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Original Research

Early-Stage Development of Novel Cyclodextrin-siRNA Nanocomplexes Allows for Successful Postnebulization Transfection of Bronchial Epithelial Cells

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Abstract

Background: Successful delivery of small interfering RNA (siRNA) to the lungs remains hampered by poor intracellular delivery, vector-mediated cytotoxicity, and an inability to withstand nebulization. Recently, a novel, cyclodextrin (CD), SC12CDClickpropylamine, consisting of distinct lipophilic and cationic subunits, has been shown to transfect a number of cell types. However, the suitability of this vector for pulmonary siRNA delivery has not been assessed to date. To address this, a series of high-content analysis (HCA) and post-nebulization assays were devised to determine the potential for CD-siRNA delivery to the lungs.
Methods: SC12CDClickpropylamine-siRNA mass ratios (MRs) were examined for size and zeta potential. In-depth analysis of nanocomplex uptake and toxicity in Calu-3 bronchial epithelial cells was examined using IN Cell® HCA assays. Nebulized SC12CDClickpropylamine nanocomplexes were assessed for volumetric median diameter (VMD) and fine particle fraction (FPF) and compared with saline controls. Finally, postnebulization stability was determined by comparing luciferase knockdown elicited by SC12CDClickpropylamine nanocomplexes before and after nebulization.
Results: SC12CDClickpropylamine-siRNA complexation formed cationic nanocomplexes of ≤ 200 nm in size and led to significantly higher levels of siRNA uptake into Calu-3 cells compared with RNAiFect-siRNA–treated cells at all MRs ($p < 0.001$, $n = 3 \times 4$), with evidence of toxicity only at MRs 50–100. Nebulization of SC12CDClickpropylamine nanocomplexes using the Aeroneb® Pro resulted in VMDs of $\sim 5\,\mu$m and FPFs of $\sim 57\%$ at all MRs. SC12CDClickpropylamine-siRNA–mediated luciferase knockdown was found to be $39.8 \pm 3.6\%$ at MR=20 before and $35.6 \pm 4.55\%$ after nebulization, comparable to results observed using unnebulized commercial transfection reagent, RNAiFect.
Conclusions: SC12CDClickpropylamine nanocomplexes can be effectively nebulized for pulmonary delivery of siRNA using Aeroneb technology to mediate knockdown in airway cells. To the best of our knowledge, this is the first study examining the suitability of SC12CDClickpropylamine-siRNA nanocomplexes for pulmonary delivery. Furthermore, this work provides an integrated nanomedicine–device combination for future *in vitro* and *in vivo* preclinical and clinical studies of inhaled siRNA therapeutics.

Key words: vibrating mesh nebulizer, RNAi, nonviral gene delivery, high-throughput screening, Calu-3 cells

Introduction

Local delivery of small interfering RNA (siRNA) to the lungs represents a promising means of treating a range of pulmonary conditions such as asthma, influenza, and lung cancer.[1–4] However, progression from the preclinical to the clinical setting remains hampered by a range of host defense mechanisms and pharmaceutical delivery issues. These include rapid clearance via the mucociliary escalator, entrapment and degradation by alveolar macrophages, and

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activation of the innate immune systems via toll-like receptors.[5–7] Coupled with this, pharmaceutical issues that are currently impeding pulmonary siRNA therapy include nanoparticle-induced toxicity, inefficient rates of siRNA nanoparticle delivery, and low levels of postdelivery siRNA stability (reviewed previously[8–10]). With these obstacles in mind, it is extremely important to confront both of these areas in order to successfully develop pulmonary siRNA therapy.

Following the selection of an appropriate platform for delivery, it is also necessary to design an siRNA delivery vector capable of withstanding the nebulization process and successfully transfecting airway cells. The use of cationic polymers for nucleic acid delivery has been well established as an efficient means of nonviral gene delivery. However, although synthetic polymers such as polyethylenimine (PEI) have demonstrated high levels of gene knockdown, they have been widely reported to be cytotoxic and immunogenic.[11–15] Following from these studies, current efforts are now turning toward alternative carrier molecules to improve toxicity while maintaining transfection efficiency.

Recently, we have developed a novel amphiphilic β-cyclodextrin (β-CD) that has been modified via ''click'' chemistry to possess cationic moieties (Fig. 1), rendering it a potential siRNA delivery vector. Studies to date have found it to be effective at delivering siRNA to both gastrointestinal–epithelial and neuronal cells[16,17]; however, its potential for pulmonary delivery of siRNA has not yet been assessed. To date, research into CD delivery to the lungs has focused primarily on enhancing solubility of preexisting small-molecule drugs,[18,19] mitigating their cytotoxic effects,[20] and improving the respirable fraction and absorption of aerosolized drugs.[21–23] CDs have also been adapted for gene delivery using a multitude of different CD conformations, including functionalization of CD–OH terminals with cell-targeting ligands and poly(ethyleneglycol) moieties, cell-penetrating peptides, cationic dendrimers, and CD block copolymers (comprehensively reviewed previously[24–27]). To date, none of these CDs have been used for transfection of airway epithelial cells.

In this study, we apply a range of high-throughput, multiparameter screening methods to obtain both qualitative and quantitative data regarding siRNA uptake efficiency and cytotoxicity in Calu-3 bronchial epithelial cells over a range of doses and time points. In addition, we investigate the ability of novel SC12CDClickpropylamine siRNA nanocomplexes to effect *in vitro* gene knockdown both before and after nebulization through the Aeroneb® Pro vibrating mesh nebulizer.

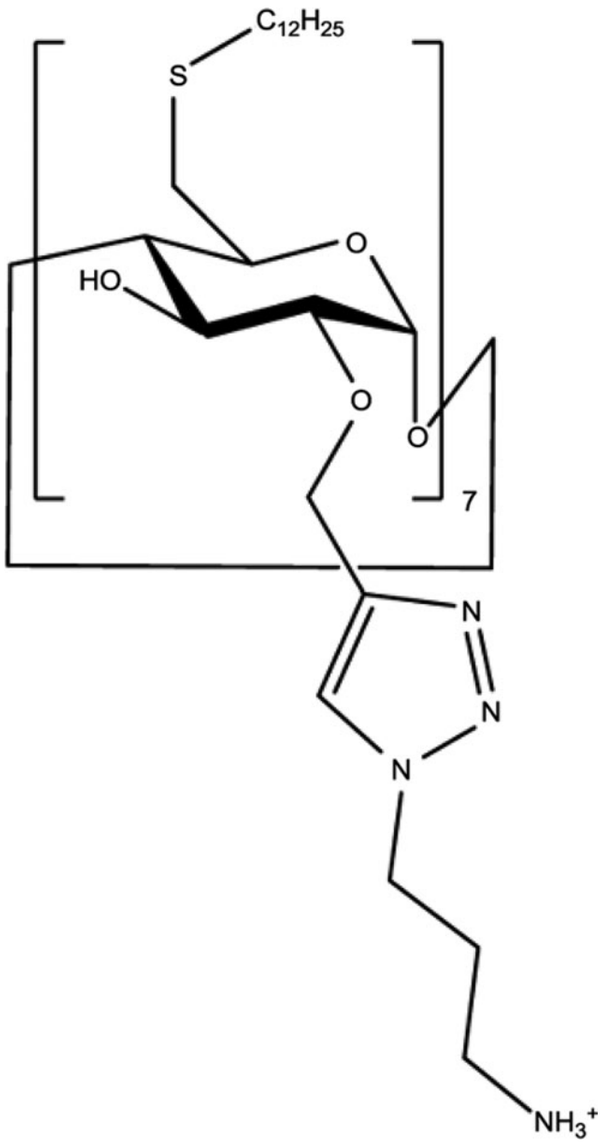

FIG. 1. Chemical structure of novel click-modified cationic CD.[39]

Materials and Methods

Materials

All cell culture and high-content analysis (HCA) reagents were obtained from Invitrogen Corporation (Carlsbad, CA), unless otherwise stated. The Calu-3 bronchial epithelial cell line was obtained from the American Tissue Type Culture Collection (ATCC, Manassas, VA) and used at passages 20–50. siGENOME Non-Targeting siRNA #2 (5′ UAAGG CUAUGAAGAGAUAC 3′) was obtained from Dharmacon (Lafayette, CO). The non-targeting sequence #2 is nonspecific for human gene sequences and specific for firefly luciferase using the Promega pGL3 cloning vector. AllStars negative control siRNA was obtained from Qiagen UK (Manchester, UK). This siRNA has no homology to any known mammalian gene and has been validated using Affymetrix GeneChip arrays and a variety of cell-based assays. AllStars negative control siRNA was also obtained from Qiagen with a fluorocein isothiocyanate (FITC) modification for cell uptake studies. The modified CD SC12CDClickpropylamine (Fig. 1) was produced in-house as previously described.[16] Aeroneb Pro vibrating mesh nebulizers were a kind gift from Aerogen Ltd. (Galway, Ireland). All other general chemicals and reagents used were of the highest grade possible and were obtained from Sigma-Aldrich Company Ltd. (Dorset, UK), unless otherwise stated.

