International Journal of Pharmaceutics 409 (2011) 307–313

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Pharmaceutical Nanotechnology

Preparation and evaluation of chitosan–DNA–FAP-B nanoparticles as a novel non-viral vector for gene delivery to the lung epithelial cells

Z. Mohammadi [a], M. Abolhassani [b], F.A. Dorkoosh [a], S. Hosseinkhani [c], K. Gilani [a], T. Amini [d], A. Rouholamini Najafabadi [a], M. Rafiee Tehrani [a,*]

[a] Department of Pharmaceutics, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran
[b] Nosco Pharmaceuticals, 105, rue de la convention, 75015 Paris, France
[c] Department of Biochemistry, Faculty of Science, Tarbiat Modares University, Tehran, Iran
[d] Nobleceuticals Ltd, Birmingham, United Kingdom

ARTICLE INFO

Article history:
Received 7 December 2010
Received in revised form 19 February 2011
Accepted 21 February 2011
Available online 26 February 2011

Keywords:
Chitosan
Fibronectin Attachment Protein of BCG
Gene delivery
Lung epithelial cells
Nanoparticles

ABSTRACT

Gene delivery using cationic polymers such as chitosan shows good biocompatibility, but reveals low transfection efficiency. Fibronectin Attachment Protein of *Mycobacterium bovis* (FAP-B) which is responsible for the attachment of many 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. In this study, chitosan–DNA nanoparticles were prepared using coacervation process. The effect of stirring speed and charge ratio (N/P) on the size and zeta potential of nanoparticles were evaluated. FAP-B ligand was added to nanoparticles at the specific condition to form chitosan–DNA–FAP-B nanoparticles via electrostatic attraction. Transfection efficiency of the final nanoparticles was investigated in A549 (alveolar epithelial cells). Cell viability was investigated using MTT assay. The optimum speed of stirring which was yielded the smallest chitosan–DNA nanoparticles with a narrow distribution (227 ± 43 nm), was 500 rpm with the corresponding N/P ratio of 20. Chitosan–DNA–FAP-B nanoparticles presented the size of 279 ± 27 nm with transfection efficiency about 10-fold higher than chitosan–DNA nanoparticles and resulted in 97.3% cell viability compared to 71.7% using Turbofect controls.

Chitosan–DNA–FAP-B nanoparticles showed good transfection efficiency without cell toxicity. They have small particle size around 279 nm which make them a promising candidate as a novel non-viral gene vector for gene delivery to lung epithelial cells.

1. Introduction

The respiratory system is an excellent target for noninvasive localized drug delivery due to its large surface area and its accessibility. The airway and the alveolar epithelium are the sites where genetic disorders such as cystic fibrosis or inherited surfactant protein B deficiency and lung cancer have their major fatal impacts. For these reasons, the lung has been considered as an attractive target for gene therapy interventions (Vadolas et al., 2002; Okamoto et al., 2003; Mao et al., 2001).

The success in gene therapeutic strategies depends on an efficient system for the delivery of nucleic acid into the target cells. Both viral and non-viral gene delivery systems have been used in clinical trials to treat maladies such as cystic fibrosis and several forms of cancer (Vadolas et al., 2002; Okamoto et al., 2003). Non-viral delivery systems have been increasingly proposed as safer alternatives to viral vectors as they could be synthesized with higher purity and quality degree and less immunogenic response, are targetable, stable in storage, and easy to produce in large quantities. These advantages have provided the impetus to continue their development (Mao et al., 2001).

Cationic polymers have been shown as promising carriers among the non-viral gene delivery systems. Many cationic polymers, such as chitosan, polylysine, polyethyleneimine, dendrimers, poly (a-(4-aminobutyl)-L-glycolic acid) as well as cationic liposomes have been investigated for gene delivery. Nevertheless, an ideal polymeric gene carrier with high efficacy of gene transfer, targeting ability, good biocompatibility and stability has yet to emerge (Mao et al., 2001; Desmedt et al., 2000; Liu et al., 2002).

In recent years, the potential of chitosan as a polycationic gene carrier has been explored (Roy et al., 1999; Leong et al., 1998; Maclaughlin et al., 1998; Mao et al., 1996; Richardson et al., 1999). Chitosan is a polysaccharide copolymer of N-acetyl-D-glucosamine and D-glucosamine. Its cationic polyelectrolyte nature provides a strong electrostatic interaction with negatively charged DNA and protects it from nuclease degradation (Mao et al., 2001; Liu et al.,

* Corresponding author. Tel.: +98 2188009440; fax: +98 2188026734.
E-mail address: rafitehr@ams.ac.ir (M.R. Tehrani).

0378-5173/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2011.02.043

2002; Borchard, 2001). Chitosan has good biocompatibility and toxicity profile and has been widely used in pharmaceutical research (Dodane and Vilivalam, 1998) however; its application in vivo has been limited due to its low transfection efficiency.

Several cell-specific ligands, such as galactose, folic acid, transferrin or epidermal growth factor have been shown to improve transfection efficiency by receptor-specific interaction and endocytosis into target cells (Liu et al., 2002; Chan et al., 2007; Ogris et al., 2003; Mansouria et al., 2006). FAP-B (Fibronectin Attachment Protein of BCG) is one of the most expressed surface proteins of *Mycobacterium bovis* (BCG) and is reported to be responsible for the attachment of mycobacteria on the Fibronectin molecule of epithelial cells membranes. It is known that Fibronectin (FAP-B receptor) is expressed on lung, intestinal and bladder human epithelial cells membranes. The molecular weight of FAP-B is about 45–47 kDa and its structure is full of Proline and Alanine. By this reason, it is called Apa (Alanin–Prolin-rich antigen) (Laqueyrerie et al., 1995; Romain et al., 1993; Zhao et al., 2000). Abolhassani et al., 2006 have shown that BCG cannot be translocated through the epithelial cell line cultures from apical side to the basolateral compartment without FAP-B interactions. FAP-B is not detectable in HK-BCG (Heat-killed BCG) extracts and, as expected, HK-BCG was not able to translocate through epithelial cells. In addition, a mutant strain of BCG deleted from the FAP-B gene (BCG Apa$^-$) could not translocate the intestinal or lung epithelial cells. On the other hand, the inert latex beads coated with the FAP-B translocated through this monolayer cells whereas those coated with ovalbumin protein or non-coated beads failed to do so (Abolhassani et al., 2006). Therefore, it was decided to investigate the novel concept of using FAP-B in gene delivery and evaluate its potential in improving the transfection efficiency of complex DNA nanoparticles.

