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In vivo transfection study of chitosan-DNA-FAP-B nanoparticles as a new non viral vector for gene delivery to the lung

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ABSTRACT

Gene therapy targeted at the respiratory epithelium holds therapeutic potential for diseases such as cystic fibrosis and lung cancer. We recently reported that Chitosan-DNA-FAP-B nanoparticles are good candidates for targeted gene delivery to fibronectin molecules (FAP-B receptors) of lung epithelial cell membrane. In this study Chitosan-DNA-FAP-B nanoparticles were nebulized to mice using air jet nebulizer. The effect of nebulization on size, zeta potential and DNA binding ability of nanoparticles were studied. The level of gene expression in the mice lungs was evaluated. Nebulization did not affect the physicochemical properties of nanoparticles. Aerosol delivery of Chitosan-DNA-FAP-B nanoparticles resulted in 16-fold increase of gene expression in the mice lungs compared with Chitosan-DNA nanoparticles. This study suggested that Chitosan-FAP-B nanoparticle can be a promising carrier for targeted gene delivery to the lung.

1. Introduction

Lung possesses inherent advantages for gene therapy since it is easily accessible via the airways, offers a large surface area for transfection and reduces the risk of systemic side-effects. Gene therapy may lead to new treatment strategies for life-threatening respiratory diseases such as cystic fibrosis, lung cancer, asthma and pulmonary fibrosis (Crystal et al., 1994; Bellon et al., 1997; Alton et al., 1999). Recombinant viral vectors, such as adenovirus or adeno-associated virus (AAV), are highly efficient but have limitations associated with their biological properties (Otake et al., 1998; Yang et al., 1996).

Non-viral vectors, based on polycation/DNA complexes, provide an attractive alternative to recombinant viral vectors since they are non-pathogenic, are less immunogenic, less restricted by the amount of DNA that can be packaged and simple to prepare and use (Cotten et al., 1992). Chitosan (1,4-2-amino-2-deoxy-b-D-glucan) is a linear biodegradable polymer derived from the natural polymer of chitin, one of the most abundant polysaccharides in nature. Chitosan and its derivatives have been widely investigated as nonviral gene carrier systems (Borchard, 2001). For this purpose, chitosan or its derivatives are complexed with pDNA to form nanosized particulates for gene delivery to the target cells. It has been shown that chitosan materials can effectively condense DNA to form complexes with stable physiochemical properties (Thanou et al., 2002; Liu et al., 2005). Furthermore, chitosan and its derivatives used in this way are reported to have excellent biocompatibility (Lee et al., 2001).

One approach to enhance the transfection efficiency of non-viral vectors is to incorporate targeting elements that bind efficiently to cell–surface receptors (Schaffer and Lauffenburger, 1998; Remy et al., 1995; Cheng, 1996) including integrins (Hart et al., 1994, 1995) and carbohydrate elements (Montier et al., 2004). The cellular targeting approach is based on cells expressing specific molecules on their surface. Many of these are known specific receptors and can be targeted to facilitate the uptake of the complexes into the cells.

Fibronectin Attachment Protein of Mycobacterium bovis (FAP-B) which is responsible for the attachment of many

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Mycobacteria on the fibronectin molecule of epithelial cell membrane can be considered as a new targeting ligand and can improve transfection rates in epithelial cells (Laqueyrerie et al., 1995; Zhao et al., 2000; Abolhassani et al., 2006).

Different application methods for gene therapy to the lung have been investigated, such as intravenous, intratracheal or even nasal administration (Bragonzi et al., 2000; Goula et al., 2000; Kichler et al., 2002). Aerosol inhalation represents a non-invasive route for gene transport into the lung. Nebulization, in particular, can target gene carriers to the peripheral regions of the respiratory tract. Air-jet and ultrasonic nebulizations are the two predominant methods utilized therapeutically to aerosolize drug solutions. Ultrasonic energy is notorious for altering or damaging some aerosolized drug substances (Rau, 2002; McCallion et al., 1995). Furthermore, ultrasonic nebulizers are usually more expensive and have not yet been investigated for the aerosolization of gene delivery systems based on cationic polymers. To date, only air-jet nebulizers have been used in inhalative gene therapy studies (Densmore et al., 2000; Gautam et al., 2001; Rudolph et al., 2002).

