An inhalable β2-adrenoceptor ligand-directed guanidinylated chitosan carrier for targeted delivery of siRNA to lung

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Abstract

siRNA-based strategies appear to be an exciting new approach for the treatment of respiratory diseases. To extrapolate siRNA-mediated interventions from bench to bedside in this area, several aspects have to be jointly considered, including a safe and efficient gene carrier with pulmonary deposition efficiency, as well as in vivo method for siRNA/nanoparticles delivery. Accordingly, in this work, (i) a non-viral DNA vector, guanidinylated chitosan (GCS) that has been developed in our previous study [X.Y. Zhai, P. Sun, Y.F. Luo, C.N. Ma, J. Xu, W.G. Liu, 2011], was tested for siRNA delivery. We demonstrated that GCS was able to completely condense siRNA at weight ratio 40:1, forming nanosize particles of diameter ~100 nm, 15 mV in surface potential. Guanidinylation of chitosan not only decreased the cytotoxicity but also facilitated cellular internalization of siRNA nanoparticles, leading to an enhanced gene-silencing efficiency compared to the pristine chitosan (CS). (ii) We chemically coupled salbutamol, a β2-adrenoceptor agonist, to GCS (SGCS), which successfully improved targeting specificity of the green fluorescent protein (GFP)-siRNA carrier to lung cells harbored with β2-adrenergic receptor, and remarkably enhanced the efficacy of gene silence in vitro and in the lung of enhanced green fluorescent protein (EGFP)-transgenic mice in vivo. (iii) It was proved that this chitosan-based polymer was able to provide both the pDNA and siRNA with the protection against destructive shear forces generated by the mesh-based nebulizers. Aerosol treatment improved the nanoparticle size distribution, which should be in favor of enhancing the transfection efficiency. We suggest a potential application of the chitosan-derived nanodelivery vehicle (SGCS) in RNA interference therapy for lung diseases via aerosol inhalation.

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1 Introduction

RNAi using small interfering RNA (siRNA) has proven to be more potent than conventional antisense strategies [1]. The potential of silencing genes implicated in diseases using siRNA has led to a rapidly growing area in drug discovery, such as viral infection [2], cancer [3], and inflammatory conditions [4]. However, unassisted delivery of siRNA to the target cells in vivo is usually frustrating as a result of its poor cellular uptake, rapid degradation by ubiquitous nuclease as well as limited blood stability. To overcome these problems, researchers have developed a variety of polymer vectors [5], among which, chitosan-based carrier has gained more and more interest due to its biocompatibility and biodegradability [6]. However, the poor water solubility and the low transfection efficiency of chitosan have posed a limitation for its use as a nucleic acid delivery vector. For the treatment of asthma, we previously developed a 12-alkylated chitosan nanoparticle vector that was able to deliver endothelin-converting enzyme (ECE)-siRNA into airway epithelial tissues of the ovalbumin-challenged mice, leading to down regulation of ECE expression to some extent [7]. But the efficiency of RNAi mediated by the 12-alkylated chitosan nanoparticle was not high enough to fully block production of active endothelin-1. The low gene-silencing efficiency of siRNA mediated by chitosan is mainly due to the poor cellular uptake across cell membrane. Taking advantage of the non-specific uptake mechanisms, such as the application of cell penetrating peptides (CPPs), seems a promising way to break through the cell membrane to deliver nucleic acid [8]. CPPs, especially arginine-rich peptides such as HIV-1 Tat, oligoarginine peptides, have been demonstrated to possess the ability to translocate the membrane [9]. It has been also found that the arginine-induced perturbation is preferentially ascribed to the presence of the guanidinium group in arginine [10]. According to these studies, we have previously developed a guanidinylated chitosan (GCS) gene carrier showing an increase in
solubility and greatly improved DNA transfection efficiency in vitro [11]. These findings imply that GCS could also serve as an effective siRNA vector.