In this study, a non-viral gene vector with improved transfection efficiency to epithelial cells was prepared via interaction of chitosan–DNA nanoparticles with FAP-B ligand. Its transfection ability and cell viability were evaluated in alveolar epithelial cells (A549). Based on our findings, it seems chitosan–DNA–FAP-B nanoparticles may be a promising carrier for targeted gene delivery to Fibronectin molecules (FAP-B receptors) of epithelial cell membrane.

2. Materials and methods

2.1. 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 (South Korea). FAP-B (Fibronectin Attachment Protein of BCG) (Mw = 45 kDa) was kindly provided by Prof G. Marchal (Institut Pasteur, Paris, France) (Romain et al., 1993). Turbofect reagent was obtained from Fermentas (Thermo Fisher Scientific, Canada). Cell culture media, Dulbecco's modified Eagle's medium (DMEM) and Penicillin–Streptomycin mixture (PS) were obtained from Gibco (Invitrogen, Singapore). Fetal Bovine Serum (FBS) was purchased from Hyclone (Research Instrument, Singapore). Sodium acetate, Sodium sulfate, Sodium hydroxide and other chemicals were purchased from Merck (Germany).

2.2. Methods

2.2.1. 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 purified using the endo free Maxi prep 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).

2.2.2. Preparation of chitosan–DNA nanoparticles

Solutions of chitosan at different concentrations were prepared in 5 mM sodium acetate buffer pH 5.5 in order to achieve N/P ratios of 0.5, 1, 2, 3, 4, 5, 6, 8, 10, 15, 20 and 40 (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 (from each concentration) and DNA solution were added (by dropping slowly) together while stirred at various speed (100–2000 rpm). The final volume of the mixture in each preparation was limited to below 500 μl in order to yield uniform nanoparticles.

2.2.3. Preparation of chitosan–DNA–FAP-B nanoparticles

The pH of chitosan–DNA nanoparticles solutions prepared above was adjusted to 8.0 using NaOH 1.0 N. A solution of FAP-B at 120 μg/100 μl concentration was prepared in sterile deionized water. FAP-B solution (varying amounts) and chitosan–DNA nanoparticles solution (equal to 1 mole chitosan) were added together in order to achieve chitosan–DNA–FAP-B solutions with different chitosan/FAP-B molar ratios (sample C = 1/0.5, sample D = 1/1 and sample E = 1/2). The mixtures were then incubated at room temperature while stirred (500 rpm) for 24 h. The resulting solutions were centrifuged at 2000, 7000 or 16,000 rpm for 6 min at room temperature. The supernatant was then poured off and the pellets were re-suspended in 500 μl sterile deionized water. These stages were repeated 3 times to purify the nanoparticles completely. For removal of possible aggregates during high speed centrifugation, a soft stirring process (speed of 500 rpm for 24 h) was also performed.

2.2.4. Characterization of chitosan–DNA and chitosan–DNA–FAP-B nanoparticles

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 it was recorded for 180 s for each 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 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. Zeta potential of chitosan–DNA nanoparticles were measured at pH 5.5, 6, 7.4 and 8 while chitosan–DNA–FAP-B nanoparticles measurements were performed at pH 7.4 and 8. Chitosan solutions in 5 mM sodium acetate buffer at various pH (pH 4, 5.5, 6, 7.4, 8) and FAP-B solutions at pH 3, 4, 6, 7.4, 8 were also measured separately.

The surface morphology of nanoparticles was examined by atomic force microscopy (AFM) (Dualscope C26, DME, Denmark) using tapping mode and high resonant frequency (F0 = 150 kHz) pyramidal cantilevers with silicon probes having force constants of 25 N/m. Scan speeds were set at 2 Hz. The samples were diluted with distilled water, and then dropped onto freshly cleaved mica plates followed by vacuum drying for 24 h at 25 ◦C.

2.2.5. Agarose gel electrophoresis

The DNA binding ability of chitosan was evaluated by agarose gel electrophoresis. The complexes containing 0.5 mg of DNA at various N/P ratios 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).

2.2.6. In vitro transfection of A549 cells with chitosan–DNA–FAP-B nanoparticles

2.2.6.1. Cell culture. The cells (A549:alveolar epithelial cells) were seeded, 48 h prior to transfection, in 24-well plates at a density of 40,000 cells/well in 1 ml of DMEM supplemented with 10% FBS and 1% PS (Elfinger et al., 2009). On the day of transfection, the culture medium in each well was replaced with 1 ml of complete medium or medium without PS, containing naked DNA (1 μg) or chitosan–DNA nanoparticles (negative controls) or chitosan–DNA–FAP-B nanoparticles (tests). The amount of nanoparticles added to each well was equivalent to 1 μg of DNA. If the medium was without any PS, after 4 h from transfection, 1% PS was added to each well. For inhibition experiments, free FAP-B was added to fresh complete media with a concentration of 100 mg/ml 20 min before the addition of the complexes (Elfinger et al., 2007). The cells were then incubated until 48 h post transfection. Turbofect was used as positive control according to the manufacturer's procedures. Each well received 2 μl of Turbofect that was complexed with 1 μg of DNA.

2.2.6.2. Luminometric assay for luciferase. After 48 h incubation, cells were rinsed with sterile phosphate buffer (pH 7.4) and permeablized with 80 μl of cell lysis buffer purchased from Promega (while the plate was placed in ice). Plate was shaken for 15 min after which the cells were centrifuged for 2 min at 12000 rpm. The pellete was sonicated for 2 s while the tube was in ice. Sonication was repeated for 4 times. The luciferase activity in cell extracts was measured using luminometer (Berthold systems GMBH, Germany) for 10 s per well. Luciferase activity in cell lysates was expressed as relative light units (RLU/10Sec) (Hofland et al., 2002; Zaric et al., 2004; Reynolds et al., 2000).