Formation of SC12CDClickpropylamine-siRNA nanocomplexes

CD nanocomplexes were formed using a film evaporation method whereby CDs were first dissolved in chloroform at 1 mg/mL in a round-bottomed flask. The solvent was then evaporated using a rotary evaporator. Evaporated films were stored at −20°C until required. For preparation of SC12CD Clickpropylamine-siRNA complexes, SC12CDClickpropylamine was rehydrated with deionized water at a final concentration of 1 mg/mL and sonicated for 1 hr at room temperature (RT). Resuspended SC12CDClickpropylamine was then mixed at various mass ratios (MRs) with 20 μM siRNA (μg of CD/μg of siRNA) in an equal volume of RNase-free water to a final volume of 1 μM siRNA and used after 20–30 min.

Size and zeta potential of SC12CDClickpropylamine-siRNA nanocomplexes

SC12CDClickpropylamine nanocomplex particle size and charge were measured with Malvern's Zetasizer Nano ZS instrument, using laser-light scattering and electrophoretic mobility measurements, respectively. CD-siRNA (3 μg of siRNA) nanocomplexes were prepared by the ''mixing method.'' The resulting mixtures were made up to 1 mL with 0.2-μm filtered deionized water (dH_2O) or phosphate-buffered saline (PBS) at pH 7.4. Five readings of Z-average size (nm), polydispersity (25°C, measurement angle 170°), and zeta potential (mV; 25°C, measurement angle 12.8°) were taken. For data analysis, the viscosity (0.8872 mPa·sec) and refractive index (1.33) of water were used to determine Z-average size. The data are presented as means ± standard error of the mean (SEM).

SC12CDClickpropylamine-siRNA nanocomplex uptake in Calu-3 cells

Calu-3 cells were seeded at 3×10^4 cells/well in a 96-well plate (Nunc). Nanocomplexes were formed as described. Cells in 125 μL of serum-free Dulbecco's modified Eagle's medium (DMEM) were treated with nanocomplexes containing 100 nM fluorescently tagged FITC siRNA at 37°C and 5% CO_2 for 2, 4, and 24 hr. Cells were washed with PBS and fixed using 4% paraformaldehyde. Cells were stained using phalloidin–tetramethylrhodamine isothiocyanate (TRITC) and Hoechst nuclear stain. Image analysis was achieved using the IN Cell® 1000 High Content Analyzer (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Four random fields were viewed per well, and the various MRs were repeated in quadruplicate. Fluorescence intensity of the dyes was monitored at the excitation and emission wavelengths specific to each dye (*i.e.*, 360 nm and 460 nm for Hoechst, 480 nm and 535 nm for FITC-siRNA, and 535 nm and 600 nm for phalloidin-TRITC). Exposure times were varied to optimize image quality for each individual experiment. After acquisition of the images, the data were analyzed using IN Cell 1000 Workstation software (GE Healthcare, UK) using multitarget analysis with a variety of settings for each of the parameters ◀AU2 shown in Table 1. Analysis was carried out on siRNA nanocomplexes found in the cytoplasm only, as this is the active site for siRNA-mediated genetic knockdown. Any complexes found outside the cell and in the nuclear area were automatically ignored using the segmentation setting available in the IN Cell 1000 Workstation software. All samples were run in quadruplicate and the experiment repeated on three independent occasions. ◀T1

Multiparameter analysis of SC12CDClickpropylamine nanocomplex toxicity in Calu-3 cells

Twenty-four hours prior to transfection, cells were seeded 3×10^4/well in a 96-well plate. SC12CDClickpropylamine-siRNA nanocomplexes were formed as previously described, and cells were treated with nanocomplexes containing 100 nM siRNA for 24 hr. Furthermore, selected wells were treated with 120 μM valinomycin or 40 μM carbobenzoxy-L-leucyl-L-leucyl-L-leucinal (MG132) for 24 hr as a positive control prior to analysis. Following incubation, cells were stained and fixed using the Cellomics® Multiparameter Cytotoxicity 3 kit (Thermo Scientific, Waltham, MA) according to its protocol. In brief, cells were live-stained for mitochondrial membrane potential (MMP) and plasma membrane permeability (PMP). Cells were then fixed using 4% paraformaldehyde before staining with Hoechst nuclear stain and fluorescent antibody labeling for cytochrome c. Image acquisition was determined using the IN Cell 1000 High Content Analyzer.

Four random fields were viewed per well, and the various MRs were repeated in quadruplicate. Fluorescence intensity of the dyes was monitored at the excitation and emission wavelengths specific to each dye (*i.e.*, 360 nm and 460 nm for Hoechst, 480 nm and 535 nm for the permeability dye, 535 nm and 600 nm for MMP dye, and 646/674 nm for DyLight 649 conjugates). Exposure times were varied between experiments to optimize image quality for each individual experiment. Following acquisition of the images, the data were analyzed using IN Cell 1000 Workstation software (GE Healthcare, UK) using multitarget analysis with a variety of settings for each of the parameters (Table 2). ◀T2 All samples were run in quadruplicate and the experiment repeated on three independent occasions.

Nebulizer droplet size characterization by laser diffraction

Droplet size distributions described by volumetric median diameter (VMD) were measured by a Malvern Spraytec

TABLE 1. SETTINGS FOR IN CELL 1000 WORKSTATION ANALYSIS OF RNAIFECT/SC12CDCLICKPROPYLAMINE-SIRNA NANOCOMPLEX UPTAKE INTO CALU-3 CELLS

Feature	*Source*	*Segmentation*	*Min. area*	*Sensitivity*	*Collar*
Nuclei	Wave 1 (Hoechst)	Top hat	50 μm^2	100%	—
Cell	Wave 2 (TRITC)	Collar	—	—	7 μm
Organelles	Wave 3 (FITC)	Cytoplasm only	0.05–0.5 μm^2	50%	—

Table 2. Settings for IN Cell 1000 Workstation Analysis of SC12CDClickpropylamine-siRNA Nanocomplex–Induced Toxicity in Calu-3 Cells

Feature	*Source*	*Segmentation*	*Min. area*	*Sensitivity*	*Collar*
Nuclei	Wave 1 (NA, NI, CN)	Top hat	50 μm^2	100%	—
Cell	Wave 2 (PMP)	Collar	—	—	8 μm
Reference 1	Wave 3 (Cyt c)	Pseudo-cells	—	—	—
Reference 2	Wave 4 (MMP)	Pseudo-cells	—	—	—
Reference 3	Wave 2 (PMP)	Pseudo-nuclei	—	—	—

particle size analyzer for several different CD-siRNA nanocomplex MRs. (Malvern Instruments Ltd., Malvern, Worcestershire, UK) with RT Sizer software (version 5.60).

A 5 L/min vacuum flow was implemented through the system, ensuring laminar flow and reducing artificial droplet size growth through collision with other droplets. The vacuum also ensured that the droplets passed through the laser beam only once. The center of the emitted aerosol plume was directed through the center of the laser beam to increase the accuracy of data acquisition.

Data acquisition began when beam obscuration exceeded 3% and continued until the end of dosing. The data acquisition rate was set to 500 Hz, that is, 500 individual readings per second were taken characterizing the droplet size distribution. The value reported for each individual measurement is an average of the individual readings recorded over the course of the dose. In order to verify the accuracy of the generated data, the Spraytec analyzer's laser diffraction apparatus was tested with a reference reticle (Malvern Instruments Ltd.). Droplet size is described by VMD (Dv_{50}). The fine particle fraction (FPF; percentage of droplets less than 5 μm in size) was also recorded.