Introduction of targeting ligands can facilitate internalization of more gene vectors to the selected cell types while reduce undesired side-effects in non-target cells. Several ligands such as transferrin [12], folate [13], mannose [14] and galactose [15] conjugated chitosans have been designed and evaluated for receptor-mediated endocytotic gene delivery. The beta2-adrenergic receptor (β2-AR) is found abundantly in bronchial smooth muscle and lung tissue. Various β2-AR agonists have been used as bronchodilators for routine treatment of chronic obstructive pulmonary disease and asthma. In previous study, we have tried to develop a liposome vector coupled with salbutamol. We found that intravenous injection of a gene carried by the liposome-salmonel increased the gene expression in the lung of the treated guinea pigs in comparison to the unmodified lipidosome gene carrier [16]. Elfinger et al. [17] as well showed that clenbuterol could be used to improve gene transfer efficiency of PEI gene vectors both in vitro and in vivo. These findings implicated that β2-AR agonists may be used as a targeting ligand to improve receptor-mediated gene delivery to lungs.

In addition, topical delivery of drugs offers the advantages of enhanced drug delivery to anatomical target site with maximum therapeutic efficacy but the minimal adverse side effect. This has led to a widespread use of inhalation therapy for lung diseases. In terms of gene therapy, direct administration of vectors/nucleic acid complexes through the trachreal route is a promising alternative to systemic delivery by which the complexes have to encounter more nuclease degradation, hepatic clearance and serum-induced aggregation [18]. In small animal models, intratracheal instillation of siRNA/carrier complexes has been used for lung transfection [18]. However, this invasive method is unlikely used for treating patients, or otherwise administration of therapeutic vector/nucleic acid complexes by aerosol inhalation is acceptable for clinical use. The issue with which we are most concerned is that many nebulization methods (ultrasonic and jet nebulization) have a negative impact on the physical stability and integrity of naked pDNA and some gene delivery systems, resulting in a marked reduction in their transfection efficiency [19,20]. Recently, a third type, namely vibrating-mesh nebulizers, have been commercially available. In its systemic design, a micropump technology (e.g., Aeroneb Pro and Aeroneb Go nebulizers) is claimed to have a better performance in the delivery of suspensions, proteins and peptides than conventional nebulizers [21]. But there is little literature about its use for gene delivery so far.

Designing an ideal non-viral nucleic acid vector system for RNAi therapy requires a well-structured, systematic investigation of several aspects ranging from the development of efficient vectors to the establishment of an available approach for targeting the therapeutic siRNA/vector complexes to specific disease sites. In present studies, we explored the application of the guanidinylated chitosan carrying siRNA for gene-silencing therapy. Meanwhile, we chemically coupled salbutamol to the guanidinylated chitosan and successfully improved pulmonary targeting specificity of the siRNA carrier, and thereby remarkably enhanced the efficacy of gene silence in lung. In addition, the effect of aerosol approach on the nanocomplexes was also investigated to determine its potential for future application in delivery of the therapeutic nanocomplexes to the lung of patients.

2. Experimental

2.1. Materials

Chitosan (CS, molecular weight 50 kDa, deacetylation degree 92%) was supplied by AK Biotech (Shandong, China). Aminominothanesulfinic acid (AIMSA), cyanamide (98%) and salbutamol sulphate (SAL) were purchased from Alfa Aesar Chemical Co. (Tianjin, China). Aminominothanesulfonic acid (AIMSOA) was prepared according to the synthetic procedure given in the literature [22]. pDNA (pEGFP-n2) encoding a red-shifted variant of wild-type green fluorescent protein (GFP) was provided by Clontech (Mountain View, CA, USA). siRNA-GFP duplex (21 bp) and negative Control #1 siRNA were prepared from Ambion, Inc. (Texas, USA). YOYO-1 iodide was acquired from Invitrogen, Inc. (Carlsbad, CA). Cell Count Kit-8 was supplied by Dojindo (Kumamoto, Japan). RIPA buffer was purchased from Cell Signaling Technology, Inc. (CST, China). All other reagents used were of analytical grade.

2.2. Synthesis of guanidinylated chitosan (GCS)

Synthesis of GCS was processed as described in previous study [11]. GCS with 8.5% guanidino substitution degree was used in present study for its substitution degree was similar to that of salbutamol modified guanidinylated chitosan (SGCS) used in this study.