2.2.6.3. Cytotoxicity assay. Cytotoxicity of nanoparticles was determined in triplicate by MTT assay. A549 cells were seeded into 96-well plates at a density of 5×10^4 cells/well. After 24 h, cell culture medium was aspirated and replaced by 200 μl fresh complete medium containing transfection solution (equal to 1 μg DNA). Cytotoxicity studies were performed 48 h after transfection. The cells received 100 μl culture medium per well containing 10 μl MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide. After 4 h incubation at 37 °C in the dark, medium was aspirated and formazan crystals were dissolved in 200 μl dimethylsulfoxide per well. Measurement was performed using an ELISA reader at wavelengths of 570 nm and 690 nm. Relative viability was calculated using 0% (wells without cells) and 100% (wells with untreated cells) controls.

2.2.6.4. 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.

3. Results and discussion

3.1. Preparation and characterization of chitosan–DNA nanoparticles

The chitosan–DNA nanoparticles were formed as a result of complex coacervation between chitosan and DNA (Mao et al., 2001; Elfinger et al., 2009; 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 size of nanoparticles is important for cellular uptake and can be affected by the speed of stirring during the formation of coacervates. To investigate such an effect, nanoparticles were prepared under various speeds of stirring (at N/P = 8). The optimum speed of stirring was found to be 500 rpm (Fig. 1) which resulted in the smallest nanoparticles (357 ± 78 nm). The nanoparticles size was increased by further increasing the speed of stirring due to aggregation. At the speeds below 500 rpm, the nanoparticles size was also increased. This was due to the insufficient stirring power to generate small nanoparticles.

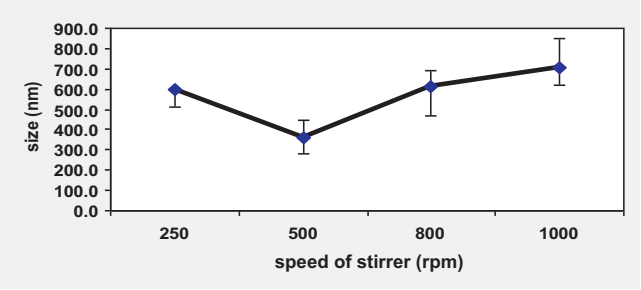

Fig. 1. Effect of speed of stirring on nanoparticles size during coaservation (at N/P = 8) (PdI < 0.5).

The effect of charge ratio on the particle size and surface charge was examined within the stirring conditions stated above. Plasmid was used with a fixed concentration of 50 μg/ml in 25 mM of sodium sulfate, while the concentration of chitosan was varied to yield different N/P ratios. At N/P ratios of 1–10, nanoparticles had large sizes. Large aggregates were formed at N/P ratio around 4 (559 nm approximately) (Fig. 2). N/P ratios below 1 and above 10 resulted in similarly small nanoparticles. Nanoparticles prepared with an N/P ratio between 15 and 40 were small and had low standard deviation. These nanoparticles are likely to have higher thermal dynamic stability (Mao et al., 2001) with an average size of 227–267 nm.

At N/P ratio below 1, the chitosan concentration was lower than plasmid concentration and was unable to condense DNA which resulted in negative zeta potential. The zeta potential of chitosan–DNA nanoparticles increased slightly with the N/P ratio (ranging from +9.3 to +21.8 mV) (Fig. 3). Although positively charged nanoparticles can attach to the cell membrane, which is negatively charged, and enter the cell more easily (Mansouria et al., 2006; Park et al., 2005), care should be taken as nanoparticles with high positive zeta potential can become cytotoxic (Park et al., 2005).

The formation of nanoparticles between plasmid and chitosan was observed by agarose gel electrophoresis (Fig. 4). It showed

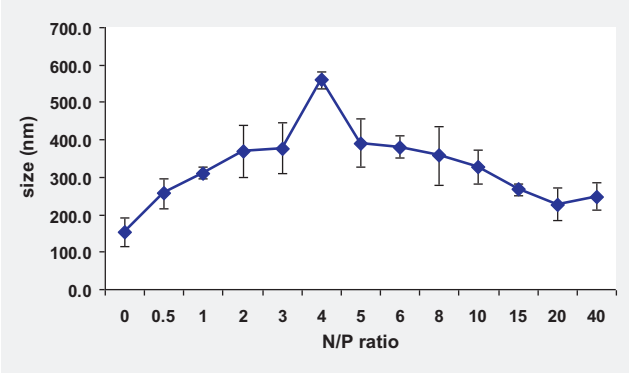

Fig. 2. Effect of N/P ratio on the mean size of the nanoparticles (PdI < 0.5).

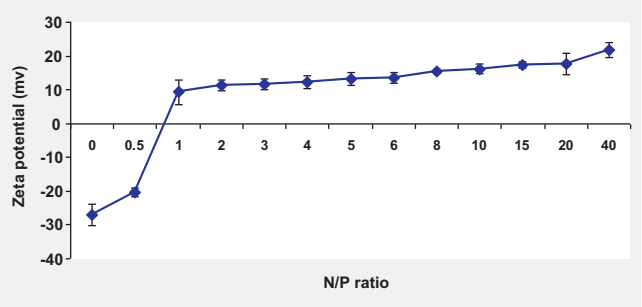

Fig. 3. Effect of N/P ratio on the zeta potential of the nanoparticles.