Luciferase knockdown in Calu-3 cells using pre- and postnebulization using SC12CDClickpropylamine-siRNA nanocomplexes

Calu-3 cells were seeded at a density of 5×10^4 in 48-well plates 24 hr prior to transfection. Following this, cells were first transfected with luciferase pGL3 control vector plasmid (Promega, Southampton, UK) and SuperFect transfection reagent (Qiagen, UK). Cells were transfected using 0.75 μg of pDNA/3 μL of SuperFect in 100 μL of serum-free DMEM per well for 4 hr. For unnebulized samples, siRNA nanocomplexes were formed in PBS following initial resuspension in dH_2O using anti-luciferase siRNA or nontargeting control siRNA as previously described and diluted to a final concentration of 100 n*M* per well in serum-containing media. Positive controls of RNAiFect™ (Qiagen, UK) siRNA nanocomplexes were formed according to the manufacturer's instructions at an siRNA:RNAiFect MR of 1:9 and diluted in serum-containing media to a final concentration of 100 n*M*. Cells were then treated with 250 μL of the final sample and incubated for 24 hr at 37°C and 5% CO_2.

For postnebulization nanocomplexes, SC12CDClickpropylamine-siRNA nanocomplexes were formed as previously described, with the exception of using PBS instead of serum-containing media, to a concentration of 500 n*M*. Nanocomplex solutions were then nebulized through the Aeroneb Pro (Aerogen) until no sample remained on the medication feed side of the nebulizer. Postnebulization samples were collected by nebulizing directly into a sealed 15-mL tube containing serum-containing media. Nebulized samples were allowed to condense before being centrifuged at 1,000 rpm for 3 min and diluted to a final concentration of 100 n*M* using serum-containing media. Cells were then treated with 250 μL of the collected sample and incubated for 24 hr at 37°C and 5% CO_2. Following incubation, all samples were analyzed for luciferase expression using the luciferase assay system (Promega) and read using a Wallac 1420 Multilabel Counter (PerkinElmer, Waltham, MA). Protein expression in each sample was examined using the Micro BCA™ protein assay kit (Pierce) according to product instructions. All samples were run in quadruplicate and the experiment repeated on three independent occasions.

Statistical analysis

Results are expressed as means ± SEM using GraphPad® Prism 5 software. Two- and one-way analysis of variance (ANOVA) for differences between treatments with $p < 0.05$ considered significant, $p < 0.01$ very significant, and $p < 0.001$ highly significant.

Results

Size and zeta potential of SC12CDClickpropylamine-siRNA nanocomplexes

In order to determine the optimal MR for nanocomplex formation in CD-siRNA nanocomplexes, a range of MRs were examined for particle size and zeta potential. For the SC12CDClickpropylamine-siRNA nanocomplexes prepared in dH_2O, MRs from 10 to 100 were examined. It was found that SC12CDClickpropylamine nanocomplexes formed compact structures under 200 nm in size at all MRs, with the most compact nanocomplexes forming at MR = 20 and 30 (Fig. 2A). Examination of corresponding polydispersity indices (PDIs) for siRNA nanocomplexes measured indicated that siRNA nanocomplexes were of a more monodisperse nature at MRs 10–30, with higher levels of heterogeneity at MR = 50 and MR = 100. However, overall, samples remained relatively polydisperse. On analysis of the dH_2O SC12CDClickpropylamine nanocomplex's corresponding zeta potentials, it was found that at all MRs 10–100 the nanocomplexes were highly cationic. Furthermore, cationic charge increased with increasing MR. However, this increase became less pronounced after the MR of 20 (Fig. 2B). On analysis of the siRNA nanocomplexes formulated in PBS, it was found that there was a very significant increase in size across all MRs to the micrometer range with PDIs

◀F2

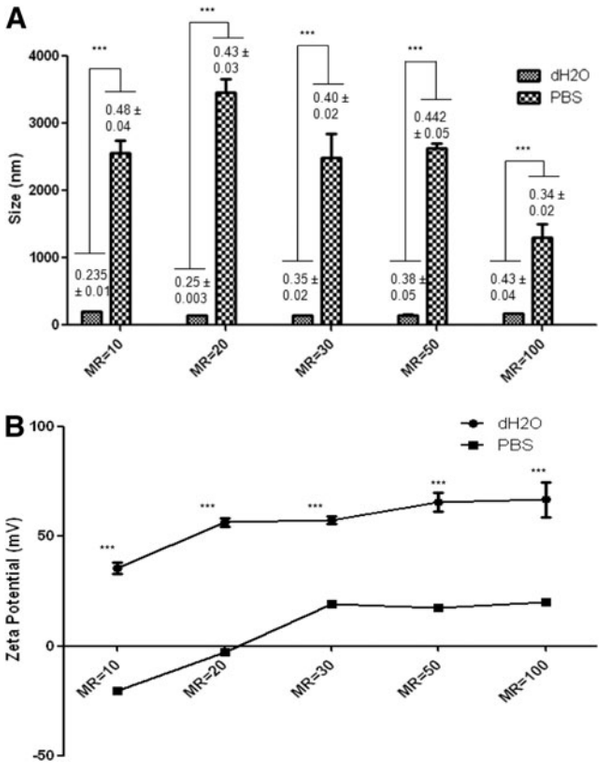

FIG. 2. **(A)** Particle size, PDI, and **(B)** zeta potential of SC12CDClickpropylamine-siRNA nanocomplexes at MRs 10–100 using either dH_2O or PBS (two-way ANOVA, $n = 5$, means ± SEM, $***p < 0.001$).

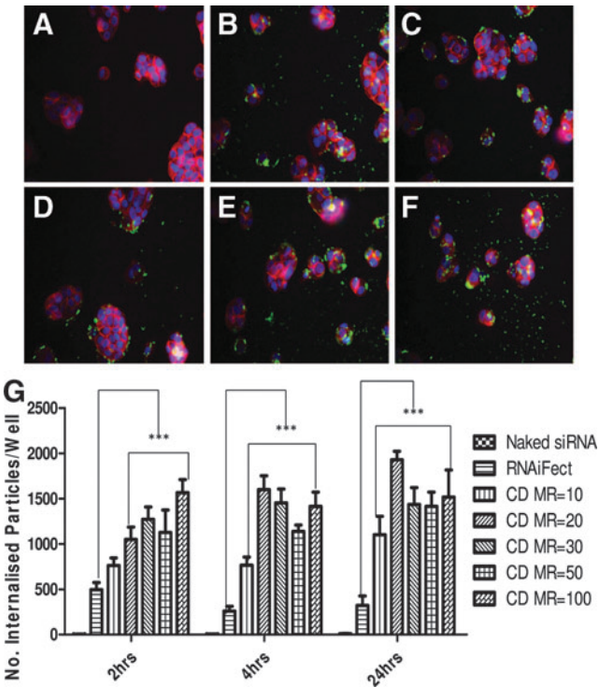

FIG. 3. HCA 20× fused image analysis of SC12CD Clickpropylamine-siRNA nanocomplex uptake in Calu-3 cells 24 hr post transfection. Cells were treated with 100 n*M* concentration of FITC-tagged siRNA nanocomplexes (green) and were subsequently stained for cell nuclei using Hoechst nuclear stain (blue) and for cell membrane using phalloidin-TRITC (red). **(A)** FITC-siRNA–treated cells. **(B)** CD MR = 10. **(C)** CD MR = 20. **(D)** CD MR = 30. **(E)** CD MR = 50. **(F)** CD MR = 100. **(G)** Quantitative HCA analysis of RNAiFect versus SC12CDClickpropylamine nanocomplex uptake in Calu-3 cells measured at 2, 4, and 24 hr post administration (two-way ANOVA, $n = 3$, means ± SEM, $***p < 0.001$).

◀4C

indicating a heterogeneous population. Examination of PBS samples also demonstrated a significant decrease in charge. However, the overall trend of increasing positive surface charge with increasing MR remained evident (Fig. 2).

HCA analysis of SC12CDClickpropylamine-siRNA nanocomplex uptake in Calu-3 cells

To adequately assess cell uptake of CD-siRNA nanocomplexes in Calu-3 cells over a wide range of MRs and incubation times, high-throughput methods were developed that allowed for both a visual qualitative and quantitative examination. Cells were treated with FITC-tagged SC12CD Clickpropylamine-siRNA nanocomplexes and incubated for either 2, 4, or 24 hr at 37°C in order to determine the optimal MR for siRNA delivery using the different SC12CDClickpropylamine MRs, as well as gaining an insight into the rates at which the different nanocomplexes were internalized into the cell.