2.3. Synthesis of salbutamol modified guanidinylated chitosan

2.3.1. Synthesis of salbutamol modified chitosan (SCS)

Chitosan (1 g) was suspended in 0.1 M HCl (150 ml), and the solution was stirred at 80 °C until chitosan was dissolved fully. After the solution was cooled to room temperature, 2 M NaOH aqueous solution (150 ml) was added. Twelve hours later, the excessive alkali solution was extracted through filtration [23]. The alkali-treated chitosan was obtained, and vacuum dried at 100 °C.

Salbutamol sulphate (0.288 g, 1 mmol) was dissolved in 20 ml DMSO, and then epoxy chloropropane (78.3 μl, 1 mmol) was added under stirring. After the solution was stirred well, 1 M NaOH aqueous solution (20 ml) was added. The reaction was kept for about 4.5 h at room temperature, followed by addition of alkali-treated chitosan thereafter for another 4.5 h. Finally, the mixture was dialyzed against water (MWCO of 3500) and lyophilized.

2.3.2. Synthesis of salbutamol modified guanidinylated chitosan (SGCS)

SCS (0.203 g, 1 mmol) was dissolved in 25 ml distilled water. After stirring thoroughly at 50 °C, AIMSOA (0.5 g, 4 mmol) was added. The reaction mixture was stirred at room temperature for about 30 min. Finally, the mixture was dialyzed against water (MWCO 3500) and lyophilized to collect salbutamol modified guanidinylated chitosan (SGCS).

2.4. Measurement of cellular internalization

Negative control siRNA (neg-siRNA) was labeled with YOYO-1 dye as follows: 1 mmol/L YOYO-1 was diluted 1:100 in PBS. Then siRNA was labeled with YOYO-1 iodide using one dye molecule per 15 bp and incubated for 2 h in the dark. YOYO-1 labeled siRNA was used to form CS/siRNA complexes with the ratio of 40. HEK293 cells were prepared as described in Supplementary Materials. Polyplexes were added in the plate (1 μg siRNA/well) and incubated for 4 h. Following transfection the cells were subjected to DAPI staining to visualize nuclei. Uptake of duplex siRNA was monitored by a laser confocal microscope.

2.5. Silencing EGFP in vitro

In the study, recombinant HEK293 cells with the constitutive EGFP expression were prepared as described in the literature [24]. In the EGFP silencing study, HEK293 green cells were plated on 24-well plates (105 cells/well) in RPMI media (containing 10% FBS and G418 selection factor) 24 h prior to transfection. Subsequently, the media was removed and replaced with 450 μl fresh media. Various polyplexes at different weight ratios were then added to the plates (50 μl in water, containing 0.5 μg siRNA/well). After 4 h, the media was replaced with 0.5 ml fresh media containing 10% FBS. The cells were left for 44 h thereafter washed twice with the pre-warmed phosphate buffered saline (PBS) before they were fixed in 4% paraformaldehyde. The fixed cells were then visualized...
under a confocal laser scanning microscopy (CLSM, Nikon, model number Japan). Using a standard trypsin protocol, the cells were removed from the culture plate and resuspended in PBS. The EGFP cell fluorescence was quantified using a Becton Dickinson FACS Calibur flow cytometer. The Geomean was taken for the measure of fluorescence intensity.

2.6. In vitro transfection in A549 cells and 16HBE

A549 (human alveolar epithelial) cells was obtained from ATCC (American Type Culture Collection). 16HBE (human bronchial epithelial) is a gift from Holgate. Cell culture was performed as described for HEH 293 cells (detailed in Supplementary Materials). For transfection, both cells were treated with GCS/pDNA and SGCS/pDNA complexes respectively. The percentage of cells showing green fluorescence was quantified by FACS.

2.7. Nebulizer device

The Aeroneb Pro nebulizer [21, 25] is a customized large aperture vibrating-mesh device and is manufactured by Aerogen, Inc., USA (detailed in Supplementary Materials).