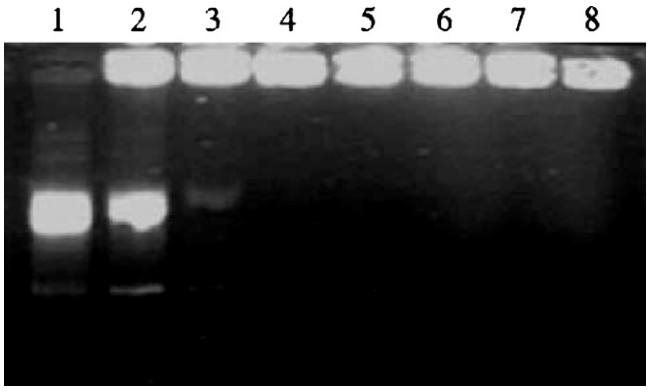

Fig. 4. Gel retarding analysis of chitosan–DNA nanoparticles. Lane 1, naked plasmid. Lanes 2–8, nanoparticles prepared at N/P ratios of 0.5, 1, 2, 4, 8, 15, and 20, respectively.

when the N/P ratio was above 1, the plasmid was totally retained within the gel loading well, which illustrated the complete coalesce of plasmid with chitosan.

The optimum nanoparticles (smallest and more positively charged nanoparticles) with a mean size of 227 ± 43 nm were prepared at the N/P ratio of 20. This could be explained by the presence of sufficient chitosan to make small and condense nanoparticles. Nanoparticles obtained under these conditions showed a spherical and polydisperse nature as revealed by their AFM image. The nanoparticles size measurement was performed using AFM imaging and Zetasizer Nano (Fig. 5). The two techniques gave different results which could be explained by different sample preparation methods. PCS was performed on nanoparticles in a fully hydrated state in solution, whereas, AFM studies were performed on samples dried on a mica surface. In addition, the PCS measurement

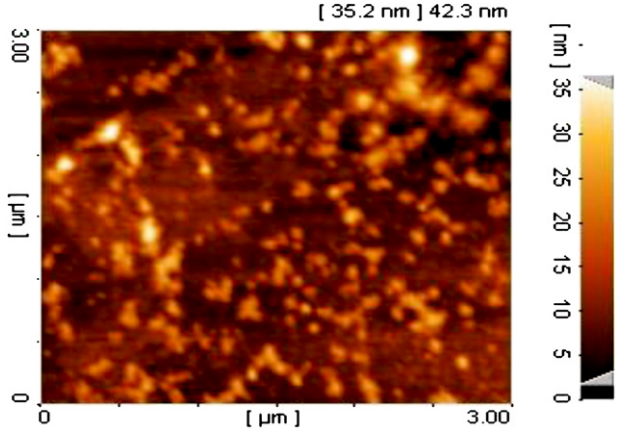

Fig. 5. . Physical characterization of chitosan–DNA nanoparticles by AFM imaging.

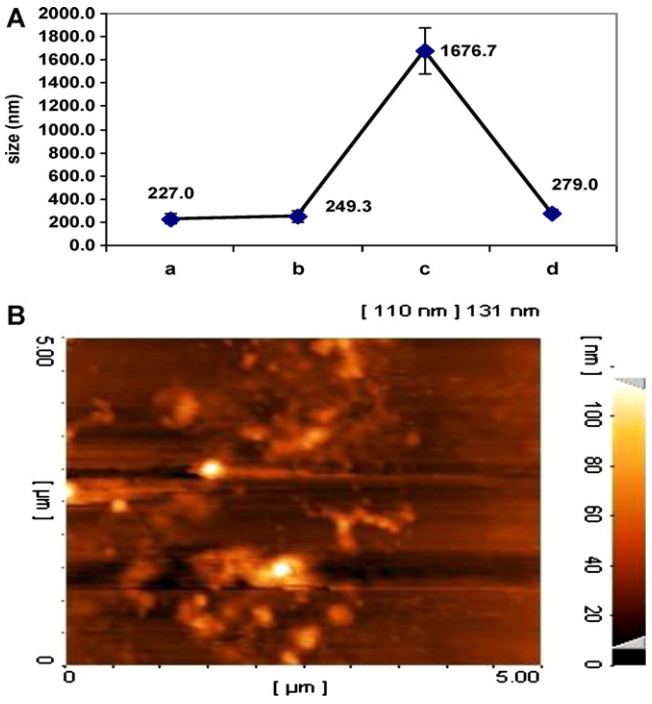

Fig. 6. (A) Effect of complexation conditions on size of nanoparticles (PdI < 0.5). (a) Nanoparticles without complexation, (b) nanoparticles immediately after complexation by FAP-B, (c) nanoparticles after centrifugation at 7000 rpm, (d) nanoparticles after additional stirring at 500 rpm. (B) AFM image of nanoparticles after additional stirring at 500 rpm.

presents an average size range whereas; AFM allows visualization of the subpopulations. However, the use of different but complementary methods allows an overall evaluation to be made of both size and morphology (Liu et al., 2007). Effect of speed of stirring on nanoparticles size during coacervation was also evaluated at N/P ratio of 20. The results were in line with the ones obtained at N/P ratio of 8 with the optimum speed of stirring being 500 rpm (data not shown).

3.2. Preparation and characterization of chitosan–DNA–FAP-B nanoparticles

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; Romain et al., 1993; Zhao et al., 2000; Abolhassani et al., 2006). In the present study, this protein was chosen as a ligand for delivery of chitosan–DNA nanoparticles to epithelial cells via receptor mediated procedure and also to improve the transfection efficiency of these nanoparticles.

Nanoparticles containing plasmids at N/P ratio of 20 as described in Section 3.1 were selected for electrostatic interaction with FAP-B. The complex of chitosan–DNA–FAP-B nanoparticles was prepared as described above. To separate the free FAP-B from the nanoparticle solutions, the solutions were centrifuged at 7000 rpm which was found to be the optimum speed as the separation was done successfully and the formed aggregates were not very strong (data not shown). A final soft stirring process (500 rpm) was performed for removal of possible aggregates upon which the size of nanoparticles became almost as it was initially (279 ± 27 nm) (Fig. 6A). AFM imaging of these complexes showed a spherical and polydisperse nanoparticles (Fig. 6B).