Using the cell organelle segmentation parameters of the IN Cell data acquisition software, it was possible to discriminate individual siRNA nanocomplexes internalized within the cell membrane to gain a quantitative understanding of nanocomplex uptake efficiency. It was found that SC12CD Clickpropylamine-siRNA nanocomplexes were capable of facilitating high levels of siRNA internalization into Calu-3 cells at all MRs studied (Fig. 3). Levels of siRNA internalization were significantly higher in SC12CDClickpropylamine nanocomplex–treated cells over all MRs and incubation times compared with RNAiFect. For example, at MR = 20, uptake versus RNAiFect siRNA nanocomplexes at 2, 4, and 24 hr post treatment was 1,049 ± 137 versus 494 ± 79, 1,598 ± 153 versus 258 ± 53, and 1,927 ± 94 versus 322 ± 103 internalized nanocomplexes per well, respectively.

F3 ▶

It was also observed that SC12CDClickpropylamine nanocomplex composition appeared to affect its cellular particokinetics in terms of both the level and the rate of internalization. For the SC12CDClickpropylamine-siRNA nanocomplexes formed at higher MRs (MR ≥ 30), it was found that there were no significant differences in the levels of internalization after 2, 4, and 24 hr of incubation. For example, MR = 100 nanocomplex uptake was 1,565 ± 145, 1,413 ± 158, and 1,516 ± 298 internalized nanocomplexes per well after 2, 4, and 24 hr of incubation. In contrast, there were significant changes in levels of internalized nanocomplexes at MR = 10 and MR = 20 at each different time point.

It would appear that nanocomplexes formed at higher MRs undergo rapid internalization and intracellular processing, whereas low-MR SC12CDClickpropylamine nanocomplexes are internalized at a slower rate but ultimately

result in higher levels of nanocomplex internalization. Evidence of this was found in the overall rate of internalization for SC12CDClickpropylamine nanocomplexes. At 2 hr post treatment, the highest level of internalization was seen in SC12CDClickpropylamine nanocomplexes at MR = 100; however, over the full 24 hr of treatment, the highest levels of internalization were seen in SC12CDClickpropylamine nanocomplexes at MR = 20. At 24 hr post transfection, levels of internalized SC12CDClickpropylamine-siRNA nanocomplexes were significantly higher using an MR = 20 formulation compared with RNAiFect and all other SC12CDClick propylamine-siRNA nanocomplexes used. This demonstrated that over 24 hr the optimal MR for intracellular delivery of siRNA delivery novel cationic CDs was at MR = 20.

Multiparameter analysis of SC12CDClickpropylamine nanocomplex toxicity in Calu-3 cells

Assessing the dose-toxicity relationship of novel transfection materials is a crucial element in their clinical and regulatory development. As with the nanocomplex uptake studies described, high-throughput screening methods were harnessed to carry out a multiparameter cytotoxicity study. Calu-3 cells were incubated with 100 n*M* SC12CDClickpropylamine-siRNA nanocomplexes per well over a range of CD:siRNA MRs for 24 hr. Following this, cell viability and nanocomplex-mediated toxicity were qualitatively and quantitatively determined using the Cellomics Multiparameter Cytotoxicity 3 kit and IN Cell 1000 Workstation software for analysis.

For these experiments, the positive toxic controls consisted of the ionophore, valinomycin, and the proteasome inhibitor MG132 (Z-Leu-Leu-Leu-Al). Valinomycin is a K^+ ionophore that selectively transports K^+ ions through the cell membrane leading to mitochondrial swelling, cytochrome c release, and autophagic processes.[28,29] In contrast, MG132 is a peptide aldehyde and is a potent inhibitor of the 26S proteasome complex that effectively blocks its proteolytic activity via the chymotrypsin degradation pathway.[30] Doses as low as 1–30 μM (compared with 120 μM for valinomycin) have been found to result in S-phase arrest in the cell cycle[31] and as such is a potent mediator of apoptosis. With this in mind, both MG132 and valinomycin were used to illustrate apoptotic effects on Calu-3 cells at different levels of potency. On analysis of the fused images of the negative (Fig. 4A) and positive (Fig. 4B and C) control cell populations, a noticeable difference in appearance was evident. Characteristics immediately noticeable in toxic controls included total cell number decreases as well as cytochrome c release and PMP increases in valinomycin-treated samples (Fig. 4B and C).

In order to analyze the level of cytotoxicity elicited by SC12CDClickpropylamine-siRNA nanocomplexes, nanocomplexes with MRs 10–100 were used to treat Calu-3 cells and compared with untreated healthy cells 24 hr post transfection. On examination of the change in cell count (Fig. 5A) in toxic positive controls, it was found that there were very significant levels of cell loss in both valinomycin (54.93% ± 5.91%)– and MG132 (85.04% ± 3.11%)–treated samples. In contrast, for SC12CDClickpropylamine-siRNA–treated cells, there was found to be a gradual decrease in cell number with increasing MRs, up to 37% ± 15.6% of total at MR = 100. However, there were no statistically significant decreases observed in cell populations at any MR examined.

Further examination of nuclear morphology characteristics for nuclear area (Fig. 5B) in positive controls demonstrated significant increases in valinomycin (19.11% ± 5.21%)– and MG132 (26.37% ± 1.70%)–treated samples. Cells treated with SC12CDClickpropylamine-siRNA–treated nanocomplexes did not demonstrate any statistically significant changes in nuclear area. Nuclear intensity analysis (Fig. 5C) of all samples did not yield any significant changes compared with untreated controls, with nuclear intensity actually decreasing in CD-treated samples. However, an increase was seen in

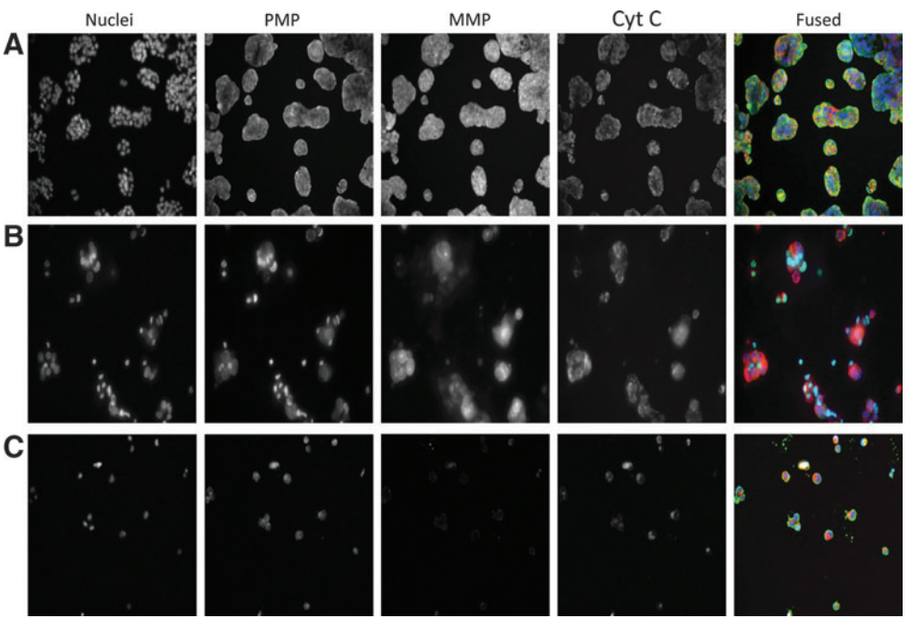

FIG. 4. 10× image of grayscale images of each toxicity parameter analyzed using the Cellomics Multiparameter Cytotoxicity kit for Calu-3 cells **(A)** untreated and **(B)** treated with 120 μM valinomycin and **(C)** 40 μM MG132. Fused images were obtained from analysis consisting of Hoechst nuclear stain (blue), PMP dye (green), and secondary antibody staining for cytochrome c (Cyt C) (red).