2.8. In vivo inhibition of EGFP gene expression

EGFP-transgenic mice (C57BL/6-Tg) (ACTb-EGFP) were provided by Cyagen Biosciences Inc. (Guangzhou, China). All animal experiments were carried out according to the National Institutes of Health Guide for Care and Use of Laboratory Animals and were approved by the Bioethics Committee of State Key Laboratory of Respiratory Disease, Guangzhou Medical University. Test polyplexes were prepared as described in Supplementary Materials (working concentration of siRNA used here was 50 μg/ml), followed by being placed into the reservoir above the domed aperture plate of the Aeroneb Pro nebulizer. The sample solution was aspirated through the nebulizer and formed an aerosol. The aerosols were collected into ice cold glass tubes. Test formulations were then sprayed directly into the lungs of mice via the endotracheal route using a MicroSprayeregizer (Penn-Century, Philadelphia, PA, USA) described by Maytal Bivas-Benita previously [26]. Administrations were conducted each day over 3 consecutive days. Lungs were isolated from the mice at day 5 after the treatment and processed for cryosectioning (on the right lobe). The left lobe was subjected to protein extract for Western blot analysis by using an anti-GFP antibody (Biolegend, USA) [27]. GAPDH was used as an internal control and the relative optical density of EGFP/GAPDH was quantified by Gel-Pro Analyzer 4.0 (Media Cybernetics, Silver Spring, MD).

2.9. Nebulization

2.9.1. pDNA integrity and polyplexes stability during nebulization

Solutions of GCS/pDNA complexes or naked pDNA were nebulized and collected as described above for siRNA polyplexes. The integrity of naked and formulated pDNA was tested using an agarose gel retardation assay. Polyplexes recovered after nebulization were dissociated with heparin (5 mg/ml) (Sigma, St Louis, MO, USA) for 2 h at room temperature before the gel assay.

2.9.2. In vitro transfection of GCS/pDNA complex before and after nebulization

HEK293 cells were cultured and GCS/pDNA complex was prepared as described in Supplementary Materials. For transfection, cells were exposed to un-nebulized and nebulized GCS/pDNA complexes (aerosol mist) respectively (containing 1 μg pDNA/50 μl water). Following transfection, cells were collected for quantification of percentage of GFP-positive cells by FACS as described above.

2.9.3. Size analysis of GCS/pDNA complexes before and after nebulization

Samples were prepared for TEM observation as described before [11]. The size distribution of complex nanoparticles after nebulization was also measured using dynamic light scattering (DLS) (detailed in Supplementary Materials).

3. Results and discussion

3.1. Synthesis and characterization of SGCS

To improve transfer specificity of GCS/nucleic acid nanoparticles for lung, coupling of salbutamol, a β2-adrenoceptor agonist, to GCS was performed as shown in the reaction scheme of Fig. 1A. The formation of SGCS can be confirmed by 13C-NMR (Fig. 1B) and 1H-NMR spectra (Fig. 1C) (detailed in Supplementary Materials). In the 13C NMR spectra of CS and SGCS, the feature bands of chitosan at 56.5(C2), 60.8(C6), 70.6(C3), 75.3(C5), 77.5(C4), and 98.0(C1) (ppm) are clearly shown. For SGCS, a new signal located at 174.8 ppm is assigned to the carbon of guanidinium group [28], which further confirms the successful introduction of guanidinium group to chitosan. The structures of CS and SGCS are confirmed by 1H-NMR spectra as shown in Fig. 1C. The 1H-NMR spectrum of CS shows the feature peak of CH3 in N-acetyl residue at 1.8 ppm and multiplets from 2.9 to 3.7 ppm corresponding to the ring methane protons [29]. For SGCS compared with SAL, new peaks locating at 1.2 and 7.2 ppm are attributed to the methylene of salbutamol and the benzene ring of salbutamol respectively, confirming the successful grafting of salbutamol. The degree of substitution (DS) of salbutamol on SGCS was calculated based on 1H-NMR spectra. Through the integral computation, we calculated that the DS of salbutamol on SGCS is about 3.5%. The degree of guanidinylation substitution for SGCS (expressed as the number of guanidinium groups per 100 anhydroglucose units of chitosan) estimated through elemental analysis is 8.2% (Table S1 in Supplementary Materials).