The complexation did not affect the size of nanoparticles significantly (before complexation: 227 ± 43 nm, after complexation:

Table 1
The zeta potential of FAP-B, chitosan, chitosan–DNA nanoparticles and chitosan–DNA–FAP-B nanoparticles at various pHs.

pH	FAP-B (mv)	Chitosan (mv)	Chitosan–DNA nanoparticles (mv)	Chitosan–DNA–FAP-B nanoparticles (mv)
3	+5.6 ± 0.5	–[a]	–	–
4	+0.97 ± 0.7	+26.6 ± 1.5	–	–
5.5	–	+19.7 ± 1.3	+12.9 ± 1.4	–
6	−10.5 ± 1.0	+14.2 ± 0.8	+10.0 ± 1.1	–
7.4	−12.3 ± 1.0	+10.0 ± 0.5	+9 ± 1.0	−2 ± 0.9
8	−14.1 ± 1.2	+8.3 ± 0.5	+7.5 ± 0.3	−2.3 ± 1.5

[a] Zeta potential measurements were performed at specific pH necessary for each experiment; [–] show that zeta potential not measured at that pH.

279 ± 27 nm) but it had a significant effect on their zeta potential. The zeta potential of chitosan–DNA nanoparticles (at pH 8) was altered from +7.5 ± 1 mV to −2.3 ± 1.5 mV after complexation with FAP-B (Table 1) (Fig. 7). This showed that complexation was done efficiently as FAP-B is negatively charged at the pH above its isoelectric point (pH ∼4.5). The zeta potential of FAP-B was altered from +5.6 at pH 3 (below its isoelectric point) to −14.1 at pH 8 (above its isoelectric point) (Table 1). Thus, FAP-B and DNA together shifted the electrokinetic behavior of the complex. Although the zeta potential of chitosan at pH 5.5 (the pH of coacervation medium) was +19.7, the zeta potential of chitosan–DNA nanoparticles became +12.9 at the same pH as a result of electrostatic complexation between chitosan and DNA (Table 1). The zeta potential of chitosan–DNA nanoparticles at pH 8 (pH of medium during complexation process) was +7.6 which after complexation with FAP-B was reduced to −2.3 (Table 1). Therefore, it can be concluded that negative charge of these nanoparticles is due to electrostatic interaction between negatively charged FAP-B and positively charged chitosan–DNA nanoparticles. At physiological pH (pH = 7.4) the zeta potential of chitosan–DNA–FAP-B nanoparticles were negative, which indicates that these nanoparticles did not degrade and were stable at such pH (Table 1).

3.3. Transfection efficiency of chitosan–DNA–FAP-B nanoparticles

The in vitro transfection ability of chitosan–DNA–FAP-B nanoparticles was first evaluated in alveolar epithelial cells (A549) using the luciferase plasmid. The results of the luminescence assay to detect the luciferase activity in the transfected cells indicated relatively high transfection efficiencies. The expressed luciferase activity was about 10-fold higher than the background level (chitosan–DNA nanoparticles) but, it was still about five to ten times lower than that of the Turbofect DNA complex group (Fig. 8).

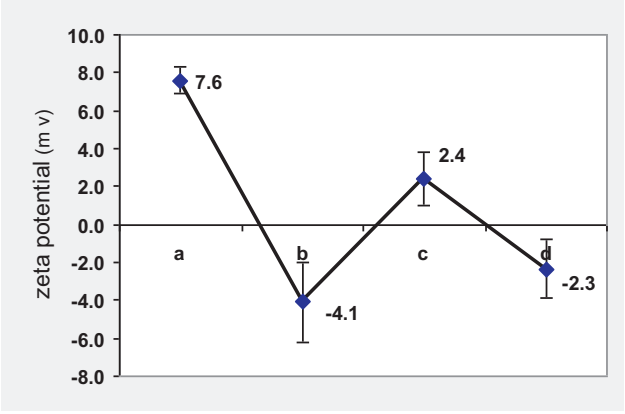

Fig. 7. Effect of complexation conditions on zeta potential of nanoparticles at pH 8. (a) Nanoparticles without complexation, (b) nanoparticles immediately after complexation by FAP-B, (c) nanoparticles after centrifugation at 7000 rpm, (d) nanoparticles after additional stirring at 500 rpm.

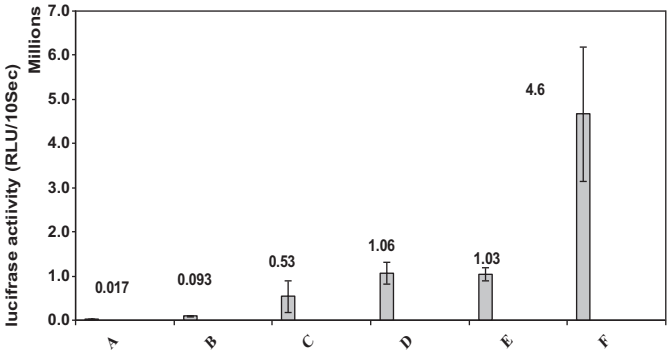

Fig. 8. In vitro transfection efficiency. (A) Naked DNA, (B) chitosan–DNA nanoparticles, (C) chitosan–DNA–FAP-B nanoparticles (chitosan/FAP-B = 1/0.5), (D) chitosan–DNA–FAP-B nanoparticles (chitosan/FAP-B = 1/1), (E) chitosan–DNA–FAP-B nanoparticles (chitosan/FAPB = 1/2), (F) Turbofect. The relative transfection efficiency was calculated as relative light units (RLU) luminescence during 10 s at constant number of cells per well. The data are given as mean ± SD (n = 3).

Transfection efficiency of the complexes examined at different ratio of chitosan/FAP-B showed that at ratio of 1/0.5, the transfection efficacy was about 2 times lower than 1/1 and 1/2 ratios. This can be due to insufficient amount of FAP-B for interaction with all of the receptors at the surface of A549 cell line. No significant difference in transfection efficiency of chitosan–DNA–FAP-B nanoparticles was observed at chitosan/FAP-B ratios of 1/1 and 1/2. This result indicates that the amount of FAP-B at chitosan/FAP-B = 1/1 is suitable for interaction with most of the receptors at the surface of A549 cell line. In addition, the presence of 1% antibiotic did not interfere with the transfection ability of chitosan–DNA–FAP-B nanoparticles (Fig. 9).