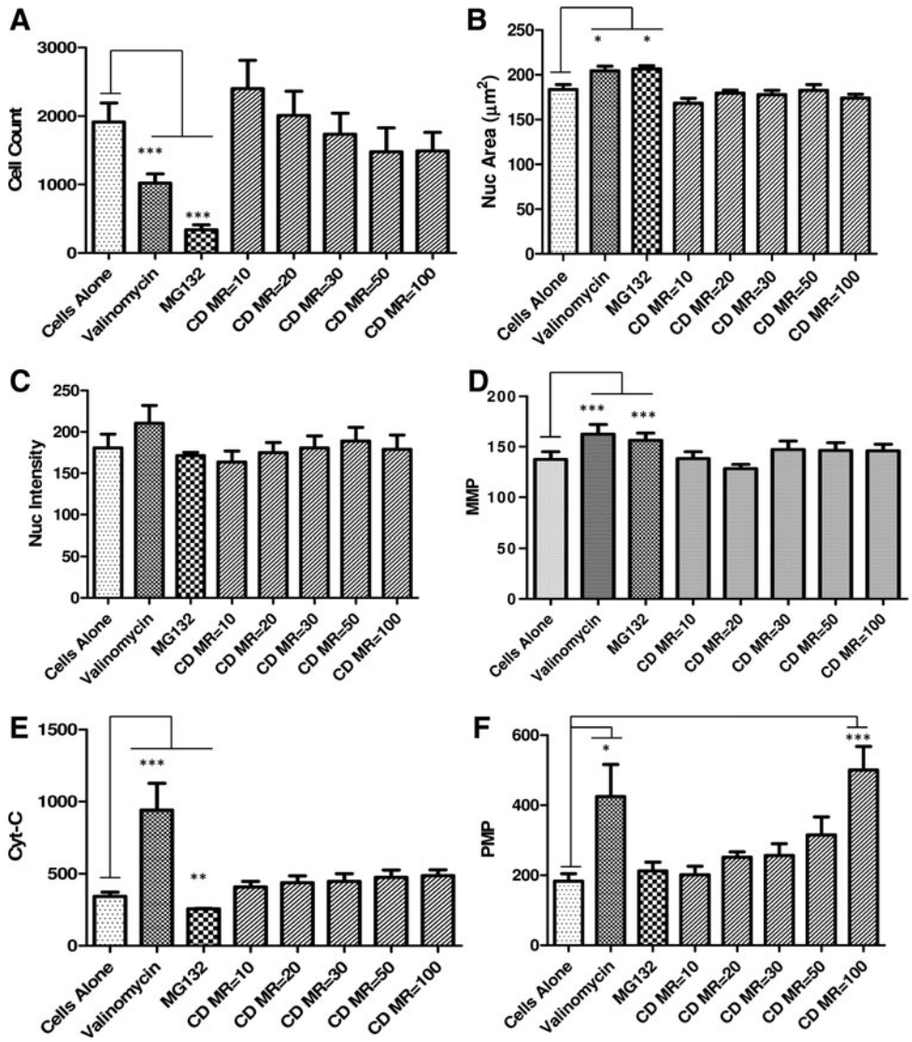

FIG. 5. Quantitative multiparameter analysis of valinomycin- and MG132-positive controls and SC12CDClickpropylamine-siRNA nanocomplex–mediated toxicity in Calu-3 cells measured at 24 hr post administration. **(A)** Cell count; **(B)** nuclear area; **(C)** nuclear intensity; **(D)** MMP; **(E)** cytochrome c (Cyt C) release; and **(F)** PMP (significance versus healthy cells, one-way ANOVA, $n=3$, means ± SEM, $*p<0.05$, $**p<0.01$, $***p<0.001$).

nuclear intensity in valinomycin-treated samples (as has previously been reported[13]), but this could not be deemed statistically significant in these experiments.

On analysis of the remaining cell death–associated characteristics, SC12CDClickpropylamine nanocomplexes were again found to be well tolerated and caused little change from untreated cells. Specifically, for both MMP (Fig. 5D) and cytochrome c release (Fig. 5E), there were again no statistically significant changes observed for CD-siRNA–treated cells when compared with untreated cells. In contrast, the positive controls for these parameters were found to exhibit significant changes in MMP with increases of 17.95% ± 6.33% and 11.74% ± 5.13% observed for valinomycin and MG132, respectively. Analysis of cytochrome c release in toxic positive controls revealed conflicting results. Cytochrome c release in valinoymcin-treated cells was found to greatly increase (173.96% ± 54.1%), whereas MG132 exhibited a significant decrease in cytochrome c levels (25.41% ± 0.98%). This is mostly likely due to the difference in potency of the two positive controls. With MG132 being the more toxic of the two, it is likely that the cytochrome c release had reached its maximum level earlier in the incubation phase and had been released into the culture media and lost following cell membrane breakdown.

The final parameter analyzed for evidence of cytotoxicity was PMP whereby an increase in PMP would indicate a reduction in the structural integrity of the cell membrane associated with apoptosis. In a similar manner to cytochrome c release, positive controls exhibited different behavior in their PMP values. Valinoymcin-treated samples demonstrated increases in PMP of 113.17% ± 35.5%, whereas the more potent MG132 demonstrated a smaller increase of 35.77% ± 15.9% but was not deemed significant. On analysis of SC12CDClickpropylamine-siRNA nanocomplex–treated cells, it was observed that the overall levels of PMP rose with increasing MRs (Fig. 5F). However, these increases did not reach statistical significance, with the exception of the highest MR = 100.

SC12CDClickpropylamine-siRNA nanocomplex droplet size analysis

To establish the potential effect of SC12CDClickpropylamine-siRNA nanocomplex formulations on droplets

Table 3. SC12CDClickpropylamine-siRNA Nanocomplex Droplet Sizes Generated and Fine Particle Fraction (FPF) Post Nebulization ($N = 3 \pm$ SD)

Sample	*VMD (Dv50) (μm)*	*FPF < 5 μm (%)*	*Flow rate (mL/min)*
Saline (0.9% w/v)	4.26 (±0.02)	58.86 (±0.46)	0.41 (±0.00)
siRNA	4.27 (±0.02)	58.78 (±0.38)	0.41 (±0.01)
CD MR = 10	4.23 (±0.04)	59.45 (±0.71)	0.39 (±0.01)
CD MR = 20	4.17 (±0.06)	60.31 (±0.96)	0.38 (±0.01)
CD MR = 30	4.13 (±0.10)	60.98 (±1.58)	0.37 (±0.02)

emitted from the Aeroneb Pro nebulizer, a range of SC12CD Clickpropylamine-siRNA nanocomplexes were formulated at the most likely working MRs and assessed for changes in VMD and % FPF compared with a 0.9% w/v saline solution and unencapsulated siRNA (Table 3). T3

On examination of the VMD and % FPF of each siRNA nanocomplex system, it was found that there were no significant changes when compared with saline and naked siRNA controls. The VMD of siRNA nanocomplex solutions remained around 4 μm in all cases, which is within the required size range for successful delivery to the deep lung.[32] In addition, the % FPF of each nebulized siRNA nanocomplex sample remained at approximately 60% for all samples. On analysis of the output rates associated with each SC12CDClickpropylamine-siRNA nanocomplex, there was no clear-cut trend apparent. Overall, output rates remained roughly in line with saline and naked siRNA samples; however, there was a slight but nonsignificant decrease in output efficiency apparent with increasing MRs in SC12CDClickpropylamine samples.

SC12CDClickpropylamine-siRNA nanocomplex–mediated luciferase knockdown in Calu-3 cells

SC12CDClickpropylamine-siRNA nanocomplexes were formulated with 100 n*M* anti-luciferase siRNA in order to assess their ability to knockdown luciferase expression in pretransfected, luciferase-expressing Calu-3 cells. Samples were incubated for 24 hr and then compared with the transfection ability of the commercially available transfection reagent RNAiFect.

In the case of Calu-3 cells transfected with SC12CD Clickpropylamine-siRNA nanocomplexes, it was found that the optimal MR for transfection was at MR = 20. At this MR, gene knockdown was found to be as high as 40% (Fig. 6) F6 with significant levels of knockdown noted in individual studies. Following this knockdown, efficiency appeared to decrease with increasing MRs. When this was compared with the percentage knockdown observed using RNAiFect, it was found that the MR = 20 SC12CDClickpropylamine-siRNA nanocomplexes exhibited slightly higher levels of knockdown, though not statistically significant.

Postnebulization SC12CDClickpropylamine-siRNA nanocomplex–mediated luciferase knockdown in Calu-3 cells

The ability of the SC12CDClickpropylamine-siRNA nanocomplexes to retain functionality post nebulization was also assessed using a luciferase knockdown assay (Fig. 7). F7 Postnebulization SC12CDClickpropylamine-siRNA nanocomplexes did not exhibit any significant changes in knockdown ability in comparison with unnebulized samples. As found previously in the siRNA nanocomplex HCA uptake studies and luciferase knockdown studies, at 24 hr post transfection, SC12CDClickpropylamine-siRNA nanocomplexes at MR = 20 remained the optimal formulation for transfection before and after nebulization.

