Le V, Nagy A, Goumas AM, Iyer RS, Del Sesto RE, Koppisch AT, Fox DT, Mitragotri S. Ionic liquids as a class of materials for transdermal

delivery and pathogen neutralization. Proceedings of the National Academy of Sciences. 2014; 111:13313–13318.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 1. Synthesis of robed-siRNA is a simple two-step process consisting of cation or anion exchange followed by acid-base neutralization

(a) Cation and anion exchange results in acidic siRNA and basic IL moieties, respectively.

(b) Acid-base neutralization results in aqueous siRNA robed with IL moieties.

Fig. 2. Transdermal delivery of siRNA1 is significantly enhanced when robed with IL moieties
(a) Penetration of FAM-siRNA1 into individual layers of porcine skin. Delivery depth increases from left to right. Naked FAM-siRNA1 (open bars), BDOA-FAM-siRNA1 (hatched bars), BDTA-FAM-siRNA1 (cross-hatched bars), and BDSA-FAM-siRNA1 (closed bars). Error bars represent mean + SD for $n = 3$. * $p < 0.05$ compared to naked FAM-siRNA1 (open bars). **(b)** Delivery enhancement of robed-FAM-siRNA1 into skin was confirmed with confocal microscopy. Porcine skin was incubated with saline (untreated control), naked FAM-siRNA1, BDOA-FAM-siRNA1, or BDSA-FAM-siRNA1. FAM-siRNA1 is indicated in green. Scale bar represents 300 μm.

Fig. 3. Robing of siRNA1 increased cell internalization into HEK293 cells

Representative images of HEK293 cells incubated with 100 nM naked FAM-siRNA1, BDOA-FAM-siRNA1, BDTA-FAM-siRNA1, and BDSA-FAM-siRNA1 are shown. FAM-siRNA1 is indicated in green. Cell nuclei stained with Hoechst 33342 is indicated in blue. Scale bar represents 25 µm.

Fig. 4. Cytotoxicity of siRNA robed with IL moieties is negligible, but shows some dependence on alkyl chain length

Cytotoxicity of **(a)** naked FAM-siRNA1 and robed-FAM-siRNA1s or **(b)** naked FAM-siRNA1 and IL moieties are shown after 4 hour incubation. Naked FAM-siRNA1 data are duplicated in both graphs for easy comparison. Error bars represent mean + SD for $n = 6$. * $p < 0.05$ relative to control (incubated with media alone).

Fig. 5. BDOA-siRNA1 shows enhanced internalization into HEK293 cells and is non-toxic following 72 hour incubation

(a) HEK293 cells were incubated with 300 nM native FAM-siRNA1 (*left*) or BDOA-FAM-siRNA1 (*right*). FAM-siRNA1 is indicated in green. Cell nuclei stained with Hoechst 33342 is indicated in blue. Scale bar represents 50 μm. (b) Cytotoxicity of BDOA-FAM-siRNA1 incubated with HEK293 cells for 72 hours. Error bars represent mean + SD for n = 6. * p < 0.05 relative to control (incubated with media alone).

Fig. 6. BDOA-siRNA1 results in significant gene silencing in HEK293 cells *in vitro*
GAPDH expression is relative to control (cells incubated with media alone). Error bars represent mean + SD for n = 6. * p < 0.05 compared to control.

Fig. 7. BDOA-siRNA does not cause skin irritation following application to MatTek Epiderm tissues

% Viability is normalized by the control (tissue not exposed to UV light). Error bars represent mean + SD for $n = 3$. No statistically significant differences were observed.

Fig. 8. Robed-siRNA significantly limited elastase upregulation and subsequent elastin degradation following MatTek Epiderm® tissue irradiation with UVB light
(a) Elastase expression is normalized by the UV control (tissue exposed to UV light and treated with saline). **(b)** Elastin expression is normalized by the no UV control (tissue not exposed to UV light). Error bars represent mean + SD for $n = 3$. ** $p < 0.01$ compared to UV; saline control. * $p < 0.05$ compared to the no UV control.

Table 1

siRNA robed with IL moieties are significantly more hydrophobic than native siRNA and native IL moiety.

Log P _{o/w}			
	Chloride	siRNA1	siRNA2
Sodium		-3.76 (0.13)	-3.80 (0.21)
BDOA	0.85 ¹	1.43 (0.04)	1.88 (0.16)
BDTA	1.81 ²	3.79 (0.38)	4.27(0.78)
BDSA	3.23 ³	2.31 (0.04)	2.75 (0.33)

Table shows Log P_{o/w} for FAM-siRNA1 and -siRNA2. Standard deviation for n = 3 is given in parentheses.

¹ Predicted value;

² Hansch, C., Leo, A., D. Hoekman. Exploring QSAR - Hydrophobic, Electronic, and Steric Constants. Washington, DC: American Chemical Society., 1995., p. 182;

³ Hansch, C. et al., American Chemical Society., 1995., p. 188.

Table 2

DLS measurements suggest siRNA robed with IL moieties form individual complexes and do not aggregate in octanol (representative of the SC), while in water aggregation is dependent on alkyl chain length of the IL moiety.

	Effective diameter (nm) in octanol	Effective diameter (nm) in water
siRNA1	Insoluble	1.06 (0.32)
BDOA-siRNA1	2.03 (0.33)	1.63 (0.16)
BDTA-siRNA1	2.68 (0.52)	182.0 (92.4)
BDSA-siRNA1	4.44 (1.29)	846.4 (176.1)
RNAiMax-siRNA1	Insoluble	88.3 (1.2)

Table shows effective complex diameters (nm) for robed-FAM-siRNA. Standard deviation for n = 3 is given in parenthesis.