3.2. Formation of polyplexes

The ability of chitosans and derivatives to form complexes with siRNA was evaluated using the gel retardation assay (detailed in Supplementary Materials). Complexes containing constant concentration of siRNA (10 μg/ml) were prepared at various weight ratios of 5–80 (vector/nucleic acid) (detailed in Supplementary Materials). As presented in Fig. 2A, CS and GCS can completely retard siRNA at the weight ratios of 20 and 40, respectively (no presence of a trailing band), suggesting that siRNA is fully complexed. The results also manifest that the effective association of siRNA with the polycations differs substantially from pDNA which is completely condensed by CS and GCS at very low weight ratios of 0.75 and 1, respectively [11], indicating that there is a stronger interactive force between polycation with large polyamionic species (pDNA) than with shorter siRNA [30]. This distinct behavior of siRNA condensation, when compared to pDNA, could be explained by some structural differences between the two molecules. In fact, pDNA is almost completely condensed when around 90% of its phosphodiester backbone charge is neutralized [31, 32], whereas siRNA molecule (with a 21 base pairs length) during the nanoparticle formation presents an excess of...
negative charges and therefore requires a greater amount of polycations for complexion [33].

Generally, with increasing the weight ratio, the particle sizes of the respective polyplexes exhibit a rising trend (Fig. 2C). At a weight ratio of 80:1, the particle sizes of GCS/siRNA are larger than those of CS/siRNA complexes, which may be due to the self-aggregation of GCS polyplexes caused by hydrogen bonding interaction between the guanidinium groups at a higher complexing ratio [34]. The best known form of endocytosis is initiated by clathrin-coated pit through a non-specific cellular internalization, which requires a smaller size than 150 nm [35]. Correspondingly, Grayson [36] recently reported that siRNA/polyethyleneimine (PEI) complexes with a diameter larger than ~150 nm were unable to mediate gene silencing in vitro. Anyway, the particle sizes of GCS/siRNA complexes remain under 150 nm (133.70±3.54) even at the weight ratio of 80, ensuring an effective cellular uptake.

The comparative positive values of surface charge (zeta potential) of both the CS and GCS polyplexes increase with the increasing concentration of polymers at a constant siRNA concentration (Fig. 2C). The appropriate net positive charge of the particles is desired to prevent particle aggregation and promote electrostatic interaction with the overall negative charge of the cell membrane [37].

From Fig. 2B, one can also see that SGCS completely retards siRNA at a similar critical complexing ratio to that of GCS/siRNA, 40, indicating that introduction of salbutamol has no significant effect on the siRNA-binding capacity of GCS. SGCS completely condensed pDNA at a very low complexing ratio (Fig. 2B) in comparable to that of GCS [11]. Comparing the particle sizes and zeta potentials between the SGCS and GCS polyplexes (Fig. 2C), no significant difference is found, suggesting that the salbutamol modification of GCS does not change their physicochemical properties.

3.3. In vitro cytotoxicity

A potential cytotoxic effect of the both CS/siRNA and GSC/siRNA complexes was tested in HEK 293 cells following the treatment with the both using a tetrazolium-based viability assay (detailed in Supplementary Materials). Those cells without the treatment of polyplexes served as a control. The control cell viability was defined as 100%. As shown in Fig. 2D, over 90% average cell viability is observed for GCS/siRNA complexes formulated at various ratios in comparison to the control cells. In contrast, the CS/siRNA exhibits a ratio-dependent cytotoxicity with decreasing cell viability (less than 80%) at the weight ratio of 80. These results suggest that guanidinylation of CS can reduce the toxicity of siRNA polyplexes due to taming electrostatic interaction with cell membrane [38]. We also found that little toxicity was attributable to the inclusion of salbutamol into GCS, which is a required characteristic for a gene delivery system. Elfinger et al. [17] also reported a PEI-g-clenbuterol gene vector with high specificity and gene transfer efficiency. However, the presence of unconjugated PEI would increase the cytotoxicity of the complex. Moreover, clenbuterol is not approved for the treatment of human diseases. Therefore, a β2-AR agonist, such as salbutamol, available for clinical therapy will be preferred as a homing device that allows receptor-mediated gene delivery.