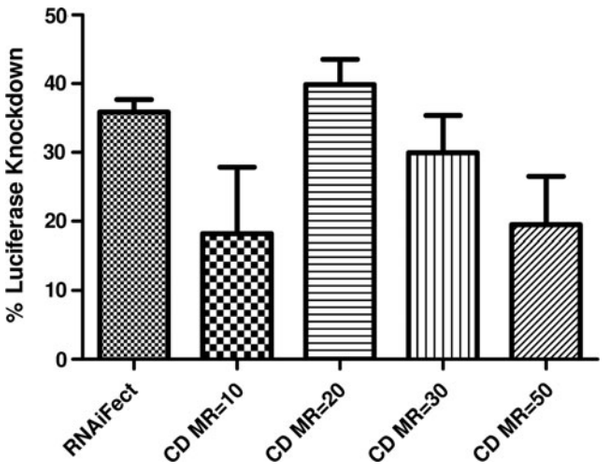

FIG. 6. Percentage of SC12CDClickpropylamine-siRNA nanocomplex–mediated luciferase knockdown in Calu-3 cells versus commercial RNAiFect control vector (knockdown normalized versus nontargeting controls, one-way ANOVA, $n = 3$, means ± SEM).

Discussion

Of key importance in the molecular kinetics of siRNA nanocomplexes is analysis of their size and zeta potential. Analysis of SC12CDClickpropylamine-siRNA nanocomplexes in this current study and previous studies has shown that they are capable of forming compact nanocomplexes between 125 and 200 nm in size and are highly cationic in nature using dH_2O.[16,17,33,34] In addition, previous gel

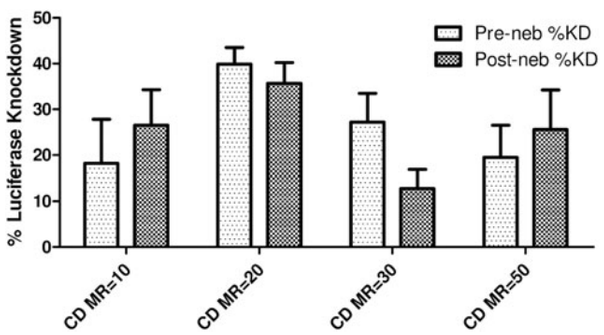

FIG. 7. Postnebulization luciferase knockdown efficiency of novel SC12CDClickpropylamine-siRNA nanocomplexes in undifferentiated Calu-3 cells [significance versus unnebulized samples, knockdown (KD) normalized versus nontargeting controls, two-way ANOVA, $n = 3$, means ± SEM].

electrophoresis analysis by our group has also demonstrated that SC12CDClickpropylamine nanocomplexes efficiently encapsulate siRNA at MR > 10.[17] In contrast, this study found that formulation of siRNA nanocomplexes in PBS resulted in marked increases in particle size. This phenomenon has been previously reported for siRNA nanoparticles and did not correlate to a loss in transfection efficiency.[35] Due to the polydisperse nature of these particles and their ability to elicit knockdown without toxicity, it is possible that they are forming ''nano-agglomerates'' that may dissociate into nano-sized particles on contact with the cell membrane. This in itself has been investigated as a means of deliverying nano-sized particles to the lung in an inhalable format.[36] Similar results in our group have previously found that whereas SC12CDClickpropylamine nanocomplexes increase in size following 24 hr of incubation in fetal bovine serum (FBS), particle size remains less than 250 nm up to 4 hr of incubation, which still allows for rapid internalization into cells.[37] This was also observed in this study using cell culture media containing FBS and high-throughput screening assays for cell uptake of SC12CDClickpropylamine nanocomplexes over a 24-hr period.

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The use of high-throughput screening methods offers a means of gaining a detailed understanding of particle-cell trafficking and drug-induced cytotoxicity. This has previously been successfully applied by our group to monitor drug delivery and excipient toxicity in a range of different cells types including colonic and lung epithelial cells as well as in alveolar macrophages.[13,38,39] Through HCA screening, the uptake of SC12CDClickpropylamine-siRNA nanocomplexes into Calu-3 cells was assessed at 2, 4, and 24 hr. Significantly enhanced levels of siRNA nanocomplex uptake were recorded compared with commercial controls as rapidly as 2 hr post treatment, which were most likely due to its highly cationic nature. It has been suggested that cationic nanocomplexes similar to SC12CDClickpropylamine nanocomplexes utilize multiple endocytic pathways in airway epithelial cells including clathrin and caveolae-mediated endocytosis.[40,41] This would allow for a rapid influx of SC12CDClickpropylamine-siRNA nanocomplexes into cells in a relatively short amount of time following administration of SC12CDClickpropylamine-siRNA nanocomplexes.

In addition, it is worth noting that at 24 hr post treatment, the greatest levels of uptake did not correspond to increases in MR. Whereas at earlier time points, rates of SC12CD Clickpropylamine-siRNA nanocomplex uptake were broadly in line with increasing MR, at 24 hr post treatment two-way ANOVA analysis of uptake rates revealed that MR = 20 SC12CDClickpropylamine-siRNA nanocomplexes achieved significantly higher levels of cell uptake compared with all other SC12CDClickpropylamine-siRNA formulations. In combination with this, it was also found that whereas levels of internalized siRNA nanocomplexes remained relatively static in MRs 30–100, the amount of internalized SC12CD Clickpropylamine-siRNA nanocomplexes at MR = 10 and 20 changed significantly over a 24-hr time period. In attempting to explain this phenomenon, it was noticed that physicochemical properties (*i.e.*, size and zeta potential) of the various MR formulations were similar and not likely to explain this difference. However, multiparameter cytotoxicity analysis had detected a proportional increase in PMP with increasing MR of SC12CDClickpropylamine-siRNA nanocomplex used. This may indicate that the rapid uptake of high levels of high-MR SC12CDClickpropylamine-siRNA nanocomplexes was being facilitated by their own increasingly cytotoxic nature. In contrast, the high levels of uptake observed over the full 24-hr time period in the MR = 10 and 20 SC12CDClickpropylamine-siRNA nanocomplexes were more likely due to normal cell endocytosis.

Using high-throughput multiparameter analysis of toxicity, it was found that the SC12CDClickpropylamine nanocomplexes were well tolerated at all MRs with no significant changes in all parameters in comparison with untreated controls (with the exception of PMP). This is in line with earlier toxicity studies of these nanocomplexes in Caco-2 colonic epithelial cells and N41 neuronal cell lines as well as primary rat embryonic neuronal cells.[16,17] It should be noted, however, that trends toward toxicity were noted as MR increased with decreases in cell population of up to 34% ± 12% and increases in cytochrome c, MMP, and PMP all evident as MR increased, with significant increases in PMP at MR = 100. Although it is expected for MMP to drop in apoptotic cells, it has previously been found that some epithelial cells will exhibit an increase in MMP prior to a loss of MMP if the dose is increased.[13,38] This would indicate a dose-dependent relationship with regard to SC12CDClickpropylamine-siRNA nanocomplex–mediated toxicity, which has also been seen in unmodified CD delivery to Calu-3 cells.[42] Finally, this high level of cell viability was found to be in contrast to previous studies indicating the cytotoxic nature of preexisting transfection agents such as PEI and RNAiFect-siRNA nanocomplexes.[13,43] With specific regard to RNAiFect, Arima *et al.* found that although well tolerated at MR ≤ 6, RNAiFect-siRNA nanocomplexes resulted in decreases in cell viability of up to 60% when MRs increased up to MR = 10.[43]

To our knowledge, this is the first investigation into the siRNA transfection efficiency of a modified cationic CD of this nature in airway epithelial cells. Calu-3 cells were selected as they are a well established cell line that is often used in early lung deposition studies.[44] Overall, it was found that SC12CDClickpropylamine nanocomplexes mediated levels of luciferase knockdown in Calu-3 cells that were comparable to knockdown levels in the RNAiFect commercial control. This optimal level of knockdown at MR = 20 was also successfully predicted using HCA analysis, which indicated optimal siRNA uptake at MR = 20. An additional contrast was noted between the significantly higher levels of uptake of SC12CDClickpropylamine-siRNA nanocomplexes compared with RNAiFect-siRNA nanocomplexes and their comparable levels of luciferase knockdown. This was mostly likely due to a previously described phenomenon whereby it is possible that the RNAi uptake and trafficking machinery can become saturated in the presence of large amounts of short-chain RNAs such as siRNA or miRNA.[45–47] This would imply that the highly efficient means by which SC12CDClickpropylamine-siRNA nanocomplexes are internalized could be managed in the future by a reduced dose, thereby reducing the potential for adverse reactions even further.