The distribution of transgene expression in the lung tissue is determined, in part, by the mode of delivery of the vector–DNA complexes. Intravenous administration leads to transfection of predominantly endothelial cells, with some transfection of the alveolar lining cells (Bragonzi et al., 2000; Goula et al., 2000; Griesenbach et al., 1998). On the contrary, intratracheal instillation leads to transfection primarily in the airway epithelial cells (Griesenbach et al., 1998; Kukowska-Latallo et al., 2000). Aerosol delivery distributes the complexes uniformly throughout the pulmonary tissues and represents a noninvasive alternative for targeting the airway epithelium (Stribling et al., 1992).

In the previous study in our laboratory Chitosan-DNA-FAP-B nanoparticles as a non-viral gene delivery system to lung epithelial cells were prepared and investigated. FAP-B was added to the Chitosan-DNA nanoparticles as a ligand for attachment to its specific receptors present at the surface of lung epithelial cells. The transfection efficiency of Chitosan-DNA-FAP-B nanoparticles was 10-fold higher than Chitosan-DNA nanoparticles (Mohammadi et al., 2011).

In the present study, chitosan-DNA-FAP-B nanoparticles were delivered to mice for specific gene delivery to the lung epithelial cell using air-jet nebulizer. The transfection efficiency of these new nanoparticles was evaluated following in vivo administration.

2. Materials

Chitosan Chitoclear (Mw = 126,000 Da/mol, deacetylation degree 98%), was purchased from Primex (Iceland). The plasmid pGL3-control vector encoding firefly lucifrase driven by an SV40 promoter was purchased from Bioneer. FAP-B (Fibronectin Attachment Protein of BCG) was kindly provided by Prof G. Marchal (Institut Pasteur, Paris, France) (Romain et al., 1993). Turbofect reagent was obtained from Fermentase. Sodium acetate, Sodium sulfate, Sodium hydroxide and other chemicals were purchased from Merck (Germany).

3. Animals

Six- to eight-week-old female BALB/c mice, obtained from Faculty of pharmacy, Tehran University of medical sciences, were maintained on ad libitum rodent feed and water at 24 ± 1 °C temperature, 55 ± 10% humidity and 12 h/12 h light/dark cycle under specific pathogen-free conditions. Animals were acclimatized for at least 7 days prior to the start of the experiments.

4. Methods

4.1. Amplification and purification of DNA plasmid

The plasmid pGL3-control vector encoding firefly lucifrase driven by an SV40 promoter was amplified in *E. coli* JM107 bacteria and was purified using the endo free Maxi preb plasmid extraction kit according to the manufacturer's instruction. The plasmid was quantified and qualified by spectrophotometer and electrophoresis in 1% agarose gel, respectively (Sato et al., 2001; Boyle et al., 2001).

4.2. Preparation of chitosan-DNA-FAP-B nanoparticles

A chitosan solution of 0.047 mg/ml in 5 mM sodium acetate buffer pH = 5.5 was prepared in order to achieve N/P ratio of 20 (An amino group to a phosphate group ratio hereafter is defined as charge or N/P ratio). A DNA solution of 50 μg/mL in 25 mM of sodium sulfate was prepared. Solutions were preheated to 50–55 °C separately. An equal volume of chitosan solution and DNA solution were added together while stirring. The final volume of the mixture was limited to below 500 μl in order to yield uniform nanoparticles. After preparation of chitosan-DNA nanoparticles, pH of solution was adjusted to 8 by adding NaOH 1 N. A FAP-B solution of 120 μg/100 μl in deionized sterile water was prepared. 1 mole of FAP-B (Mw = 45 kDa) was added to nanoparticles solution equal to 1 mole chitosan (Mw = 126 kDa). The mixture was incubated at room temperature while stirred (500 rpm) for 24 h. The resulting solution was then centrifuged for 6 min at room temperature under speed of 7000 rpm. To remove possible aggregates during high speed centrifugation, a soft stirring process (speed of 500 rpm for 24 h) was performed (Mohammadi et al., 2011).

4.3. Preparation of chitosan-DNA-FAP-B nanoparticles for aerosol delivery

A solution of chitosan-DNA-FAP-B nanoparticles at N/P ratio of 20 and chitosan/FAP-B ratio of 1/1 was prepared as described previously. Total volume of solution was 20 ml which was equivalent to 1 mg DNA. Turbofect was used as positive control according to the manufacturer's procedure.