3.4. Cellular uptake and gene silencing

We had previously demonstrated that modification of chitosan with guanidinium groups could significantly improve the cellular uptake of plasmid DNA [11]. Using YOYO-1 labeled-siRNA prior to the complex formulation for tracking the intracellular siRNA distribution [39], we investigated if the modified chitosan for siRNA delivery could present the same effect as pDNA delivery. Under confocal fluorescence microscope, it is shown that there is a significant increase in accumulation of fluorescence signals in the cytosol of the cells treated with GCS/siRNA-YOYO-1 complex, compared to those cells treated with YOYO-1-labeled CS/siRNA (Fig. 3A), indicating an enhanced
cellular uptake of siRNA by using GCS carrier compared to unmodified CS.

We next tested the gene-silencing efficacy of the GCS/siRNA nanocomplexes. A HEK293 cell line containing stably integrated EGFP expression cassette was subjected to performance of the cell transfection with either CS/GFP-siRNA or GCS/GFP-siRNA complexes. We observe, using confocal microscopy, that the intensity of the cell fluorescence diminishes significantly in the cells exposed to GCS/GFP-siRNA complexes, as compared to the untreated control, or those cells exposed to CS/GFP-siRNA, even though CS/GFP-siRNA could down regulate EGFP to some extent (Fig. 3B). GCS nanoparticles containing mismatch siRNAs do not knockdown EGFP expression on the cells in comparison to the control (Fig. 3B), confirming GFP-siRNA specificity. We further quantified this trend using flow cytometric measurements. As shown in Fig. 3C, the highest level of GFP knockdown (34.9%) is observed in cells treated with GCS/GFP–siRNA complex with a weight ratio of 40. Although siRNA functions in the cytoplasm, while DNA is expressed in the nucleus, the delivery of siRNA faces almost the same obstacles as DNA delivery, from cell targeting to internalization and endosomal escape [40]. Efficient nucleic acid delivery requires a fine balance between protection and release of cargo following internalization. In the present study, a low weight ratio (vector/nucleic acid) of less than 20 did not provide sufficient protection for siRNA, resulting in a lower cell uptake of GFP-siRNA, while an excessive vector, at a weight ratio of 80, could hinder the siRNA release. Nanoparticle formulation with weight ratio of 40 used in this study to efficiently deliver siRNA-EGFP therefore achieves an optimal intermediate stability profile, simultaneously providing nuclease protection and intracellular disassembly for efficient endosomal escape and high GFP silencing efficiency (Figs. 2A and 3C).

3.5. Specificity of salbutamol-mediated receptor binding

Given that \( \beta_2 \)-AR is present on the surface of A549 cells but not on the surface of 16HBE cells [41], the both lung epithelial cell lines were subjected to the transfection experiments for evaluation of the selectivity of the salbutamol modified GCS carrier in the cells with or without \( \beta_2 \)-AR. As shown in Fig. 4A and B, at a complexing ratio of 1:1, the EGFP expression in A549 cells transfected with pEGFP-n2/SGCS is significantly increased with EGFP positive cells up to 10%, compared to 5.1% EGFP positive cells of those transfected with pEGFP-n2/unmodified GCS. In contrast, no significant difference in transfection efficiency is observed in 16HBE cells transfected with the pEGFP–n2 using either carrier. These results indicate the specificity of SGCS.

\( \beta_2 \)-AR belongs to the family of G-protein-coupled receptors (GPCRs). Binding of agonists with GPCRs leads to endosomal internalization of receptor/ligand complexes via a clathrin-mediated process [42]. Therefore, gene vectors conjugated with \( \beta_2 \)-AR agonists could be additionally internalized by cells via this route besides the non-specific adsorptive endocytosis. Taken together, it is reasonable to believe that SGCS has the ability to target receptor for receptor-mediated gene delivery, thereby to further improve gene transfection.