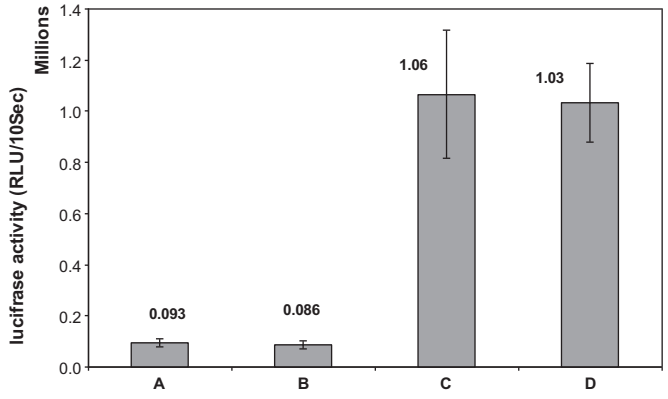

Fig. 9. Effect of presence of 1% antibiotic on transfection ability of chitosan–DNA and chitosan–DNA–FAP-B nanoparticles. (A) chitosan–DNA nanoparticles with antibiotics, (B) chitosan–DNA nanoparticles without antibiotics, (C) chitosan–DNA–FAP-B nanoparticles with antibiotics, (D) chitosan–DNA–FAP-B nanoparticles without antibiotics. The relative transfection efficiency was calculated as relative light units (RLU) luminescence during 10 s at constant number of cells per well. The data are given as mean ± SD (n = 3).

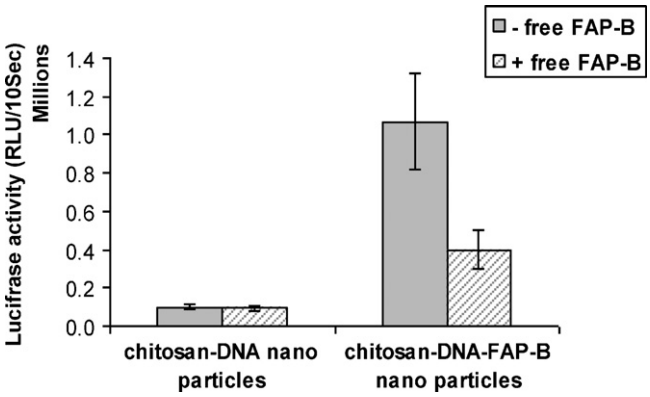

Fig. 10. In vitro transfection efficiency of chitosan–DNA nanoparticles (N/P = 20) and chitosan–DNA–FAP-B nanoparticles (chitosan/FAP-B = 1/1) in the absence (gray bars) or presence (hatched bars) of excess of free FAP-B. The relative transfection efficiency was calculated as relative light units (RLU) luminescence during 10 s at constant number of cells per well. The data are given as mean ± SD (n = 3).

Since the complex of chitosan–DNA–FAP-B is slightly negatively charged (−2 mV approximately) and the cell membrane is also negatively charged, it seems overall attraction between this ligand (FAP-B) and receptors on the surface of A549 (alveolar epithelial cell line) has a dominant attraction energy and can overcome the repulsion of negatively charged nanoparticles and cells. This can explain the transfection of the complex despite having a negative surface charge. These mechanistic data are in agreement with the results obtained by Park et al. who also showed negatively charged particles can penetrate into the cells by ligand–receptor endocytosis (Park et al., 2010). Inhibition experiments with 100 mg/ml free FAP-B showed a significantly 2.5-fold decrease of gene expression for chitosan–DNA–FAP-B complexes (chitosan/FAP-B ratio of 1/1), whereas chitosan–DNA nanoparticles were not influenced (Fig. 10). These results show that entrance of chitosan–DNA–FAP-B nanoparticles to the A549 cell line is via ligand–receptor endocytosis by attachment of FAP-B to its receptors at the surface of the epithelial cells. Chitosan–DNA–FAP-B nanoparticles showed good transfection efficiency in alveolar epithelial cell line (A549).

Other researchers have employed and proposed alternative ligands for targeted lung gene delivery. Lactoferrin (Lf) has been reported to target airway epithelial cells however it is not a good candidate for gene delivery to alveolar epithelial cells. This is due to the fact that Lactoferrin receptors are only expressed on the apical side of bronchial epithelial cells and not in alveolar epithelial cells (Elfinger et al., 2007) hence it does not offer the same opportunities as FAP-B. Other ligands which have been used for gene delivery to the lung are Transferrin (Tf) and transforming growth factor alpha (TGFα) (Kakimoto et al., 2007). None of these ligands are specific for epithelial cells, because Tf and TGFα receptors are over expressed at the surface of all tumor cells (Prost et al., 1998; Rusch et al., 1996). Unlike Tf and TGFα receptors, Fibronectin (receptor of FAP-B) is expressed only at the epithelial cells (Laqueyrerie et al., 1995; Romain et al., 1993; Zhao et al., 2000). Therefore, Tf and TGFα can be utilized for gene delivery only to lung tumor cells while FAP-B can be used for gene delivery to lung epithelial cells, both tumoral and non-tumoral. All these point to the promise of FAP-B as a potential ligand for targeted delivery to lung epithelial cells.

3.4. Cytotoxicity

For the concerns of efficient gene delivery and biocompatibility, nanoparticles should exhibit minimal cytotoxicity. To investigate the potential cytotoxic effect of chitosan–DNA complexes after coating of FAP-B, the viability of A549 cells was tested in the presence of chitosan–DNA nanoparticles and chitosan–DNA–FAP-B nanoparticles at N/P ratio = 20. Naked DNA, Turbofect–DNA complex were used as controls. Cells without treatment of DNA complexes were considered as a positive control with a cell viability of 100%. Several studies (Borchard, 2001; Sato et al., 2001; Lee et al., 2005) reported that no significant decrease in viability was observed for cells incubated with chitosan–DNA complexes. The results obtained in this study are in good agreement with those reported in the literature. Average cell viability of over 97% was obtained with naked DNA and chitosan–DNA nanoparticles and chitosan–DNA–FAP-B nanoparticles (Fig. 11).