4.4. Design of the aerosol device

For the nebulization procedure based on a whole body device, mice were placed in a sealed 9.8 × 13.2 × 21.5 cm plastic box which was connected directly to the nebulizer (Fig. 1A). Two small holes were made on the opposite side of the plastic box to allow aerosol flow through the box (Fig 1B) (Elfinger et al., 2009; Rudolph et al., 2005)

4.5. Aerosol application of nanoparticles

Six mice were housed in whole body chamber. Solution was nebulized in two portions of 10 ml using a nebulizer (Hudson Rci large volume nebulizer). Each 10 ml was nebulized during 30 min. There was a 20 min rest between two deliveries.

4.6. Characterization of chitosan-DNA and chitosan-DNA-FAP-B nanoparticles before and after nebulization

The size of the nanoparticles was determined by photon correlation spectroscopy (PCS) and zeta potential by laser doppler velocimetry (LDV) using Zetasizer Nano ZS ZEN 3600 (Malvern Instruments, Malvern, UK). The size measurement was performed at 25 °C, using 633 nm red laser and was recorded for 180 s for each

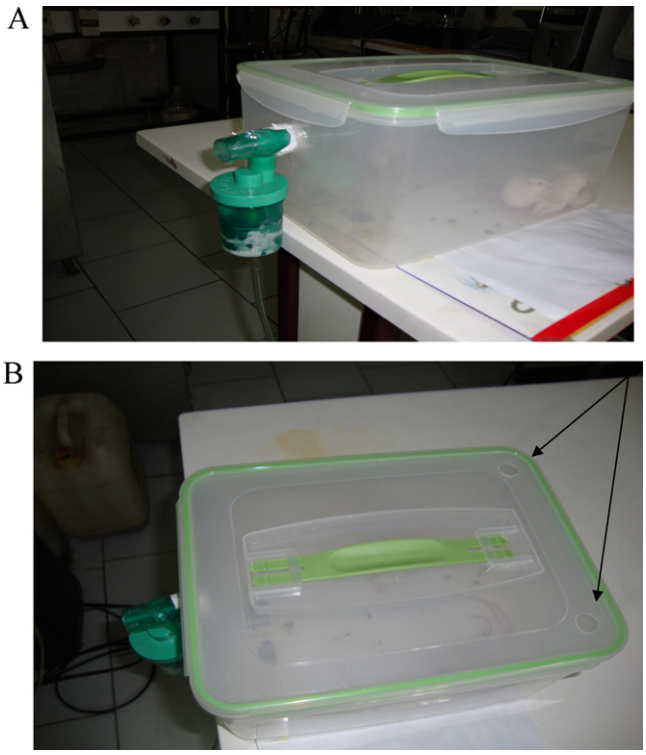

Fig. 1. Plastic box designed for aerosol delivery of nanoparticles to the mice. (A) Direct connection of the nebulizer to the box. (B) Two small holes for aerosol flow through the box.

measurement. The viscosity of samples was 0.88 cP and refractive index of the solvent was 1.33. The mean hydrodynamic diameter was generated by cumulative analysis using Malvern software. The results were acceptable if only they had Polydispersity indices below 0.5 (PdI < 0.5). The Zeta potential measurements were performed using an aqueous dip cell in the automatic mode.

4.7. Agarose gel electrophoresis

The DNA binding ability of chitosan was evaluated by agarose gel electrophoresis. The complexes containing 0.5 mg of DNA were loaded into individual wells of 1.0% agarose gel in 1-Tris–boric acid–EDTA buffer, electrophoresed at 80 V for 45 min, and stained with 0.5 mg/ml ethidium bromide. The resulting DNA migration pattern was revealed under UV irradiation (Chan et al., 2007).

4.8. Confirmation of gene expression levels in the lungs

Mice were sacrificed after 48 h. After opening the peritonea by midline incisions, lungs were dissected from animals and washed twice with ice-cold phosphate buffered saline (PBS) and homogenized with a lysis buffer using homogenizer (IKA, T25). The volume of the lysis buffer was 4 ml/g tissue. The homogenate was incubated for 20 min on ice and centrifuged at 15,000 rpm for 5 min at 4 °C. Twenty microliters of each supernatant were subjected to the luciferase assay with luminometer (Berthold systems GMBH, Germany) for 10 s (Zaric et al., 2004).

4.9. Statistical analysis

All experiments were repeated at least three times, and measurements were collected in triplicate. Data are expressed as mean ± standard deviations. Statistical analysis was performed using Student's *t*-test with $p < 0.05$ considered as a statistically significant difference.