3.6. In vivo gene silencing

We next evaluated gene-silencing efficiency with the SGCS polymer for siRNA delivery in vivo. CS/GFP-siRNA, GCS/GFP-siRNA and SGCS/GFP-siRNA complexes were separately prepared at the ratio of 20 or 40, with which either of the polyplexes attained the optimized gene-silencing efficiency in vitro. Following administrations with the
test complexes (5 μg of siRNA one time each day for 3 consecutive days), the lungs of the transgenic mice which ubiquitously express EGFP were isolated for assessing the expression of GFP in lungs. The results observed by a confocal laser scanning microscopy show that a significant inhibition of GFP expression in the bronchial epithelial cells is evident in the mice treated with either GCS/GFP-siRNA or SGCS/GFP-siRNA complexes, compared to the control mice or the mice treated with CS/GFP-siRNA complex (Fig. 5A). It also appears that the green fluorescence intensity is much lower in the lung of the mice treated with SGCS/GFP-siRNA complex than that of the mice treated with GCS/GFP-siRNA complex. This difference is not only observed in the airway epithelial cells but also in the alveolar epithelial cells. Western blotting analysis of quantified GFP in the lung of the mice verifies the same results as above (Fig. 5B and C).

Salbutamol is a short-acting β2-adrenergic receptor agonist used to treat or prevent bronchial spasm in people with reversible obstructive airway disease as well as to prevent exercise-induced bronchospasms. The usual nebulizer dose is 0.63, 1.25 or 2.5 mg 3–4 times a day. The recommended dosing for tablets is 2 or 4 mg given 3–4 times daily and the dose of extended release tablets is 4 or 8 mg every 12 h. Given that 1 mg of SGCS only contained about 40 μg salbutamol, actually, there is no observed difference in airway resistance between normal mice treated with SGCS and untreated mice (Fig. S1). It seems that the bronchodilational effect of salbutamol on penetrating deeper into the lung could be ignored and the increased knockdown efficiency of SGCS is mainly due to its improved specificity. We suggest

Fig. 3. Cellular uptake and gene silencing. (A) Confocal microscopy was used to visualize cellular uptake and translocation of YOYO-1-labeled siRNA within CS and GCS. Images showed fluorescent overlay of siRNA (green, YOYO-1-labeled) and nuclei (blue, Dapi-labeled). (B) A set of representative images for EGFP-HEK293 cells respectively transfected with different complexes at weight ratio of 40. (C) Flow cytometry analysis of the EGFP-HEK293 cells transfected with different complexes at various weight ratios. Values shown are geometric mean of the fluorescence intensity (normalized to % control untreated cells). *P<0.05 vs. control, †P<0.05 vs. cells treated with CS/GFP-siRNA complex at weight ratio of 40 (n=4).
that coupling of a β2-adrenoceptor agonist to guanidinylated chitosan makes it a promising siRNA vector for gene silence therapy in lung diseases. In addition, the bronchodilator action of salbutamol might serve as an additional advantage for SGC/siRNA application in subjects with bronchospasm if it was retained to some extent in the airway.