Furthermore, levels of gene knockdown in Calu-3 cells were similar to those seen using other cationic polysaccharides such as chitosan in more easily transfected cells

lines such as HeLa and HEK where knockdown levels of approximately 50% were reported.[48,49] Finally, knockdown levels observed in this study were also comparable to levels of the gold standard, branched PEI (25 kDa) *in vitro* siRNA knockdown, which ranged from 30% to 60% depending on the cell type used,[13,50] with none of the PEI-associated toxicity issues. The variations observed in knockdown efficiency using this particular SC12CDClickpropylamine are likely due to the different microenvironments within each cell type and target gene examined. A variety of knockdown levels have also been reported in other studies using several different modified CD constructs, ranging from $\sim$40% to 80%[33,51–53] using both *in vitro* and *in vivo* models.

In this study, an additional step was under taken to examine the behavior of these SC12CDClickpropylamine nanocomplexes following nebulization. Expelled droplets containing SC12CDClickpropylamine-siRNA nanocomplexes were found to be of appropriate diameter for deep lung delivery, with the % FPF indicating that 50–60% of expelled particles were $<5\,\mu$m. Overall, these results were in agreement with previous studies involving nebulized CDs whereby nebulization of CD-drug combinations resulted in no loss in output performance and an increase in emitted drug in cases.[18,54] Coupled with this, postnebulization transfection ability appeared to be unaltered using the Aeroneb Pro vibrating mesh nebulizer. The use of a nebulizer in early screening studies for siRNA delivery is also of high importance, as this is a clinically relevant mode of siRNA delivery as opposed to solely investigatory modes such as intratracheal microsprayers and whole-bolus nasal delivery. Investigations using clinically relevant siRNA delivery models remain in the minority with only one previous study undertaking similar experiments.[35] It is encouraging to note, however, that this study also reported high levels of siRNA stability using the Aeroneb Pro system. With this in mind, this new generation of nebulizer technology, coupled with the appropriate siRNA delivery vector appears to have great potential in maintaining siRNA stability. This will allow for successful future scale-up and testing in more complex *in vitro* and *in vivo* models for a more comprehensive and in-depth examination of therapeutic potential.

Conclusions

Novel gene and siRNA delivery vector designs and innovations based on existing molecules are constantly being developed, and the need for rapid and accurate evaluation is of paramount importance if they are to reach their clinical and commercial potential. Evaluations must, of course, be designed with specific regard to the particular environment within the body to which the siRNA is to be directed and any interface with devices that are required. With this in mind, the high-throughput methods that we have successfully optimized in our previous studies were harnessed to examine a completely novel vector for delivering siRNA to airway epithelial cells. This gene delivery vector, a modified cationic CD, was assessed over a range of parameters in order to rapidly and comprehensively screen the various formulations of CD-siRNA nanocomplex for transfection to airway epithelial cells.

These studies revealed that SC12CDClickpropylamine was capable of forming highly cationic nanocomplexes with siRNA, which traversed the cell membrane at high levels, were well tolerated by epithelial cells, and mediated levels of knockdown at MR = 20 that were comparable to commercial controls. Aerosol delivery is a device-dependent approach, and by using Aeroneb vibrating mesh nebulizers, the transfection ability of the SC12CDClickpropylamine-siRNA nanocomplexes was undiminished following nebulization. Following this study, it was found that SC12CD Clickpropylamine-siRNA nanocomplexes can be effectively nebulized for pulmonary delivery of siRNA using Aeroneb technology to mediate knockdown in epithelial cells. Herein an integrated nanomedicine-device platform was developed for potential application in preclinical and clinical studies of inhaled siRNA therapeutics.

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Author Disclosure Statement

At the time of preparation of this document, Ronan MacLoughlin was employed as a Senior Scientist in Aerogen Limited. All other authors have no conflicts of interest to declare.

References

1. de Fougerolles A, and Novobrantseva T: siRNA and the lung: research tool or therapeutic drug? Curr Opin Pharmacol. 2008;8:280–285.
2. Taratula O, Garbuzenko OB, Chen AM, and Minko T: Innovative strategy for treatment of lung cancer: targeted nanotechnology-based inhalation co-delivery of anticancer drugs and siRNA. J Drug Target. 2011;19:900–914.
3. Ge Q, Filip L, Bai A, Nguyen T, Eisen HN, and Chen J: Inhibition of influenza virus production in virus-infected mice by RNA interference. Proc Natl Acad Sci U S A. 2004;101:8676–8681.
4. Seong J-H, Lee K-M, Kim ST, Jin S-E, and Kim C-K: Polyethylenimine-based antisense oligodeoxynucleotides of IL-4 suppress the production of IL-4 in a murine model of airway inflammation. J Gene Med. 2006;8:314–323.
5. Birchall J: Pulmonary delivery of nucleic acids. Expert Opin Drug Deliv. 2007;4:575–578.
6. Weiss DJ: Delivery of DNA to lung airway epithelium. Methods Mol Biol. 2004;246:53–68.
7. Judge AD, Sood V, Shaw JR, Fang D, McClintock K, and MacLachlan I: Sequence-dependent stimulation of the mammalian innate immune response by synthetic siRNA. Nat Biotechnol. 2005;23:457–462.
8. Aneja MK, Geiger J-P, Himmel A, and Rudolph C: Targeted gene delivery to the lung. Expert Opin Drug Deliv. 2009;6:567–583.
9. Whitehead KA, Langer R, and Anderson DG: Knocking down barriers: advances in siRNA delivery. Nat Rev Drug Discov. 2009;8:129–138.
10. Patton JS, and Byron PR: Inhaling medicines: delivering drugs to the body through the lungs. Nat Rev Drug Discov. 2007;6:67–74.
11. Kichler A: Gene transfer with modified polyethylenimines. J Gene Med. 2004;6:S3–S10.
12. Kircheis R, Wightman L, and Wagner E: Design and gene delivery activity of modified polyethylenimines. Adv Drug Deliv Rev. 2001;53:341–358.