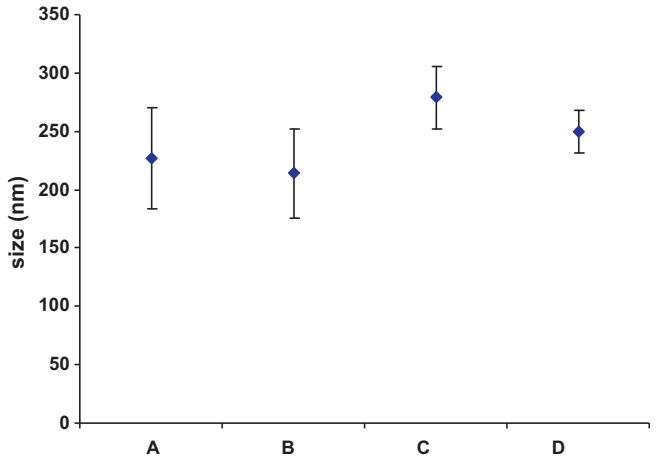

Fig. 2. Effect of nebulization on the mean size of the nanoparticles (PdI < 0.5). (A) Chitosan-DNA nanoparticles pre nebulization, (B) chitosan-DNA nanoparticles post nebulization, (C) chitosan-DNA-FAP-B nanoparticles pre nebulization, and (D) chitosan-DNA-FAP-B nanoparticles post nebulization.

5. Results and discussion

5.1. Preparation and characterization of chitosan-DNA-FAP-B nanoparticles

The chitosan-DNA nanoparticles were formed as a result of complex coacervation between chitosan and DNA (Elfinger et al., 2009; Mao et al., 2001; Schmitz et al., 2007; Issa et al., 2006). The cationic characteristic of chitosan is a crucial parameter for the complex formation with DNA bearing negative charges. The chitosan-DNA nanoparticles were prepared at N/P ratio of 20 which proved to result in the optimum nanoparticles with regards to size (227 ± 43 nm) and positive surface charge (+17.8 ± 3.2 mv) as presented previously (Mohammadi et al., 2011).

FAP-B is an important protein on the surface of *Mycobacterium bovis* which makes this bacterium capable of entering the epithelial cells via interaction with its specific receptor on the membrane of the epithelial cells (Laqueyrerie et al., 1995; Zhao et al., 2000; Abolhassani et al., 2006, Romain et al., 1993). Chitosan-DNA-FAP-B nanoparticles, prepared and evaluated as previously described (Mohammadi et al., 2011). Nanoparticles containing plasmids at N/P ratio of 20 were complexed with FAP-B via electrostatic interaction. After complexation, the size and zeta potential of resulted nanoparticles were about 279 nm and −2.3 mv, respectively.

5.2. Characterization of chitosan-DNA and chitosan-DNA-FAP-B nanoparticles after nebulization

A solution of chitosan-DNA-FAP-B nanoparticles at N/P ratio of 20 and chitosan/FAP-B ratio of 1/1 was nebulized in two portions of 10 ml during 30 min.

The size of nanoparticles did not change significantly pre and post nebulization (279 ± 27 nm and 250 ± 18 nm, respectively) (Fig. 2) confirming that nebulization did not degrade the nanoparticles. Moreover, the nebulization did not affect the zeta potential of nanoparticles significantly (before nebulization: −2.3 ± 1.5 mv, after nebulization: −1 ± 1 mv) (Fig. 3). FAP-B is negatively charged at the pH above its isoelectric point (pH~4.5) and chitosan-DNA nanoparticles are positively charged at pH 8 (pH of medium during complexation process). The negative charge of chitosan-DNA-FAP-B nanoparticles can be attributed