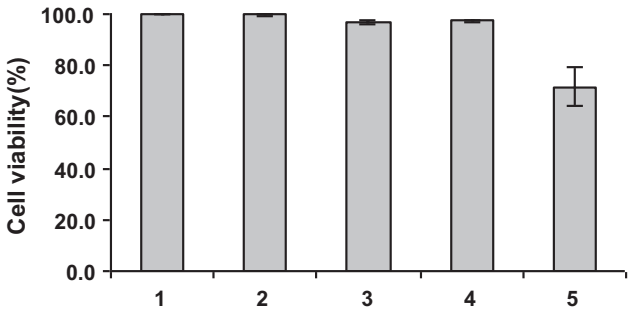

Fig. 11. Cytotoxicity of (1) non-treated control cells, (2) naked DNA, (3) chitosan–DNA nanoparticle, (4) chitosan–DNA–FAP-B nanoparticles and (5) Turbofect.

No significant decrease in viability was found for A549 cells treated with polyplexes when compared to naked DNA. Chitosan–DNA nanoparticles and chitosan–DNA–FAP-B nanoparticles carried lower cytotoxicity than the commercial carriers such as Turbofect transfection reagents.

4. Conclusions

Chitosan–DNA nanoparticles were successfully prepared by complex coacervation process under defined conditions. The optimum speed of stirring during coacervation was found to be 500 rpm with an ideal N/P ratio of 20 which resulted in the smallest nanoparticles. FAP-B was added to the chitosan–DNA nanoparticles as a ligand for attachment to its specific receptors present at the surface of epithelial cells. Chitosan–DNA–FAP-B nanoparticles had similar and small particle size as chitosan–DNA nanoparticles, however their zeta potential was different (negative). The transfection efficiency of chitosan–DNA–FAP-B nanoparticles was 10-fold higher than chitosan–DNA nanoparticles; however, it was still about five times lower than that of the Turbofect DNA complex group. In addition, the presence of FAP-B did not show any significant cytotoxicity against A549 cells. This study suggested that chitosan–FAP-B nanoparticles can be a promising carrier for targeted gene delivery to Fibronectin molecules (FAP-B receptors) of epithelial cells membranes.

Acknowledgements

This work would not have been possible without the invaluable excellent technical assistant of following colleagues: Dr. Zare, Dr. Khanahmad, Mr. Rahimi, and Miss Sohrabi. This work has been supported by Pasteur Institute of Iran and Tehran University of Medical Sciences (grant 57-6858).

References

Abolhassani, M., Lagranderie, M., Caminshi, I., Romain, F., Balazuc, A., Wagner, M., Tanguy, M., Fiette, L., Sobhani, I., Milon, G., Marchal, G., 2006. Similar functional activity of dendritic cells recruited to the mesenteric lymph nodes of newborn and adult mice after the rectal delivery of *Mycobacterium bovis* BCG. Microb. Infect. 8, 2341–2351.