13. Hibbitts A, Lieggi N, McCabe O, Thomas W, Barlow J, O'Brien F, and Cryan S-A: Screening of siRNA nanoparticles for delivery to airway epithelial cells using high-content analysis. Ther Deliv. 2011;2:987–999.
14. Godbey WT, and Mikos AG: Recent progress in gene delivery using non-viral transfer complexes. J Control Release. 2001;72:115–125.
15. Plank C, Mechtler K, Szoka FC Jr, and Wagner E: Activation of the complement system by synthetic DNA complexes: a potential barrier for intravenous gene delivery. Hum Gene Ther. 1996;7:1437–1446.
16. O'Mahony AM, Ogier J, Desgranges S, Cryan JF, Darcy R, and O'Driscoll CM: A click chemistry route to 2-functionalised PEGylated and cationic β-cyclodextrins: co-formulation opportunities for siRNA delivery. Org Biomol Chem. 2012;10:4954–4960.
17. O'Mahony AM, Godinho BM, Ogier J, Devocelle M, Darcy R, Cryan JF, and O'Driscoll CM: Click-modified cyclodextrins as nonviral vectors for neuronal siRNA delivery. ACS Chem Neurosci. 2012;3:744–752.
18. Tewes F, Brillault J, Couet W, and Olivier J-C: Formulation of rifampicin–cyclodextrin complexes for lung nebulization. J Control Release. 2008;129:93–99.
19. Thi THH, Azaroual N, and Flament M-P: Characterization and in vitro evaluation of the formoterol/cyclodextrin complex for pulmonary administration by nebulization. Eur J Pharm Biopharm. 2009;72:214–218.
20. Salem LB, Bosquillon C, Dailey LA, Delattre L, Martin GP, Evrard B, and Forbes B: Sparing methylation of β-cyclodextrin mitigates cytotoxicity and permeability induction in respiratory epithelial cell layers *in vitro*. J Control Release. 2009;136:110–116.
21. Cabral-Marques H, and Almeida R: Optimisation of spray-drying process variables for dry powder inhalation (DPI) formulations of corticosteroid/cyclodextrin inclusion complexes. Eur J Pharm Biopharm. 2009;73:121–129.
22. Zhang Y, Jiang XG, and Yao J: Nasal absorption enhancement of insulin by sodium deoxycholate in combination with cyclodextrins. Acta Pharmacol Sin. 2001; 22:1051–1056.
23. Jalalipour M, Najafabadi AR, Gilani K, Esmaily H, and Tajerzadeh H: Effect of dimethyl-β-cyclodextrin concentrations on the pulmonary delivery of recombinant human growth hormone dry powder in rats. J Pharm Sci. 2008;97: 5176–5185.
24. Chaturvedi K, Ganguly K, Kulkarni AR, Kulkarni VH, Nadagouda MN, Rudzinski WE, and Aminabhavi TM: Cyclodextrin-based siRNA delivery nanocarriers: a state-of-the-art review. Expert Opin Drug Deliv. 2011;8:1455–1468.
25. Mellet CO, Fernandez JMG, and Benito JM: Cyclodextrin-based gene delivery systems. Chem Soc Rev. 2011;40: 1586–1608.
26. Loftsson T, Jarho P, Másson M, and Järvinen T: Cyclodextrins in drug delivery. Expert Opin Drug Deliv. 2005; 2:335–351.
27. O'Mahony AM, O'Neill MJ, Godinho BMDC, Darcy R, Cryan J, and O'Driscoll C: Cyclodextrins for non-viral gene and siRNA delivery Pharm Nanotechnol. 2013;1:6–14.
28. Klein B, Worndl K, Lutz-Meindl U, and Kerschbaum HH: Perturbation of intracellular K^+ homeostasis with valinomycin promotes cell death by mitochondrial swelling and autophagic processes. Apoptosis. 2011;16:1101–1117.
29. Lofrumento D, La Piana G, Abbrescia D, Palmitessa V, La Pesa V, Marzulli D, and Lofrumento NE: Valinomycin induced energy-dependent mitochondrial swelling, cytochrome c release, cytosolic NADH/cytochrome c oxidation and apoptosis. Apoptosis. 2011;16:1004–1013.
30. Lee DH, and Goldberg AL: Proteasome inhibitors: valuable new tools for cell biologists. Trends Cell Biol. 1998;8:397–403.
31. Han YH, and Park WH: MG132, a proteasome inhibitor decreased the growth of Calu-6 lung cancer cells via apoptosis and GSH depletion. Toxicol In Vitro. 2010;24:1237–1242.
32. Heyder J: Deposition of inhaled particles in the human respiratory tract and consequences for regional targeting in respiratory drug delivery. Proc Am Thorac Soc. 2004;1: 315–320.
33. O'Mahony AM, Doyle D, Darcy R, Cryan JF, and O'Driscoll CM: Characterisation of cationic amphiphilic cyclodextrins for neuronal delivery of siRNA: effect of reversing primary and secondary face modifications. Eur J Pharm Sci. 2012;47:896–903.
34. O'Mahony A, Desgranges S, Ogier J, Quinlan A, Devocelle M, Darcy R, Cryan J, and O'Driscoll C: In vitro investigations of the efficacy of cyclodextrin-siRNA complexes modified with lipid-PEG-octaarginine: towards a formulation strategy for non-viral neuronal siRNA delivery. Pharm Res. 2012;1–13.
35. Steele TWJ, Zhao X, Tarcha P, and Kissel T: Factors influencing polycation/siRNA colloidal stability toward aerosol lung delivery. Eur J Pharm Biopharm. 2012;80:14–24.
36. Tsapis N, Bennett D, Jackson B, Weitz DA, and Edwards DA: Trojan particles: large porous carriers of nanoparticles for drug delivery. Proc Natl Acad Sci U S A. 2002; 99:12001–12005.
37. O'Mahony AM, Ogier J, Darcy R, Cryan JF, and O'Driscoll CM: Cationic and PEGylated amphiphilic cyclodextrins: co-formulation opportunities for neuronal siRNA delivery. PLoS ONE. 2013;8:e66413.
38. Rawlinson LA, O'Brien PJ, and Brayden DJ: High content analysis of cytotoxic effects of pDMAEMA on human intestinal epithelial and monocyte cultures. J Control Release. 2010;146:84–92.
39. Lawlor C, O'Sullivan MP, Sivadas N, O'Leary S, Gallagher PJ, Keane J, and Cryan S-A: The application of high-content analysis in the study of targeted particulate delivery systems for intracellular drug delivery to alveolar macrophages. Mol Pharm. 2011;8:1100–1112.
40. Rejman J, Bragonzi A, and Conese M: Role of clathrin- and caveolae-mediated endocytosis in gene transfer mediated by lipo- and polyplexes. Mol Ther. 2005;12:468–474.
41. von Gersdorff K, Sanders NN, Vandenbroucke R, De Smedt SC, Wagner E, and Ogris M: The internalization route resulting in successful gene expression depends on both cell line and polyethylenimine polyplex type. Mol Ther. 2006;14:745–753.
42. Matilainen L, Toropainen T, Vihola H, Hirvonen J, Järvinen T, Jarho P, and Järvinen K: *In vitro* toxicity and permeation of cyclodextrins in Calu-3 cells. J Control Release. 2008;126:10–16.
43. Arima H, Tsutsumi T, Yoshimatsu A, Ikeda H, Motoyama K, Higashi T, Hirayama F, and Uekama K: Inhibitory effect of siRNA complexes with polyamidoamine dendrimer/α-cyclodextrin conjugate (generation 3, G3) on endogenous gene expression. Eur J Pharm Sci. 2011;44:375–384.
44. Sporty JL, Horalkova L, and Ehrhardt C: *In vitro* cell culture models for the assessment of pulmonary drug disposition. Expert Opin Drug Metab Toxicol. 2008;4:333–345.

45. Grimm D, Streetz KL, Jopling CL, Storm TA, Pandey K, Davis CR, Marion P, Salazar F, and Kay MA: Fatality in mice due to oversaturation of cellular microRNA/short hairpin RNA pathways. Nature. 2006;441:537–541.
46. Yi R, Doehle BP, Qin Y, Macara IG, and Cullen BR: Overexpression of Exportin 5 enhances RNA interference mediated by short hairpin RNAs and microRNAs. RNA. 2005;11:220–226.
47. Castanotto D, Sakurai K, Lingeman R, Li H, Shively L, Aagaard L, Soifer H, Gatignol A, Riggs A, and Rossi JJ: Combinatorial delivery of small interfering RNAs reduces RNAi efficacy by selective incorporation into RISC. Nucleic Acids Res. 2007;35:5154–5164.
48. Lee DW, Yun K-S, Ban H-S, Choe W, Lee SK, and Lee KY: Preparation and characterization of chitosan/polyguluronate nanoparticles for siRNA delivery. J Control Release. 2009;139:146–152.
49. Raviña M, Cubillo E, Olmeda D, Novoa-Carballal R, Fernandez-Megia E, Riguera R, Sánchez A, Cano A, and Alonso M: Hyaluronic acid/chitosan-g-poly(ethylene glycol) nanoparticles for gene therapy: an application for pDNA and siRNA delivery. Pharm Res. 2010;27:2544–2555.
50. Malek A, Czubayko F, and Aigner A: PEG grafting of polyethylenimine (PEI) exerts different effects on DNA transfection and siRNA-induced gene targeting efficacy. J Drug Target. 2008;16:124–139.
51. Bartlett D, Su H, Hildebrandt I, Weber W, and Davis ME: Impact of tumor-specific targeting on the biodistribution and efficacy of siRNA nanoparticles measured by multimodality *in vivo* imaging. Proc Natl Acad Sci U S A. 2007;104:15549–15554.
52. Hu-Lieskovan S, Heidel JD, Bartlett DW, Davis ME, and Triche TJ: Sequence-specific knockdown of EWS-FLI1 by targeted, nonviral delivery of small interfering RNA inhibits tumor growth in a murine model of metastatic Ewing's sarcoma. Cancer Res. 2005;65:8984–8992.
53. Bartlett DW, and Davis ME: Impact of tumor-specific targeting and dosing schedule on tumor growth inhibition after intravenous administration of siRNA-containing nanoparticles. Biotechnol Bioeng. 2008;99:975–985.
54. Yang W, Chow KT, Lang B, Wiederhold NP, Johnston KP, and Williams RO 3rd: In vitro characterization and pharmacokinetics in mice following pulmonary delivery of itraconazole as cyclodextrin solubilized solution. Eur J Pharm Sci. 2010;39:336–347.

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