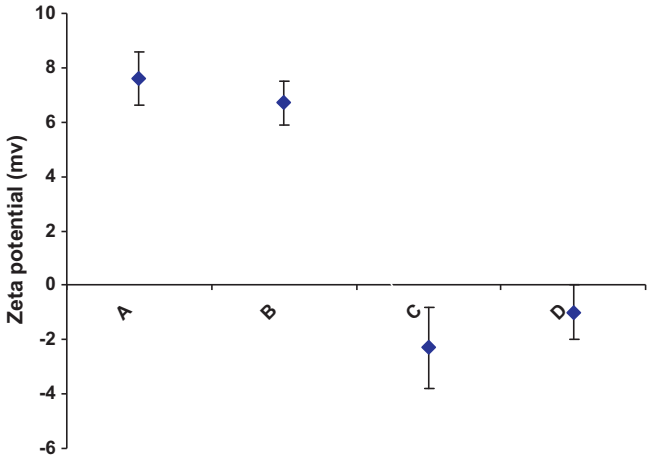

Fig. 3. Effect of nebulization on the zeta potential of the nanoparticles (PdI < 0.5). (A) Chitosan-DNA nanoparticles pre nebulization, (B) chitosan-DNA nanoparticles post nebulization, (C) chitosan-DNA-FAP-B nanoparticles pre nebulization, and (D) chitosan-DNA-FAP-B nanoparticles post nebulization.

to electrostatic interaction between negatively charged FAP-B and positively charged chitosan-DNA nanoparticles indicating that FAP-B was still present at the surface of nanoparticles as these nanoparticles were negatively charged after nebulization.

According to the agarose gel electrophoresis studies, in both chitosan-DNA and chitosan-DNA-FAP-B nanoparticles, plasmid could retain within the loading well after nebulization (Figs. 4 and 5) which showed that chitosan did not lose its condensing ability during nebulization.

5.3. Confirmation of gene expression levels in the lungs

Gene vectors were delivered to the lungs of mice via aerosol application. Aerosol delivery of Chitosan-DNA-FAP-B nanoparticles resulted in 16-fold increase of gene expression in the lungs of mice compared with unmodified chitosan gene vectors (Fig. 6).

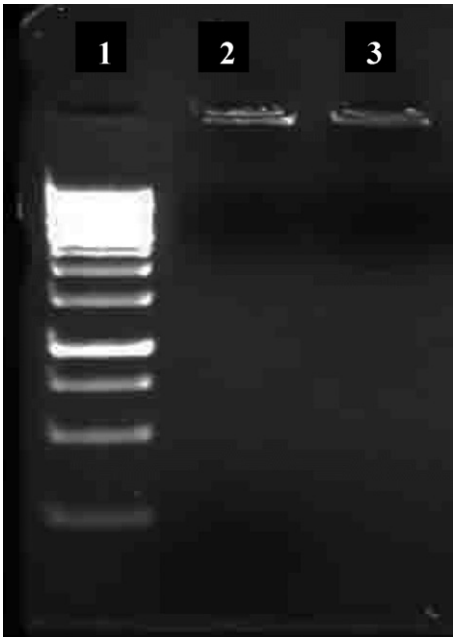

Fig. 4. Gel retarding analysis of chitosan-DNA nanoparticles. Lane 1: Ladder, Lane 2: before nebulization, and Lane 3: after nebulization.

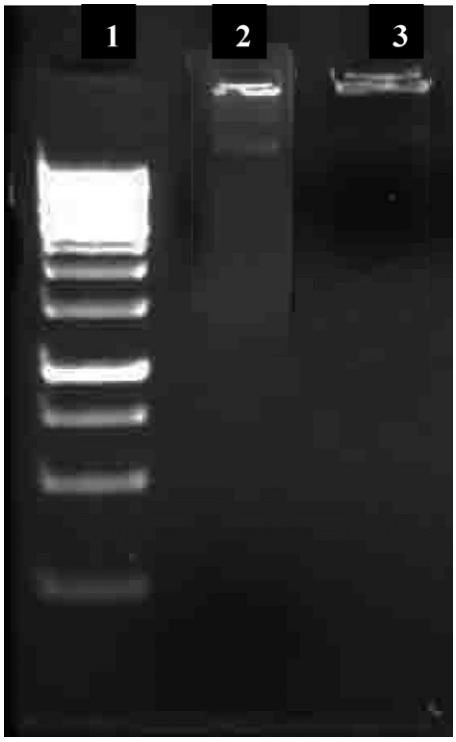

Fig. 5. Gel retarding analysis of chitosan-DNA-FAP-B nanoparticles. Lane 1: Ladder, Lane 2: before nebulization, and Lane 3: after nebulization.

Since FAP-B has specific receptor on the surface of lung epithelial cells, this significant increase of gene expression level can be due to ligand–receptor interaction which was previously demonstrated by inhibition experiments on A549 cell line (Mohammadi et al., 2011). This result can indicate that FAP-B receptors are suitable targets for in vivo gene delivery.