3.7. Effect of aerosol treatment on the siRNA polyplexes

An ideal approach for targeting the siRNA nanocomplexes to specific disease sites is required for RNAi therapy. There are a number of advantages to aerosol inhalation including local targeting (e.g., for respiratory disease), noninvasiveness, immediate availability and decreased systemic toxicity [43]. The Aerogen Aerneb Nebulizer is among the new generation of nebulizers employing a vibrating mesh or plate with multiple apertures to generate a liquid aerosol [21]. The Aerneb has a three- to fivefold higher efficiency for delivering drug to the lungs than conventional jet or ultrasonic nebulizers [44,45]. However, another two commercial mesh-based nebulizers (eflow [46] and MicroAir 47) have been reported to damage extensively plasmid DNA. We therefore investigated the physical stability of naked and formulated pDNA during the aerosol procedure via a third type vibrating-mesh nebulizer that is commercially available. A 3.7-kb pDNA (pEGFP-n1) was complexed with GCS and subjected to nebulization. Gel retardation assay (Fig. 6A) was performed on the pDNA/GCS complex before and after nebulization. The structural integrity of the unprotected naked pDNA is soundly destroyed after the aerosol treatment, leaving varying lengths of fragments (smeared band, Fig. 6A, lane 3). In contrast, following nebulization, dissociation of the polyplexes by heparin shows that the integrity of pDNA within GCS is maintained (Fig. 6A, lane 2). In addition, combination of the pDNA with GCS was perfectly retained after nebulization, as evidenced by a definite fluorescence in the loading well of the corresponding lane (Fig. 6A, lane 1). Cell culture was used to determine whether the polyplexes collected in the aerosol mist retained its transfection efficiency. Compared to the corresponding un-nebulized formulations, the transfection efficiency with the nebulized GCS/pDNA complex is dramatically increased (Fig. 6B). It was also reported that pDNA condensed within PEI could tolerate shear force experienced during aerosol formation [47]. Our study, for the first time, proves that chitosan-based polymers can provide the same protection against destructive shear forces generated by the mesh-based nebulizers as well. In the case of siRNA, gene-silencing efficiency is almost the same between polyplexes before and after nebulization (data not shown). This demonstrates that the mesh-based nebulizer is a promising candidate for siRNA nanocomplexes delivery to lung. The possible reason for the observed higher transfection efficiency for the nebulized nanoparticles could be due to the difference in total number of GCS/pDNA particles internalized into the cells for the same mass of nanoparticles administered. It has been suggested that the larger particles are too voluminous to be taken up by the cell via endocytosis resulting in lower transfection, whereas the smaller particles display a much lower DNA loading [48,49]. Observational evidence by TEM shows significant changes of the nanoparticle size distribution with more than 50% of particles ranging between 70 and 80 nm, and a more uniform particle size distribution (Fig. 6C) after nebulization, compared to those without aerosol treatment where more than 50% of particles cover a range between 20 and 30 nm, concomitant with a broader particle size distribution. This is further confirmed by DLS, which displays the particle dispersion index (PDI) of GCS/pDNA nanoparticle decreases from 0.623 to 0.464 after nebulization (Table S2 in Supplementary Materials).

Fig. 5. Optimal gene-silencing activity in the lungs of GFP transgenic mice by tracheal spray of SGC/GFP-siRNA. Test polyplexes was respectively administrated to the lungs of GFP transgenic mice. Twenty-four hours after the last administration, GFP expression in lungs was assayed by CLSM (A) and western blot (B). Quantification by densitometric analysis was performed using Gel-Pro software (C). *P < 0.05 vs. untreated mice, #P < 0.05 vs. CS/GFP-siRNA treated mice, $P < 0.05 vs. GCS/GFP-siRNA treated mice (n = 4).
We therefore suggest that the nebulized nanoparticles have a more uniform distribution of optimal particle size, which should be in favor of enhancing the transfection efficiency [Fig. 6B].

4. Conclusion

In the present studies, we have demonstrated that SGCS can be used as a non-viral carrier for siRNA delivery to lung via formulating a stable siRNA/nanocomplexes targeting β2-adrenoceptor. The inhalable chitosan-based carrier for pulmonary siRNA delivery exhibited improved biological activity, resulting from a high efficient non-specific cellular uptake synergized with a simultaneous ligand-induced internalization, via introduction of guanidinium group to chitosan and conjugation with a β2-adrenoceptor agonist, thereby greatly facilitating the targeted gene silencing in the lung of the mice. The modified chitosan shows no toxicity against HEK293 cells even at a high weight ratio of 80 (carrier/siRNA), in comparison to unmodified chitosan. We suggest that SGCS can be a promising carrier for siRNA delivery to lung, such as for viral gene-specific siRNA delivery to treat viral respiratory infection. In addition, we confirm that aerosol is a feasible approach for the delivery of the nanocomplexes to the targeted gene in lung.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jconrel.2012.06.005.

References