Borchard, G., 2001. Chitosans for gene delivery. Adv. Drug Deliv. Rev. 52, 145–150.
Boyle, M.P., Enke, R.A., Adams, R.J., Guggino, W.B., Zeitlin, P.L., 2001. In utero AAV-mediated gene transfer to rabbit pulmonary epithelium. Mol. Ther. 4, 115–121.
Chan, P., Kurisawa, M., Chung, J.E., Yang, Y., 2007. Synthesis and characterization of chitosan-g-poly(ethylene glycol)-folate as a non-viral carrier for tumor-targeted gene delivery. Biomaterials 28, 540–549.
Desmedt, S.C., Demeester, J., Hennink, W.E., 2000. Cationic polymer based gene delivery systems. Pharm. Res. 17, 113–126.
Dodane, V., Vilivalam, V.D., 1998. Pharmaceutical applications of chitosan. Pharm. Sci. Technol. Today 1, 246–253.
Elfinger, M., Geiger, J., Hasenpusch, G., Üzgün, S., Sieverling, N., Aneja, M.K., Maucksch, C., Rudolph, C., 2009. Targeting of the β2-adrenoceptor increases nonviral gene delivery to pulmonary epithelial cells in vitro and lungs in vivo. J. Control. Release 135, 234–241.
Elfinger, M., Mauckscha, C., Rudolph, C., 2007. Characterization of lactoferrin as a targeting ligand for nonviral gene delivery to airway epithelial cells. Biomaterials 28, 3448–3455.
Hofland, H.E.J., Masson, C., Iginla, S., Osetinsky, I., Reddy, J.A., Leamon, C.P., Scherman, D., Bessodes, M., Wils, P., 2002. Folate-targeted gene transfer in vivo. Mol. Ther. 5, 739–744.
Issa, M.M., Köping-Höggård, M., Tømmeraas, K., Vårum, K.M., Christensen, B.E., Strand, S.P., Artursson, P., 2006. Targeted gene delivery with trisaccharide-substituted chitosan oligomers in vitro and after lung administration in vivo. J. Control. Release 115, 103–112.
Kakimoto, S., Moriyama, T., Tanabe, T., Shinkai, S., Nagasaki, T., 2007. Dual-ligand effect of transferrin and transforming growth factor alpha on polyethyleneimine-mediated gene delivery. J. Control Release 120, 242–249.
Laqueyrerie, A., Miltzer, P., Romani, F., Eiglmeier, K., Cole, S., Marchal, G., 1995. Cloning, sequencing, and expression of the apa gene coding for the *Mycobacterium tuberculosis* 45/47-KDa secreted antigen complex. Infect. Immun. 63, 4003–4010.
Lee, M.K., Chun, S.K., Choi, W.J., Kim, J.K., Choi, S.H., Kim, A., 2005. The use of chitosan as a condensing agent to enhance emulsion-mediated gene transfer. Biomaterials 26, 2147–2156.
Leong, K.W., Mao, H.Q., Truong-Le, V.L., 1998. DNA–polycation nanospheres as non-viral gene delivery vehicles. J. Control. Release 53, 183–193.
Liu, X., Howard, K.A., Dong, M., Andersen, M., Rahbek, U.L., Johnsen, M.G., Hansen, O.C., Besenbacher, F., Kjems, J., 2007. The influence of polymeric properties on chitosan/siRNA nanoparticle formulation and gene silencing. Biomaterials 28, 1280–1288.
Liu, W.G., Yao, K.D., 2002. Chitosan and its derivatives a promising non-viral vector for gene transfection. J. Control. Release 83, 1–11.
MacLaughlin, F.C., Mumper, R.J., Wang, J.J., 1998. Chitosan and depolymerized chitosan oligomers as condensing carriers for in vivo plasmid delivery. J. Control. Release 56, 259–272.
Mansouria, S., Cuieb, Y., Winnikb, F., Shia, Q., Lavignea, P., Benderdoura, M., Beaumonta, E., Fernandes, J.C., 2006. Characterization of folate–chitosan–DNA nanoparticles for gene therapy. Biomaterials 27, 2060–2065.
Mao, H., Roy, K., Troung-Le, V., Janes, K.A., Lin, K.Y., Wang, Y., August, J.T., Leong, K.W., 2001. Chitosan–DNA nanoparticles as gene carriers: synthesis, characterization and transfection efficiency. J. Control. Release 70, 399–421.
Mao, H.Q., Roy, K., Walsh, S.M., 1996. DNA–chitosan nanospheres for gene delivery, in: Proc. Int. Symp. Control. Rel. Bioact. Mater., Kyoto, Japan, vol. 23, pp. 401–402.
Ogris, M., Walker, G., Blessing, T., Kircheis, R., Wolschekd, M., Wagner, E., 2003. Tumor-targeted gene therapy: strategies for the preparation of ligand–polyethylene glycol–polyethylenimine/DNA complexes. J. Control. Release 91, 173–181.
Okamoto, H., Nishida, S., Todo, H., Sakakura, Y., iida, K., danjo, K., 2003. Pulmonary gene delivery by chitosan–pDNA complex powder prepared by a supercritical carbon dioxide process. J. Pharm. Sci. 92, 371–380.
Park, M.R., Han, K.O., Han, I.K., Cho, M.H., Nah, J.W., Choi, Y.J., Cho, C.S., 2005. Degradable polyethylenimine-alt-poly(ethylene glycol) copolymers as novel gene carriers. J. Control. Release 105, 365–380.
Park, W., Kim, K.S., Bae, B., Kim, Y., Na, K., 2010. Cancer cell specific targeting of nanogels from acetylated hyaluronic acid with low molecular weight. Eur. J. Pharm. Sci. 40, 367–375.
Prost, A.C., Menegaux, F., Langlois, P., Vidal, J.M., Koulibaly, M., Jost, J.L., Duron, J.J., Chigot, J.P., Vayre, P., Aurengo, A., Legrand, J.C., Rosselin, G., Gespach, C., 1998. Differential transferrin receptor density in human colorectal cancer: a potential probe for diagnosis and therapy. Int. J. Oncol. 13, 871–875.
Reynolds, P.N., Zinn, K.R., Gavrilyuk, V.D., Balyasnikova, I.V., Rogers, B.E., Buchsbaum, D.J., Wang, M.H., Miletich, D.J., Grizzle, W.E., Douglas, J.T., Danilov, S.M., Curiel, D.T., 2000. A targetable, injectable adenoviral vector for selective gene delivery to pulmonary endothelium in vivo. Mol. Ther. 2, 562–578.
Richardson, S.C.W., Kolbe, H.J.V., Duncan, R., 1999. Potential of low molecular mass chitosan as a DNA delivery system: biocom-patibility, body distribution and ability to complex and protect DNA. Int J. Pharm. 178, 231–243.
Romain, F., Laqueyrerie, A., Militzer, P., Pescher, P., Chavarot, P., La-granderie, M., Auregan, G., Gheorghiu, M., Marchal, G., 1993. Identification of a *Mycobacterium bovis* BCG 45/47-KDa antigen complex, an immunodominant target for antibody response after immunization with living bacteria. Infect. Immun. 61, 742–750.
Roy, K., Mao, H.Q., Huang, S.K., Leong, K.W., 1999. Oral gene delivery with chitosan–DNA nanoparticles generates immunologic protection in a murine model of peanut allergy. Nat. Med. 5, 387–391.
Rusch, V., Mendelsohn, J., Dmitrovsky, E., 1996. The epidermal growth factor receptor and its ligands as therapeutic targets in human tumors. Cytokine Growth Factor Rev. 7, 133–141.
Sato, T., Ishii, T., Okahata, Y., 2001. In vitro gene delivery mediated by chitosan. Effect of pH, serum, and molecular mass of chitosan on the transfection efficiency. Biomaterials 22, 2075–2080.
Schmitz, T., Bravo-Osuna, I., Vauthier, C., Ponchel, G., Loretz, B., Bernkop-Schnurch, A., 2007. Development and in vitro evaluation of a thiomer-based nanoparticulate gene delivery system. Biomaterials 28, 524–531.
Vadolas, J., Williamson, R., Ioannou, P.A., 2002. Gene therapy for inherited lung disorders: an insight into pulmonary defence. Pulm. Pharmacol. Ther. 15, 61–72.
Zaric, V., Weltin, D., Erbacher, P., Remy, J., Behr, J., Stephan, D., 2004. Effective polyethylenimine-mediated gene transfer into human endothelial cells. J. Gene Med. 6, 176–184.
Zhao, W., Schorey, J.S., Bong-Mastek, M., Ritchey, J., Brown, E.C., Ratliff, T.L., 2000. Role of a bacillus Calmette–Guerin fibronectin attachment protein in BCG-induced antitumor activity. Int. J. Cancer 86, 83–88.