Gene delivery to the lung has some limitations. One drawback is inhalation apparatus; especially with animals. Although whole body chamber for nebulization is easier and more practical compared to other systems such as nose only systems, it has some disadvantages. Accurate control on administrated dose is impossible as some of nebulized nanoparticles can escape the chamber, may be absorbed by the skin of animal or be swallowed. Therefore, in this study it can be concluded that with a well and accurate designed apparatus, the in vivo transfection ability of Chitosan-DNA-FAP-B nanoparticles could be improved.

The particle size of droplets made by nebulizer is important for deposition of particles in the lung. There is a maximum in

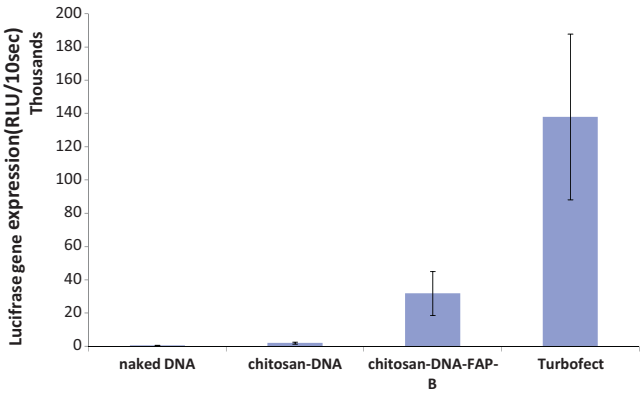

Fig. 6. Lucifrase gene expression in mice lungs after nanoparticles nebulization.

alveolar deposition of about 60% for aerodynamic diameter of about 3 μm (Hickey, 2004). But it is noticeable that at such optimum size for alveolar deposition a significant amount of material is also deposited in tracheobronchial (~10%) (Hickey, 2004). Based on previous studies in our laboratory, the size of droplet obtained by nebulizer and used in this study was about 3–5 μm. Therefore, it could be expected that these droplets deposit in alveoli.

One of the most important obstacles to lung gene transfer is the problem with immune system. There are several mechanisms that limit the effectiveness of gene transfer systems in the lung. Understanding these mechanisms may lead to the development of novel strategies to circumvent these biological barriers. The first barrier is the extracellular mucus gel. By virtue of their extended filamentous structure and negatively charged surface, mucins can bind to various inhaled particles. Many of these particles can be displaced by mucociliary transport to the pharynx where they exit the lung by coughing or swallowing (Sleigh, 1988).

Another barrier to lung gene transfer is surfactant proteins. The removal of pathogens from the lung is associated with the binding of surfactant proteins to different carbohydrate molecules on the surface of invading pathogens (LeVine and Whitsett, 2001). Therefore, these molecules may also be the imperative substances of defense encountered by non viral vectors which contain carbohydrates. Alveolar macrophages are another essential constituent of the lung immunity system which play an important role in host defense (van Rooijen et al., 1996). By virtue of their phagocytic nature, abundance and location (in the alveoli and alveolar ducts) (van Rooijen et al., 1996), alveolar macrophages appear to be one of the main barriers to lung gene transfer. It is likely that delivery of viral and synthetic vectors will be rapidly eliminated by alveolar macrophages (Kuzmin et al., 1997).

Chitosan-DNA and chitosan-DNA-FAP-B nanoparticles should pass all of these barriers to reach the lung epithelial cells after in vivo administration. At first these nanoparticles may be removed by mucociliary transport or positively charged chitosan-DNA nanoparticles may bind to negatively charged mucins. Since chitosan is a carbohydrate polymer, surfactant proteins may bind to chitosan and remove the nanoparticles from the lung. Moreover, alveolar macrophages may also prevent the chitosan-DNA and chitosan-DNA-FAP-B nanoparticles from playing their roles as non viral vectors for gene delivery to the lung. Therefore, it seems that chitosan-DNA-FAP-B nanoparticles could be more effective for gene delivery to the lung epithelial cells if strategies to temporarily eliminate or suppress the lung immune system are suggested in future studies.

6. Conclusions

Chitosan-DNA-FAP-B nanoparticles were nebulized to mice for targeted gene delivery to the lung. Nebulization did not affect the size and zeta potential of nanoparticles. The level of gene expression of Chitosan-DNA-FAP-B nanoparticles in the mice lung was 16-fold higher than chitosan-DNA nanoparticles. This study suggested that Chitosan-FAP-B nanoparticles can be a promising carrier for targeted gene delivery to the lung. However, additional studies will be necessary for improving in vivo gene transfer.

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